Target in context : molecular pathology of pediatric ependymoma and high grade glioma

Felipe Andreiuolo

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Felipe Andreiuolo

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TARGET IN CONTEXT: MOLECULAR PATHOLOGY OF PEDIATRIC EPENDYMOMA AND HIGH GRADE GLIOMA

JURY :

Pr Leila Chimelli	Président
Pr Dominique Figarella-Branger	Rapporteur
Pr Richard Grundy	Rapporteur
Pr Gilles Vassal	Directeur de Thèse
Dr Jacques Grill	Co-directeur de Thèse

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Table of contents

"The origin is the target" Karl Kraus

General Introduction

1. Historical perspective

Rudolf Virchow was a pioneer in the systematic description of histological characteristics of brain tumors back in the first half of the 19th century. Until the beginning of the 20th century many different authors reported on brain tumors, providing with outstanding contributions, such as Deiters, Jastrowitz, Klebs and Golgi among others; for historical notes see (Zulch, 1986). The strive for defining cells of origin of brain tumors and subsequent controversies regarding their classification dates from those early days, when gliomas were initially separated from brain sarcomas by Virchow: Interestingly, such issue was still pertinent until much later, as for instance glioblastomas were definitely classified as astrocytic tumors as opposed to mesenchymal/vascular tumors only in the 1993 version of the World Health Organization (WHO) guidelines.

The current WHO classification scheme for tumors of the central nervous system is universally used. It is largely based on the work of Bailey and Cushing, which dates back to almost 100 years ago. It has also been strongly influenced by the works by Kernohan and Sayre, particularly the "Armed Forces Institute of Pathology (AFIP) Fascicle—Tumors of the Central Nervous System" published in 1952 (Zulch, 1986; Scheithauer, 2009). The histological four-tiered system with increasing grades of malignancy for brain tumors, still in use today, was developed by Kernohan et al based mostly on his studies on autopsy specimens, and was itself inspired by the pioneer studies of Broders et al on grading of skin squamous cell carcinoma (Broders, 1921). Since its proposal by Kernohan, the grading criteria for gliomas have been successively changed and further developed, aiming at a better correlation with clinical outcome (Kernohan et al., 1949; Louis et al., 2007; Zulch, 1986).

As pointed out by Scheithauer in his interesting review of the successive editions of the WHO classification for tumors of the central nervous system "arriving at prognostically meaningful "grades" has proven to be a challenge", with both clinical and histological malignancy being taken into consideration (Scheithauer, 2009). Particularly true for patients with central nervous system tumors, mechanisms of death are not necessarily correlated with histological grade, location having also a pivotal influence on outcome. In particular, infiltrative neoplasms, such as in those the brainstem or more diffuse ones such as gliomatosis cerebri, have a dismal outcome despite not necessarily exhibiting criteria of malignancy on histological evaluation. The concept of clinical malignancy continues to impact the histoprognostic classification of gliomas.

Even so, the actual grading system, albeit trying to integrate clinical correlation, does not predict outcome reliably in a number of cases. This is particularly striking for tumors affecting pediatric populations. To name a few examples, a subset of epileptogenic tumors among children are diagnosed as non-specific forms of dysembryoplastic neuro-epithelial tumors, which are far from being consensus among neuropathologists, and which may show worrisome cytological pleomorphism to those not acquainted with such particular lesions. Pilocytic astrocytomas are attributed a grade I, but there is a subset of patients with these tumors that will develop progressive disease, in the complete absence of histological "anaplastic" changes (Fernandez et al., 2003; Mazloom et al., 2012). WHO grades II and III do not discriminate well the outcome for ependymoma, especially among infants. Myxopapillary ependymomas, by definition grade I lesions according to the WHO guidelines often show aggressive clinical course with cerebrospinal fluid dissemination in children (Fassett et al., 2005).

Would this limitation to predict clinical evolution in children be particularly related to the fact that the WHO classification of glial/neuroepithelial tumors has actually been based and established in a mostly adult population, in which the incidence of high-grade glial tumors exceeds by far that observed in children? In the last WHO classification, less than ten percent of cases used for the classification of malignant gliomas were from patients under the age of 15 (Louis et al., 2007).

Interestingly, if we consider medulloblastomas, tumors affecting a highly predominant pediatric population, histological subtypes such as desmoplastic/nodular and large cell/anaplastic are largely correlated with significant favorable and poor outcome, respectively. More strikingly than for other brain tumors, for medulloblastomas, pathologists are also able to provide with a molecular classification: B-catenin mutations, which can be assessed by IHC status, allow us to identify a group with significantly better prognosis. *MYC* amplifications, which can be routinely detected by FISH analysis on paraffin–embedded material defines a subgroup with poor prognosis. As recently published, molecular subtypes of medulloblastoma, which were largely defined by genetic studies, can be reliably characterized by their histological and immunohistochemical profile (Ellison et al., 2011).

More than ever pathologists are striving for a more precise classification of tumors, with better prognostic and predictive input, and molecular diagnosis has become an important part of the daily practice in a Pathology laboratory.

2. Contextualizing Molecular diagnosis in Cancer Pathology

Any classification system has by definition an inherent limitation as it is based on grouping according to similarity, which is an approximation. In the field of biology and

particularly in oncology there is a plethora of new data produced and published each day. The practice of molecular pathology has become very complex, as it should integrate such a wide array of information.

The following table, extracted from the review article of Harris and McCormick (Harris and McCormick, 2010) lists the numerous patient profiling technologies mostly available to the present:

Host genomic DNA	Tumor nucleic acids	Tumor imaging	Serum and body fluid tests
Germline mutations	Gene-expression profiling	Size and location by X-ray and CAT scan	Specific proteins (PSA/CEA)
Disomy/trisomy analysis	Mutation analysis	Surface marker expression	Selected proteomic patterns
Genotypes at multiple loci for susceptibility alleles	Tumor-specific microRNA	Histopathology	Phosphoprotein profiles
Small insertions and deletions	Copy number variation and loss of heterozygosity	Tissue infrastructure	Peptide profiles
Gene amplification for specific genes	DNA insertions or deletions	Immunohistochemistry	Other post-translational protein profiling
Epigenetic modifications (e.g. methylation of CpG islands)	DNA translocations	PET scanning for tumor metabolism	Metabolomic analysis (e.g. hormone metabolites)
Chromatin modification by acetylation	–	–	–

Abbreviations: CAT, computerized axial tomography; CEA, carcinoembryonic antigen; PET, positron emission tomography; PSA, prostate specific antigen.

Probably, the most difficult task is not necessarily to succeed in obtaining consistent data, but rather finding how new data should be integrated with what is already known, and validated on a clinical setting, which will allow for a real improvement to the patients' prevention, diagnosis and therapy. For instance, the recent and herculean achievement of sequencing the human genome, was nonetheless far from presenting definitive solutions to understanding pathogenesis and evolution of all diseases and their specific mechanisms, and has urged the scientific community to focus on the integration, correlation and translation of data.

We briefly discuss a few examples to illustrate this point, in which one alteration is not necessarily associated with the same effect on different cancers, and could have even opposite effects. Moreover clinical and histological subgroups show particularities that should be considered and attentively evaluated in the context of targeted therapies.

The activating mutation of the *BRAF* (V600E) kinase is found in around 50% of melanomas and in many other cancers; in some of them, this alteration is associated with an epithelial-to-mesenchymal transition (EMT) (Knauf et al., 2011; Lin et al., 2010; Makrodouli et

al., 2011). This multi-step process results in the loss of cell-to-cell adhesive properties, loss of cell polarity, and the gain of invasive and migratory mesenchymal properties. The recently developed therapeutic agent (PLX4032) specifically targets this mutant form of *BRAF*, and some encouraging results of response in 81% of metastatic melanoma have been observed with this drug (Bollag et al., 2010). Unfortunately resistance develops rapidly and frequently in melanoma, by multiple mechanisms linked to melanoma oncogenesis (Poulikakos et al., 2011; Su et al., 2012). Other types of cancer also harbor *BRAF* V600E mutations, such as papillary thyroid carcinoma, colon carcinoma, ovarian carcinoma and some gliomas to name a few (Cantwell-Dorris et al., 2011; Schindler et al., 2011). The *BRAF* gene is part of the mitogenic pathway downstream of KRAS and activating mutations in *BRAF* could, therefore, interfere with drugs acting upstream in the pathway, which suggests that PLX4032 could be useful for treating cancers other than melanoma. Interestingly, our group has observed that a subgroup of patients with diffuse intrinsic pontine glioma (DIPG) shows EMT which seems associated with a worse prognosis in this group (Puget et al., 2012), but *BRAF* mutations were not found in these patients as we show in chapter 2 (Grill et al., 2012). It is possible that the effect of PLX4032 could therefore be different depending on the type of tumor considered.

The NOTCH pathway provides another striking example of the distinct and even opposed biological roles of one same system in different cellular contexts. The role of *NOTCH* as an oncogene in T-acute lymphoblastic leukemia is well characterized and further supported by a high frequency of activating *NOTCH1* mutations in patients with this disease (Lobry et al., 2011). Our group has also shown a high frequency of *NOTCH1* mutations and activation in pediatric ependymoma (Puget et al., 2009). Several other types of cancer such as head and neck squamous cell carcinoma, breast cancer, melanoma and medulloblastoma show activation of NOTCH pathways and corroborate its role as an oncogene (Ranganathan et al., 2011). However it has been shown that in the skin and particularly in basal cell carcinoma *NOTCH* acts as a tumor-suppressor gene (Nicolas et al., 2003) and recently also in hepatocellular carcinoma (Viatour et al., 2011), B-cell malignancies (Zweidler-McKay et al., 2005) and neuroblastoma (Zage et al., 2012) Therefore in these latter diseases normal NOTCH activity could have a potentially protective role against oncogenesis.

Anti-EGFR targeted therapies for non small-cell lung cancer (NSCLC) are an interesting setting in which subgroup analyses from the initial clinical trials showed that patients with certain clinical and histological characteristics (specifically, women, patients of east Asian descent, with no history of smoking, and those with adenocarcinomas) who received erlotinib or gefitinib had higher rates of response and overall survival (Fukuoka et al., 2003; Shepherd et al., 2005; Thatcher et al., 2005). Although the benefit in overall

survival was not statistically confirmed in Asian cohorts, a clear benefit in the progression-free survival was confirmed in the anti-EGFR branch as compared to classical chemotherapy regimens (Maemondo et al., 2010; Mitsudomi et al., 2010; Mok et al., 2009). There is strong evidence that EGFR mutated NSCLC, particularly those bearing deletions in exon 19 and the L858R point mutation in exon 21, the most common activating *EGFR* mutations in NSCLC, are both associated with improved outcomes with erlotinib or gefitinib therapy (Jackman et al., 2006; Lynch et al., 2004; Maemondo et al., 2010; Paez et al., 2004). Interestingly, some authors have shown that the presence of *KRAS* mutations is a better predictor of response to gefitinib than the presence of *EGFR* mutations, and also that *KRAS* and *EGFR* mutations in the same tumor are mutually exclusive (Cataldo et al., 2011; Ding et al., 2008). However, responses exist also in patients without *EGFR* mutation but with a specific gene expression profile (Balko et al., 2006; Bryant et al., 2012). The role of EGFR tyrosine kinase inhibitor-based therapies for NSCLC remains yet to be further refined and further translational studies are needed (Cataldo et al., 2011).

These various findings prove that the context in which a distinct molecular abnormality occurs will dictate the response to specific inhibitor and the mechanism of resistance. Moreover, the biologic profile associated to the response to a given targeted agent may not always be the consequence of a unique molecular event.

3. Conclusion

Biomarkers for the classification and management of pediatric glial brain tumors in the specific context of clinico-pathological characteristics and molecular pathology are lacking. The work in this thesis is part of an effort to address this issue. Biomarkers will be explored in view of classification (neuronal differentiation), prognostication (TNC), target identification (PI3KCA) and prediction of the efficacy of a treatment (erlotinib). Whenever possible, biomarkers will be discussed in the particular context of a given tumor type or subtype.
The first chapter focuses on ependymoma and the second on high grade-glioma, including DIPG.

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Chapter 1:

EPENDYMOMA

1. Some historical notes

As mentioned by Zülch and other authors (Zülch, 1986; Ernestus et al., 1991) Virchow was the first to describe ependymal tumors in 1863-65. In 1899 Störch described the perivascular pseudo-rosettes and ependymal rosettes, which are still current major histological criteria for the diagnosis of ependymoma. The relation of ependymomas to normal ependyma was further stressed by Mallory (Mallory, 1902), based on the identification of cytoplasmic structures derived from centrioles named blepharoplasts, alternatively named basal bodies, also present in normal ependyma, and which form the base for cilia and flagella that extend out of the cell (Kobayashi and Dynlacht, 2011). Interestingly very recent studies have shown particular subsets of ependymoma with upregulation of genes involved in ciliogenesis (Witt et al., 2011) and the over-expression of proteins associated with centrioles (Peyre et al., 2010).

Ependymomas and ependymoblastomas were set apart as distinct entities by Bailey and Cushing in 1926, and since then there has been controversy regarding their relationship and even the real existence of the latter (Zülch, 1986; Judkins and Ellison, 2010), which is currently included among embryonal tumors in the WHO classification scheme, while ependymomas are included among neuroepithelial lesions (Louis et al., 2007). Roussy and Oberling distinguished ependymocytomas “ependymoma of Bailey”, ependymoblastomas and ependymogliomas, the latter “consisting of ependymal and astrocyte elements”, already acknowledging the difficulties in defining a cell of origin versus dedifferentiation or even divergent differentiation in these tumors (Roussy et al., 1924; Roussy and Oberling, 1932).

2. Pathology

Histologically, the main hallmarks of ependymoma are:

- Perivascular pseudo-rosettes, which consist of nucleus-free mantles, surrounded by a radial disposition of cells around blood vessels. These may be not easy to identify in lesions or areas with very high or very low cellularity.
- True ependymal rosettes and ependymal canals, composed of columnar cells arranged around a central lumen or cavity. These are very characteristic of ependymoma, but are not found in all cases.

Immunohistochemical stains in ependymoma generally show strong cytoplasmic positivity for glial fibrilary acidic protein (GFAP), vimentin and CD56 (Godfraind, 2009; Louis et al.,2007). Epithelial membrane Antigen (EMA) usually highlights the luminal surface of true rosettes and canals, and often shows intra-cytoplasmic dots (Hasselblatt and Paulus, 2003). OLIG2 is usually absent or stains only few nuclei in ependymoma, and can be a useful marker in the

differential diagnosis in favor of other glial tumors such as oligodendroglioma or pilocytic astrocytoma (Godfraind, 2009; Preusser et al., 2007).

According to the WHO classification scheme for central nervous system tumours, ependymal tumors can be classified as follows:

-Subependymoma (WHO grade I), generally presenting in adults and associated with minimal morbidity.

-Myxopapillary ependymoma (WHO grade I), extremely rare in children but which shows a tendency for CSF dissemination in this population (Fassett et al., 2005)

-Besides the classic histological form, different morphological variants are distinguished, which do not have prognostic implications per se (cellular, clear cell, tanicytic and papillary ependymoma) (Louis et al., 2007). Intracranial pediatric ependymomas are mostly represented by either classic (WHO grade II) or anaplastic (WHO grade III) tumors (Ellison et al., 2011; Tihan et al., 2008).

The diagnosis of anaplastic ependymoma should be based on the presence of "increased cellularity, brisk mitotic activity, often associated with microvascular proliferation and pseudopalisading necrosis" (Louis et al., 2007), criteria classically developed for astrocytic and oligodendroglial tumors.

There is considerable histopathological variation among and within tumors -for instance, well demarcated nodules of densely cellular mitotically active cells can be found in around one third of posterior fossa lesions (Tihan et al., 2008), which results in great difficulty to grade them reliably (Figarella-Branger et al., 2000; Korshunov et al., 2002; Pollack et al., 1995; Robertson et al., 1998). Such difficulty is reflected by studies of clinically similar cohorts of children with intracranial ependymoma that report ratios of grade II to grade III tumors ranging between 17:1 and 1:7, a striking discordance that could be attributed to various different reasons such as intratumoral heterogeneity, inter-observer variability, variations in definitions, the use of different histological criteria, the uneven application of criteria for anaplasia by review pathologists, and idiosyncratic small patient cohorts as it has been pointed out in excellent works dealing with this subject (Ellison et al., 2011; Tihan et al., 2008). Moreover, attempts from efforts by groups of neuropathologists with experience in pediatric brain tumors have not yet presented an alternative classification scheme that would be easily reproducible (Ellison et al., 2011; Figarella-Branger et al., 2000; Tihan et al., 2008).

More importantly, in particular among pediatric ependymoma it is not clear whether lesions classified as grade II or grade III according to either WHO classification schemes or alternative grading systems have a clearly distinct outcome (Bouffet et al., 1998; Figarella-Branger et al., 2000; Gerszten et al., 1996; Grill et al., 2001; Grundy et al., 2007; Massimino et al., 2011; McGuire et al., 2009a; Ross and Rubinstein, 1989; Schiffer et al., 1991; Shu et

al., 2007; Sutton et al., 1990).

3. Origin

In a classification where tumors are diagnosed according to the appearance of the main cellular component, ependymomas are neoplasms thought to originate from the ependymal layer of the ventricular system. Therefore, they may develop in the third or lateral ventricles and in the spinal cord, but also without direct adhesion to the ventricular system, in the white matter, and some rare cases of ependymomas have even been referred to as "cortical" in the literature (Lehman, 2008; Roncaroli et al., 2005; Van Gompel et al., 2011). Characterization of tumor stem cells in ependymoma points to an origin from radial glia cells (Johnson et al., 2010; Taylor et al., 2005). Indeed, these tumor initiating cells harbor specific markers of radial glial progenitor cells such as BLBP (Brain Lipid-Binding Protein) or RC2 (Taylor et al., 2005).

4. Epidemiology

Ependymomas represent 6 to 12% of all childhood brain tumors, and are the third most common pediatric brain tumors after astrocytomas and medulloblastomas. In this population more than 50% cases occur before 5 years of age. Almost 90% of pediatric ependymomas are intracranial, around 2/3 of cases located in posterior fossa (Duffner et al., 1998; Grill et al., 2001; McGuire et al., 2009b), spinal lesions being relatively rare in children and predominant in the adult population (McGuire et al., 2009b; Merchant and Fouladi, 2005). Extra-axial ependymomas are intriguing and anecdotal, and have been reported in the ovary, broad ligament, sacrococcygeal region, lung, and mediastinum both in children and adults (Aktug et al., 2000; Hirahara et al., 1997; Mallory, 1902). These lesions show some clinical similarities with germ cell tumors, inside which they may also occur, and seem to exhibit different both morphology and immunophenotype from primary central nervous system ependymomas, suggesting that they derive from distinct precursors and/or differentiate along distinct pathways (Idowu et al., 2008).

Some cases of ependymoma have been described associated with genetic predisposition syndromes. Patients with Neurofibromatosis type 2 have germline mutations in the NF2 gene (22q12.2), which is associated with an increased incidence of vestibular schwannomas, and less frequently meningiomas and spinal ependymomas. More rarely ependymomas can be associated with other familial syndromes such as multiple endocrine neoplasia type 1 (MEN1) and Turcot syndrome (Louis et al., 2007). Most pediatric cases of ependymoma appear to be sporadic.

5. Imaging

Ependymomas are usually well circumscribed. Most frequent computed tomography findings are of a mass with solid and cystic components and heterogeneous signal, often calcified and which may show signs of hemorrhage (Yuh et al., 2009). Tumors usually display iso to low T1, iso to high T2, and intermediate-to-high FLAIR signal intensity relative to both gray and white matter. The signal tends to be heterogeneous within a same tumor, irrespectively of the type of acquisition, and this is particularly observed in supratentorial lesions, which show greater propensity for cyst formation. FLAIR sequences can show a sharp interface between tumor, surrounding brain and cerebrospinal fluid. On postgadolinium T1-weighted images, there is usually heterogeneous enhancement, alternating strongly and poorly enhancing or nonenhancing areas. A minority of lesions demonstrates little or no contrast enhancement. Posterior fossa lesions have a tendency to squeeze out fourth ventricle, projecting inferiorly through Magendie foramen and laterally through Lushka foramina into cerebellopontine angle cisterns. (Yuh et al., 2009)

6. Treatment

The main treatment of pediatric intracranial ependymomas is surgery, followed by adjuvant radiotherapy. The extent of surgical resection has been consistently identified as a critical determinant of outcome (Bouffet et al., 1998; Grill et al., 2001; Merchant et al., 2009), however this was not found in other pediatric series and most particularly with posterior fossa tumors (Akyuz et al., 2000; Goldwein et al., 1990; Grundy et al., 2007). The role of chemotherapy is controversial, but its use alongside radiotherapy has been the focus of several clinical trials, especially in the setting of attempts to avoid or to defer radiotherapy in infants (Grill et al., 2001; Grill et al., 2003; Grundy et al., 2007; Merchant and Fouladi, 2005).
To our knowledge, at the moment we started the study, biomarkers predictive of response to treatment were unknown.

7. Prognosis

Despite progress in imaging techniques, surgery and radio/chemotherapy the prognosis for children with ependymomas is relatively poor compared to those with other brain tumors, with a median overall survival of around 50% and a progress free survival at five years varying from 30% to 60% (Massimino et al., 2009; Merchant et al., 2009). In addition, late relapses up to 20 years after initial treatment have reported (Bouffet et al., 2012; Paulino et al., 2002).
Except extent of surgery, to our knowledge validated prognostic factors are lacking for this disease.

8. Recent findings and controversies

Lessons from comparison with adult counterparts: the influence of localization on oncogenesis

Differences between ependymomas in children and adults have been constantly reported. Besides different preferential locations as mentioned above, ependymomas in children have been largely known to have a worse prognosis (Witt et al., 2011). Before the advent of recent technologies in genetic studies, it was difficult to explain these differences on a biological basis. Comparative genomic hybridization (CGH) and array CGH (aCGH) studies have allowed a finer analysis of genetic alterations in these tumors (Grill et al., 2002; Jeuken et al., 2002; Puget et al., 2009). A comprehensive meta-analysis of 13 CGH studies and literature review on biomarkers has shown striking differences between pediatric and adult ependymoma on a genetic level (Kilday et al., 2009), which were initially not identified in earlier karyotype-based studies.

Pediatric ependymoma present more frequently gains of 1q, 7 and 9 and losses of chromosomes 22, 3, 9p, 13q 6p, 1p 17 and 6 and its adult counterparts display more often gains of chromosome 7, 9 12, 5, 18 x, 2 and loss of 22/22q, 10, 13q 6 and 14q. Statistically most relevant differences are the higher frequency of 1q gains among children, above 20% vs. 8%, ($p< 0.0040$) and higher prevalence of gains of chromosomes 7, 9, and 12 in adults ($p< 0.001$)(Kilday et al., 2009). Another important difference between pediatric and adult ependymoma pertains to the number and complexity of genomic aberrations. Adult lesions show more frequent chromosomal aberrations; a balanced genomic profile, without chromosomal gain or loss, can be seen in 36% to 58% of pediatric ependymomas and is significantly associated with children under 3 years of age and is found in less than 10% of adult cases (Kilday et al., 2009). Chromosomal aberrations in adults involve more often whole chromosomal rearrangements, unlike the partial and complex imbalances frequently seen in pediatric cases, these latter having been associated with worse prognosis also in other types of cancer such as colorectal and breast malignancies (Birkbak et al., 2011; Isola et al., 1995; Rooney et al., 2001)

Dyer et al in a study including 42 primary and 11 recurrent pediatric ependymomas showed a subgroup of 5 patients with copy number alterations, more similar to those found in adults and spinal lesions. Moreover in this study 14 of 14 children under 3 years of age displayed a balanced profile while 24 of 28 children (86%) diagnosed older than 3 years of age showed a non-balanced profile (Dyer et al., 2002).

Two different studies from a large cohort of over 120 adult and pediatric ependymoma described by CGH analysis a more balanced profile was described in tumors from younger

patients (Korshunov et al., 2010; Witt et al., 2011).
In a recent study on adult and pediatric paraffin embedded infratentorial ependymomas the authors established a 10-gene signature that was correlated with both worse overall survival and worse progression-free survival, and which was significantly correlated with younger age at diagnosis (Wani et al., 2012).

There is convincing evidence that such diverse biological characteristics and that the particular clinical behavior distinguishing ependymomas in children from those in adult populations also reflects major location-specific differences amongst these groups. Several recent works have shown these region-associated differences on a chromosomal and transcriptomic level. (Modena et al., 2006; Palm et al., 2009; Peyre et al., 2010; Schneider et al., 2009; Taylor et al., 2005). Johnson et al studied a series of pediatric and adult ependymoma showed that DNA-copy number alterations (n=204), messenger RNA (n=83) and micro RNA expression (n=64) allow to cluster ependymomas in 9 similar subgroups in agreement with age and location. The authors compared data from human ependymoma and murine neural stem cells, either wild type or null for the genes Ink4a/Arf, (locus frequently deleted in supratentorial ependymoma that code for CDKN2A et CDKN2B). Only one specific group of supratentorial ependymoma with amplification of Ephrin B2 (EPHB2), an oncogene selectively amplified in supratentorial ependymoma, matched the murine Ink4a/Arf null neural stem cells by transcriptome analysis. They generated the first mouse-model of supratentorial ependymoma derived from neural stem cells, combining deletion of Ink4a/Arf and viral induced expression of EPHB2, which corresponds at a genomic level to a subgroup of supratentorial ependymoma from their large cohort. This unique mouse model of ependymoma is an important basis for the study of physiopathological mechanisms and signaling pathways in the genesis of ependymoma. This cross-species study has allowed the identification of the specific oncogene EPHB2 and the deletion of Ink4a/Arf in the genesis of supratentorial ependymoma.
Their work provided additional evidence that supratentorial ependymoma are derived from radial glia as had been indicated previously by the same group: In a mouse model overexpressing NOTCH1 in radial glial cells under the control of the promoter Blbp. Ink4a/Arf- null mice developed ependymoma in a much shorter period than wild-type mice (Gottardo et al., 2008).

9. Natural history: oncogenesis and progression

Much beyond than a mere cartographical description of genes in pediatric ependymoma, studies based on CGH techniques have brought us interesting information concerning not only the genesis and (to some extent) prognosis, but have also helped to elucidate some aspects of the natural history of this disease.

Puget et al. analyzed the genomic profile of 59 pediatric ependymoma by array-CGH, of which 33 at diagnosis and 26 at relapse (Puget et al., 2009). Comparing genetic profiles at diagnosis and at relapse our group described a significant increase in genomic imbalances at relapse compared with diagnosis, such as gain of 9qter and 1q (54% v 21% and 12% v 0%, respectively) and loss of 6q(27% v 6%). Supervised classification showed that gain of 9qter was associated with tumor recurrence, age older than 3 years, and posterior fossa location. Through a candidate-gene strategy two potential oncogenes were found overexpressed at the locus 9qter in comparison to normal adult or fetal brains: *NOTCH1* and *Tenascin-C* (TNC).

The gene *NOTCH1* is involved in cell growth and differentiation and its pathways are implicated in several types of cancer originating from the skin, lung, prostate, breast, uterine cervix, brain and neuroblastoma (Allenspach et al., 2002; Stockhausen et al.). Modena et al and Taylor et al have previously described activation of NOTCH pathway in ependymoma (Modena et al., 2006; Taylor et al., 2005). Further analysis of NOTCH pathway analysis by qPCR revealed overexpression of NOTCH ligand DLL-1, its receptor, and its target genes (Hes-1, Hey2, and c-Myc), and the downregulation of its repressor Fbxw7. *NOTCH1* missense mutations were detected in 8.3% of the tumors (all located in the posterior fossa and in cases of 9q33-34 gain), to our knowledge the first description of *NOTCH* mutations in brain tumors. Furthermore, inhibition of NOTCH pathway with a γ-secretase inhibitor impaired the growth of ependymoma stem cell cultures. The overexpression of NOTCH effector Hes-1 and TNC were confirmed by immunohistochemistry (Puget et al., 2009).

Dyer et al. were the first to propose a classification scheme for pediatric ependymoma based on genetic alterations, which is also associated with prognosis. They described three subgroups showing 1) a more “balanced” profile 2) “structural” aberrations with gain or loss of specific chromosomal regions and worse outcome 3) copy number alterations most commonly found in adult disease and best prognosis (Dyer et al., 2002).

The CGH profile from a large cohort of 122 ependymomas allowed Korshunov et al. to define genomic alterations characterizing three different groups (Korshunov et al., 2010). The first group with the worst prognosis showed 1q gain and deletion of CDKN2. A second group essentially comprising deletion of chromosome 6, gains of chromosome 9, 15q and 18 with a better prognosis. A third group with an intermediate outcome showed a balanced

profile. However these data should be interpreted cautiously since in this study the stratification does not take into account age or tumor location. The great number of cases included in this work does not make up for these methodological flaws.

These data were confirmed and further advanced by the recent work by Witt et al. Departing from a transcriptome analysis of 177 posterior fossa ependymomas from adult and pediatric patients from two different countries two subgroups were distinguished, namely A and B, and this was reproducible in another independent patient cohort. Subtype A tumors occurred in younger patients, of which 70% male, and tended to be more laterally located. These tumors had relatively lower genomic instability (most often gain of chromosome 1 or loss of chromosome 22) and showed a worse prognosis. Patients from subgroup B were older, displayed tumors with more central location in the posterior fossa which harbored higher genomic instability and better prognosis. The authors further characterized the genes and pathways associated with each of these subgroups and validated the differences on a protein level (Witt et al., 2011).

These findings have been recently validated by another group, also working on a mixed adult/pediatric posterior fossa ependymomas by gene expression studies (Wani et al., 2012). Working exclusively with paraffin-embedded material two gene expression subgroups were defined, group 1 overexpressing genes associated with mesenchyme and group 2 showing no distinct gene ontologies. Groups 1 and 2 were highly correlated with subgroups A and B, from the work by Witt et al, respectively (Witt et al., 2011).

Few studies have explored the evolution of ependymomas. An earlier work suggested that distinct pathways of progression might be involved from the cytogenetic point of view (Grill et al., 2002). Stepwise acquisition of cytogenetic imbalances was further confirmed in later studies (Peyre et al., 2010). The comparison of the gene expression profile at relapse with the one at diagnosis showed that the pattern of progression was different in supratentorial tumors than in infratentorial tumors. Supratentorial relapsed ependymoma expressed more genes involved in mesenchymal transition, corresponding to a more invasive behavior while in the infratentorial compartment relapses expressed more ribosomal genes associated with an increased proliferation rate. Interestingly, some common findings were present irrespective of the location or treatment: metallothioneins, especially type 3, were down regulated at relapse both at the transcriptional and immunohistochemical level, suggesting that these proteins may work as tumor suppressor genes. Conversely, several genes from the kinetochore including Erg5 or ASPM were upregulated at relapse compared to diagnosis (Appendix 1.1).

Few studies on epigenetic changes (e.g. methylation profiles) have been performed in ependymoma, using a candidate-gene approach, with hypertmethylation leading to silencing

of the different genes in adult populations. However no definite relation between gene silencing and prognosis has been reported (Alonso et al., 2004; Hamilton et al., 2005; Michalowski et al., 2006; Rousseau et al., 2003; Waha et al., 2004). To the present only one study on epigenetics was carried on a pediatric ependymoma cohort (Rogers et al., 2011); and revealed a hypermethylated profile of tumor-suppressor genes in supratentorial and spinal but not in posterior fossa lesions, further supporting the differences in biology according to location.

10. Prognostic markers at a molecular level

The ideal prognostic marker should easy to perform and quickly available in clinical practice, it should be robust, standardized, and validated by a solid prospective study with strict statistical criteria. Data should be reproducible. Most studies aiming to identify novel predictive or prognostic biological markers for pediatric ependymoma have been performed in a retrospective fashion, were based on small cohorts or used non-standardized immunohistochemical analysis, often yielding conflicting results. Furthermore, a great number of studies analyzed a mixed cohort also including adult patients. However, the identification of new prognostic markers, even if based on retrospective cohorts, could help us define the high-risk patients that should potentially benefit from more intense treatment schemes and those for whom a less aggressive therapeutic approach would be suitable, resulting in considerably less associated side-effects. A relatively high number of studies on candidate prognostic markers for ependymoma have been published and have been reviewed by (Kilday et al., 2009). We have selected to present here only a selection of the most important ones.

A. hTERT and Nucleonin

An immunohistochemical study on 65 pediatric ependymomas has shown positivity in 58% of these tumors. Multivariate analysis on this cohort identified hTERT as a potent prognostic predictor of survival in childhood ependymoma (Tabori et al., 2006). In a retrospective cohort of 83 pediatric patients with 133 ependymomas, of which 31 at relapse, the same group showed that hTERT expression was correlated with proliferation index assessed by MIB-1 and the mitotic count. These data suggest that hTERT could be a marker of aggressive behavior in these tumors (Tabori et al., 2008). However, it was subsequently shown that the antibody used in this study in fact was directed against Nucleolin, a protein with the function of nuclear chaperone for hTERT (Ridley et al., 2008). This retrospective study of 97 intracranial tumors from 74 patients with pediatric intracranial ependymoma which evaluated several « candidate » prognostic markers including Nucleolin, KI67, members of the RTK1

family and survivin and nucleolin, showed only low nucleolin expression (<50% cells) as a significant prognostic factor, with a favorable impact on OS and PFS (Ridley et al., 2008). In their series, patients with tumors with high immunohistochemical expression of nucleolin had a 5-year PFS at 31%, versus 74% for those with low expression. The unavailability of commercial antibodies (withdrawn from the market) and difficulties for establishing a threshold for positivity have considerably hampered the further utilization of this marker.

B. 1q gain, 22q loss, 6q loss

In the meta-analysis mentioned above Kilday et al showed that the most frequent genetic alteration described in childhood ependymoma is the 1q gain, also described as a marker of bad prognosis in other types of pediatric cancer, such as nephroblastoma, neuroblastoma and Ewing sarcoma (Hing et al., 2001; Hirai et al., 1999; Ozaki et al., 2001). This highlights the role of this genetic abnormality in the progression and recurrence in different types of pediatric cancer, and which could be a frequent characteristic of pediatric neoplasia in general. Different studies report the loss of 1q as a marker of bad prognosis in ependymoma (Carter et al., 2002; Dyer et al., 2002; Korshunov et al., 2010; Mendrzyk et al., 2006). One study analyzed 68 tumors also from a mixed adult and pediatric cohort and identified the gain of the region 1q25 to be associated with relapse and to be an independent marker of overall survival (Mendrzyk et al., 2006). In the work by Korshunov et al in adult and pediatric patients (Korshunov et al., 2010) the subgroup of patients harboring 1q gain and deletion of CDKN2 had a worse prognosis. In the study by Witt including posterior fossa ependymoma from all ages 1q gains had an increase occurrence the group A, which had also a worst prognosis. Interestingly in the validation of the gene expression data performed on an independent cohort, patients from group A with 1q gains assessed by FISH exhibited no difference in survival compared with group A patients who did not posses this aberration (Witt et al., 2011). A recent multicentric international study on well clinically characterized pediatric cohorts showed that 1q25 gain significantly predicts shorter time for disease progression (Kilday et al., 2012; Appendix 1.2). Although several candidate genes have been proposed, especially in the regions 1q21-31 and 1q42.13 the oncogene on 1q definitively implicated in ependymoma tumorigenesis remains yet to be identified (Johnson et al., 2010; Karakoula et al., 2008; Korshunov et al., 2003; Mendrzyk et al., 2006; Rand et al., 2008).

The loss of 22q has also been reported as a recurrent abnormality in ependymomas (Ebert et al., 1999; Johnson et al., 2010; Modena et al., 2006), often observed in spinal lesions. A retrospective study on 47 pediatric ependymomas showed that the gene RAC2, deleted from the region 22q13.3 is associated with an inferior overall survival (Karakoula et al., 2008). The loss of 6q seems also to be a factor of bad prognosis (Korshunov et al., 2010; Monoranu et

al., 2008). However, the deletion of 6q23 was associated with a superior event free survival in a mixed adult and pediatric cohort (Rajaram et al., 2005). Further studies are needed to validate this observation and to confirm 6q alteration as a robust prognostic marker.

C. Pro-inflammatory markers

As reported in other types of cancer the immune system seems to influence the prognosis of childhood ependymoma. A recent study on 19 samples from pediatric ependymoma by Donson et al showed the overexpression of immune function-associated genes with non-recurrent lesions (Donson et al., 2009). Of genes associated with both nonrecurrent phenotype and positively correlated with time to progression, 95% were associated with both innate and acquired immune function. The authors showed the overexpression of innate immune function genes in microglial cells and more numerous CD4 lymphocytes, mediators of adaptative immune response, in nonrecurrent lesions. These interesting data should be confirmed on a larger series but point out to an alternative direction in the study of prognostic factors in ependymoma.

D. Proliferation markers

Numerous studies have focused on the assessment of the proliferation index in pediatric ependymomas by immunohistochemistry for Ki67 and its relation with prognosis. Although most authors have shown an inverse and significant correlation between proliferation and prognosis (Bennetto et al., 1998; Figarella-Branger et al., 2000; Gilbertson et al., 2002; Preusser et al., 2008; Zamecnik et al., 2003), other groups did not confirm these data (Prayson, 1999; Ridley et al., 2008; Shuangshoti et al., 2005). All were retrospective studies and for the great majority the association was not confirmed in multivariate analysis. Moreover the labeling index used as a cutoff in these various studies was extremely variable (from 1 to 25 %), which renders definite conclusions difficult as to its prognostic value in pediatric ependymoma.

E. LAMA2, NELL2

Witt et al identified two molecularly distinct subgroups of posterior fossa ependymoma with different clinical outcome (Witt et al., 2011). The authors successfully validated their signature using immunohistochemistry. As mentioned above, in their stratification the subgroup A had worse prognosis, and showed over-expression of genes of extracellular matrix assembly, and group B overexpressed genes involved in ciliogenesis and microtubule assembly. Two markers of extracellular matrix signaling, Laminin-alpha2 (LAMA2) were tested as positive markers for subgroup A, while Neural Epidermal Growth factor-Like 2

(NELL2) as a marker for group B. These antibodies currently commercially available could be used for stratification of low and high risk subgroups of ependymoma departing from paraffin embedded material.

11. Conclusion

Therapeutic targets have yet to be identified in ependymomas. In addition to the target, it is necessary to define its context because it may influence importantly the effect of a given drug on this specific target. In this respect, classifying the tumors is key to define homogenous groups of lesions with the same biological characteristics, e.g. neuronal differentiation, that could influence the way relevant therapeutic targets could be identified (mutations driving the phenotype being more likely to be driver mutations) and the way targeted therapies should be designed efficiently (drugs need to be in a certain context to act efficiently).

The relatively rare occurrence of ependymoma in children is one of the factors hampering advances in research, since tumor material, specially frozen tissue, can be quite difficult to access, and large prospective clinical cohorts are unavailable. However collaboration between different groups has allowed us to refine some of our knowledge regarding its origin, and to some extent the biology of both progression and relapse.

Biomarkers for both prognosis and prediction of response to therapy of ependymoma in children are lacking, and would be of great benefit to define a more adapted clinical approach.

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Introduction to first article

As mentioned in the previous section, from a histopathological perspective, major problems concern the diagnosis of pediatric ependymomas. Some of the remarkable issues are:

1) The low reproducibility of grading among different observers.

2) The limitations of the WHO grading system with respect to prognosis; this is especially true in young children.

3) Despite a higher incidence of clear cell variant described in the supratentorial compartment and myxopapillary and tanicytic ependymoma in the spine, the occurrence of all variants in any location.

All these constitute evidence of the limitations of histopathology and its current ancillary techniques (e.g. the disparate thresholds proposed for Ki67 labeling index), hampering the clinical management of ependymomas. Moreover these limitations became progressively more outstanding as successive studies came out confirming the different clinical behavior and particular biological characteristics of ependymoma from different locations.

Taylor et al showed that the cell of origin of ependymomas is the radial glial cell (Taylor et al., 2005). In their study the gene expression signature distinguishing supratentorial, posterior fossa and spinal ependymomas included genes that regulate neural precursor cell proliferation and differentiation in the corresponding region of the central nervous system. For instance spinal ependymomas expressed Homebox (HOX) family members that coordinate anteroposterior tissue patterning during development and have an increased expression in caudal CNS regions. Conversely supratentorial ependymomas overexpressed members of the Ephrin and Notch family, which are very important in the maintenance of neural stem cells in the subventricular zone. The authors further identified a population of radial glia-like cells expressing BLBP and RC2 in primary neurospheres generated from fresh pediatric supratentorial and spinal ependymoma, and showed that RGC-like ependymoma stem cells form morphologically typical ependymoma in nude mice.

As radial glial cells are neural stem cells and also give rise to mature ependymal cells we hypothesized that cells forming ependymomas could possibly exhibit differentiation along neuronal lines.

Striving for a refined characterization of adult malignant infiltrative gliomas, the Sainte-Anne group had previously shown that with clinico-radiological and immunohistochemistry, it was possible to define a subset of patients with “pseudo- well circumscribed” tumors mostly situated superficially, often attached to the meninges and which co-expressed by IHC glial and to some extent neuronal markers such as

Neurofilament light polypeptide (NEFL) and/or synaptophysin, which were called malignant glioneuronal tumors. Patients with such lesions tended to be younger, and when benefiting from a total gross resection seemed to show a more favorable prognosis (Varlet et al., 2004). Interestingly, it was confirmed later in the pioneer work by Phillips et al (Phillips et al., 2006) that among glioblastomas a "pro-neural" signature, enriched with neuronal differentiation genes, was characteristic of a group with better prognosis.
Therefore, it seemed to us that neuronal markers could possibly help to delineate a particular subgroup of ependymomas.

We analyzed an exclusively pediatric and clinically well documented cohort of patients with supratentorial (SE) (n=34) and infratentorial ependymoma (IE) from posterior fossa (n=32), with emphasis on the expression of neuronal expression/differentiation by IHC on formalin fixed paraffin embedded material and quantitative PCR from snap frozen samples from SE (n=10) and IE (n=11).
We were able to show that there was a significant overexpression of neuronal markers in SE compared with IE, most notably NEFL. Of 34 SE, 23 exhibited NEFL positive cells whereas among 32 IE only 4 were positive for this marker ($p<0.001$). Between NEFL-positive SE, high immunoexpression defined as over 5% positive cells was found in 10/34 cases and was correlated with better progression-free survival ($p=0.009$). The immunoexpression of NEFL was positively correlated with positivity for Olig2, Chromogranin A and synaptophysin ($p=0.038$, 0.008 and 0.04 respectively).
Quantitative PCR results confirmed the upregulation of neuronal markers in SE as compared to IE. Genes overexpressed in ependymomas from various locations selected from the literature were NEFL, lim homebox protein 2 (LHX2), forkhead boxG1B (FOXG1), neuronal pentraxin receptor (NPTXR), reelin (RLN), tenascin c (TNC) and Notch1.
This study, besides providing further evidence that SE and IE are distinct biological entities, pointed to NEFL as a immunohistochemical marker that should be further validated in larger series and multivariate analyses, and which could be potentially integrated in the clinical setting, for the prognosis of supratentorial lesions.

Interestingly, although evidence from the literature regarding ependymomas indicates very strongly an origin from the radial glia, which presents the capacity to differentiate into glial cells and are also the source for "adult neurons", some questions are still not completely answered:
Is neuronal differentiation in ependymomas irrefutable evidence that these tumours originate from a glioneuronal progenitor rather than a committed glial progenitor (Ever and Gaiano, 2005; Spassky et al., 2005)? Given the fact that some rare ependymomas display

morphological and ultra-structural evidence of neuronal differentiation (Gessi et al., 2005; Rodriguez et al., 2007) and that also other tumor types such as ganglioglioma can rarely show an "ependymal component" (Varlet et al., 2009), would the upregulation of genes involved in neuronal differentiation and the relatively frequent expression of neuronal proteins in childhood ependymoma represent differentiation of neuronal precursors? Of note, this phenomenon has been well described in medulloblastomas and supratentorial PNETs. However as these are embryonal tumors it seems less surprising that they present a broader potential of diverse, heterogeneous lines of differentiation (Louis et al., 2007), than ependymomas.

Would the greater plasticity and divergent neuronal and glial phenotype observed more often in supratentorial tumors in children reflect an origin from a more undifferentiated neuroepithelial stem cell precursor, situated above the radial glia in the ontogenesis?

Back in 1928, Roussy and Cornil criticized Bailey and Cushing's classification scheme for tumors of the CNS, as the concept of cytogenesis seemed insufficiently substantiated (Roussy and Cornil 1928). Although Roussy and Oberling's classification system had many similarities to the pioneer work by Bailey and Cushing, they argued that cellular anaplastic transformation was different from the unlikely arrest at different stages of development (Roussy and Oberling, 1932). Indeed, the concept of cell of origin in the context of the somatic mutation theory of carcinogenesis and metastasis, centered at the cellular level of biological organization is undoubtedly the most widely accepted among researchers in the field (Hanahan and Weinberg, 2011). However some authors are very critical as to the pertinence and to the possibility of pragmatically characterizing features in a cancer cell that would identify it from a normal one, viewing a cancer rather as a deregulation on tissular or a "field" scale (Sonnenschein and Soto, 2011). One of the frequent characteristics of cancer cells of a given tissue is to re-express proteins of completely different lineage, e.g. testicular genes in recurrent ependymomas (Peyre et al., 2010). While insisting that location is driving the oncogenesis (Gilbertson & Gutmann, Cancer Res 2007), one other driving force is divergence, i.e. the stochastic acquisition of new features that do not necessarily belong to the cell of origin.

Histogenetical and conceptual controversies aside, phenotype does confirm singularities in ependymomas from different locations. We present another pertinent angle to look from: neuronal differentiation in these central nervous system lesions.

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Neuronal differentiation distinguishes supratentorial and infratentorial childhood ependymomas

Felipe Andreiuolo, Stephanie Puget, Matthieu Peyre, Carmela Dantas-Barbosa, Nathalie Boddaert, Cathy Philippe, Audrey Mauguen, Jacques Grill, and Pascale Varlet

INSERM UMR 8203 "Vectorology and Anticancer Therapies," Institut Gustave Roussy, Universite´ Paris 11, Villejuif, France (F.A., S.P., M.P., C.D.-B., C.P., J.G.); Department of Neurosurgery, Hoˆpital Necker Enfants Malades, Universite´ Paris Descartes, Paris, France (S.P.); Department of Radiology, Hoˆpital Necker Enfants Malades, Paris, France (N.B.); Department of Biostatistics and Epidemiology, Institut Gustave Roussy, Villejuif, France (A.M.); Department of Pediatric and Adolescent Oncology, Institut Gustave Roussy, Villejuif, France (J.G.); Department of Neuropathology, Hoˆpital Sainte-Anne, Paris, France (P.V.); Inserm UMR U894; Universite´ Paris 5, Paris, France (P.V.)

Ependymomas are glial neoplasms occurring in any location throughout the central nervous system and supposedly are derived from radial glia cells. Recent data suggest that these tumors may have different biological and clinical behaviors according to their location. Pediatric supratentorial and infratentorial ependymoma (SE and IE) were compared with respect to clinical and radiological parameters and immunohistochemistry (IHC). Neuronal markers were specifically assessed by IHC and quantitative PCR (qPCR). No single morphological or radiological characteristic was associated with location or any neuronal marker. However, there was a significant overexpression of neuronal markers in SE compared with IE: neurofilament light polypeptide 70 (NEFL)-positive tumor cells were found in 23 of 34 SE and in only 4 of 32 IE ($P < .001$). Among SE, 10 of 34 exhibited high expression of NEFL, defined as more than 5% positive cells. qPCR confirmed the upregulation of neuronal markers (NEFL, LHX2, FOXG1, TLX1, and NPTXR) in SE compared with IE. In addition, strong NEFL expression in SE was correlated with better progression-free survival (P 5 .007). Our results support the distinction of SE and IE. SEs are characterized by neuronal differentiation, which seems to be associated with better prognosis.

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Corresponding Author: Felipe Andreiuolo, MD, INSERM UMR 8203 "Vectorology and Anticancer Therapies," Institut Gustave Roussy, 39 rue Camille Desmoulins, Villejuif, 94805, France (felipe.andreiuolo@igr.fr).

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Ependymomas represent the third most common intracranial tumors in children and are defined as neoplasms exhibiting glial and/or epithelial morphology.[1] Only 25%–35% of them occur in a supratentorial location.[2,3] According to the WHO 2007 classification,[4] histological variants include classic, cellular, clear cell, papillary, tanicytic, and myxopapillary ependymoma. In the supratentorial compartment, tanicytic or myxopapillary variants are exceptional. The current WHO 2007 classification distinguishes grade II from grade III, which does not accurately predict clinical outcome.[5] The extent of surgery remains the most important prognostic indicator,[3] although children with supratentorial ependymoma (SE) seem to have a better outcome.[6]

Ependymomas are neoplasms thought to originate from the ependymal layer of the entire ventricular system.[4] Therefore, SE may develop in the third or lateral ventricles, but also without direct adhesion to the ventricular system, in the white matter, and some rare cases of ependymomas have even been referred to as "cortical" in the literature.[1,7] Ependymomas are morphologically similar in every CNS location but seem to display distinct chromosomal imbalances or genomic abnormalities.[8–10] Interestingly, recent comparative gene expression profiles support the idea that pediatric ependymomas exhibit the patterns of gene expression recapitulating those of radial glia cells in the corresponding CNS regions.[8] As radial glia cells are now considered neural stem cells[11] and as they give rise to mature

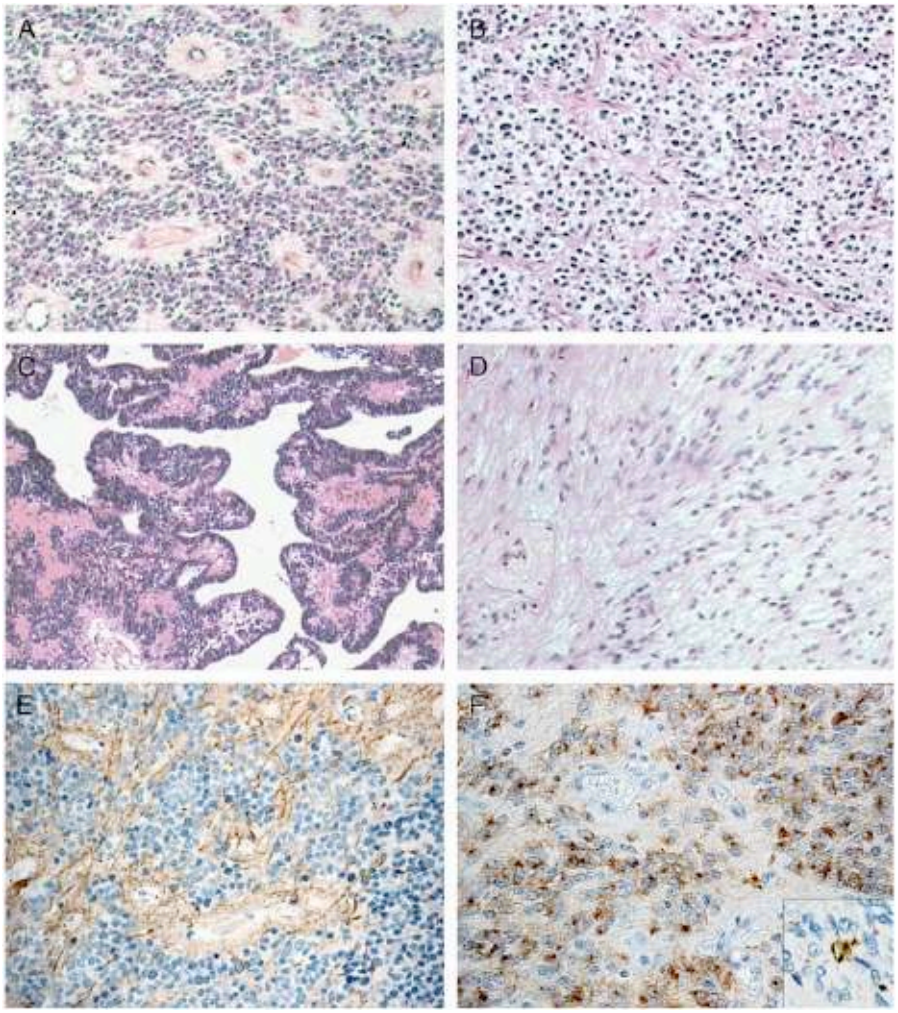

Fig. 1. Histological variants and IHC findings in SE: (A) classic ependymoma histology; (B) clear cell ependymoma; (C) papillary ependymoma; (D) tanicytic ependymoma; (E) GFAP immunostaining showing a classical perivascular enhancement; and (F) EMA staining, with positivity in dots, some cases showing an apical cell-membrane staining in true rosettes (insert). Original magnifications: $_{\times}$200 (A, B, and E), $_{\times}$100 (C), and $_{\times}$400 (F).

ependymal cells,[12] it is thus possible to hypothesize that tumor cells forming ependymomas may not only express glial markers but may rarely exhibit differentiation along neuronal lines.

The aim of the present study was to compare SE and infratentorial ependymoma (IE) by gene expression studies and immunohistochemistry (IHC), with emphasis on neuronal expression and/or differentiation.

Material and Methods

Pathology Review

We reviewed the pathological features of 43 SE and 32 IE resected from children in the years from 1994 to 2007 at the Necker Enfants Malades Hospital. Subependymomas, myxopapillary ependymomas, and ependymoblastomas were excluded. Slides from all paraffin blocks were diagnosed and graded according to the WHO 2007 classification[4] by 2 neuropathologists (P.V. and F.A.). The following histological characteristics were evaluated: ependymal rosettes, perivascular pseudorosettes, number of mitotic figures per 10 high-power fields, cellular density, necrosis, and endothelial proliferation.

After histological review, 9 cases were excluded: 1 atypical teratoid/rhabdoid tumor (reclassified on the base of the loss of nuclear INI1 expression), 2 papillary glioneuronal tumors, and 3 gangliogliomas with ependymoma as the glial component.[13] Three ependymomas were excluded because the residual tissue for complementary IHC studies was insufficient. The remaining 34 SEs were separated into subcategories: classic (n ¼ 27), clear cell (n ¼ 3), papillary (n ¼ 3), and tanycytic (n ¼ 1; Fig. 1A–D). These were compared with a

Table 1. Clinical and IHC characteristics of childhood ependymomas

	Supratentorial tumors (34)	Infratentorial tumors (32)	P value
Median age at diagnosis (yrs)	6.35 (0.2–14.9)	4.6 (0.6–12.6)	.08
Sex	16 M, 18 F	13 M, 18 F	NS
Gross total resection	26 (76%)	22 (69%)	NS
Subtotal resection	8 (23%)	10 (31%)	NS
Radiotherapy/chemotherapy	8 (23%)/12 (35%)	13 (40%)/19 (60%)	NS
Histological grade	4 II, 30 III	5 II, 27 III	NS
NEFL expression	23 (67.6%)	4 (12.5%)	,.001
NeuN expression	14 (41.2%)	8 (25%)	NS
Synaptophysin expression	6 (18%)	3 (9%)	NS
Chromogranin expression	10 (32%)	0	.001
Olig2 expression	22 (64.7%)	29 (90.6%)	.018
Recurrence/progression	19 (56%)	21 (70%)	NS
Progression-free survival (2/5 yrs)	55%/46.5%	45.3%/27.9%	NS
Overall survival (5/10 yrs)	68.3%/54.7%	58%/33.7%	NS
Dead	10 (28.6%)	16 (50%)	NS
Follow-up (yrs)	4 (1–14)	4.2 (0.8–14)	NS

Abbreviations: M, male; F, female; NS, not significant; NEFL, neurofilament light polypeptide 70; NeuN, neuronal nuclei; Olig2, oligodendrocyte transcription factor 2.

group of 32 IEs from posterior fossa, separated as follows: classic (n ¼ 30) and clear cell (n ¼ 2).

Clinical Characteristics of the Population

Relevant clinical and follow-up data were obtained from patients' records or eventually by contacting patients' practitioners. Extent of surgical resection was assessed from the surgeon's report and immediate postoperative contrast-enhanced CT scan or on magnetic resonance imaging (MRI) performed before adjuvant therapy. A recurrence was defined as a new lesion that appeared in situ, after gross total resection. Tumor progression was defined as an enlargement of a residual tumor. Progression-free survival (PFS) was defined as time from first surgery to recurrence or progression. Overall survival (OS) was calculated from the time of first surgery to death or time to last follow-up appointment of surviving patients. Clinical features of children included in supratentorial and infratentorial control groups are shown in Table 1.

These patients were treated according to age groups. After surgery, children younger than 5 years received chemotherapy. In the case of relapse, they were reoperated and received focal radiotherapy (50–55 Gy). Children older than 5 years received postoperative radiotherapy. At relapse, these patients received chemotherapy after a reoperation when feasible. Adjuvant therapy was used for all patients who had an incomplete resection.

IHC Analysis

Four-micrometer sections were deparaffinized and subjected to microwave antigen retrieval for 30 minutes at 988C. After blocking of nonspecific endogenous peroxidase by H_2O_2 and nonspecific antibody-binding sites, sections were incubated with one of the following primary antibodies: MIB-1 (1/10, Zymed), antineuronal nuclei (NeuN) (1/500, clone VMA377, Abcys), anti-neurofilament light polypeptide 70 (NEFL) (1/50, clone 2F11, Dako), antichromogranin A (1/75, clone LK2H10, Immunotech), antisynaptophysin (1/50, clone SY38, Progen), antiglial fibrillary acidic protein (GFAP) (1/200, clone 6F2, Dako), antioligodendrocyte transcription factor 2 (Olig2) (1/25, polyclonal goat antihuman, R&D), antiepithelial membrane antigen (EMA) (clone E29, 1/1, Dako), and anti-INI1 (1/50, clone BAF47, BD Biosciences) for 1 hour at room temperature. The reaction was revealed using the diaminobenzidine chromogen (kit DAB K3468, Dako).

To evaluate neuronal differentiation, we examined the expression of a panel of 4 immunomarkers: NEFL, chromogranin A, synaptophysin, and NeuN, besides the proliferation index MIB-1 and the oligodendroglial lineage marker Olig2.[14,15] Immunostains for GFAP (Fig. 1E), EMA (Fig. 1F), and INI1 were performed in some cases to confirm the diagnosis of ependymoma. For NEFL immunostaining, a semiquantitative analysis was used with a staining score scale: score 0, negative in all blocks containing a viable tumor, including sonic aspiration specimens; score 1, positive in ,5% tumor cells; and score 2, positive in .5% of tumor cells. The MIB-1 proliferation index was scored as a percentage of positive cells (as of most positive areas, total 200 cells counted per area).

Quantitative PCR

For quantitative PCR (qPCR), the following genes involved in neurogenesis/neuronal differentiation were selected based on the literature review: NEFL, T-cell leukemia homeobox 1 (TXL1), LIM homeobox protein 2 (LHX2), forkhead box G1B (FOXG1), neuronal pentraxin receptor (NPTXR), reelin (RLN), tenascin C

(TNC), and NOTCH1. RNA was extracted from 10 SE and 11 IE snap-frozen fresh samples using the QIAGEN Microkit (Qiagen). Approximately 1 mg of total RNA was used to synthesize cDNA using random hexamers and the SuperScript Vilo kit (Invitrogen). qPCR was carried out using Taqman Gene Expression Assays on Demand (Applera) and ABI Prism 7700 Sequence Detector (Applied Biosystems). Expression profile in each specimen was assessed by using the comparative threshold cycle (2^{2ddCt}) method. The TBBP gene was used as an endogenous control and normal whole brain RNA (Stratagene) as a calibrator, as shown previously.[9]

Imaging Analysis

Radiological features were assessed by a pediatric neuroradiologist (N.B.) and 2 neurosurgeons (S.P. and M.P.) who were blinded to the histopathological data and outcome. Preoperative MRI scans were available for all patients.

The following image features were analyzed: location, edema, gadolinium enhancement, and ventricular contact on MRI sequences.

Statistical Analysis

The chi-square test was performed for binomial procedures concerning location, histological features, and radiological presentation. The nonparametric Mann–Whitney rank-sum test was also performed to test the differences between the groups, and the Kaplan–Meier analyses were performed for survival data using the log-rank test. The level of significance was P , .05. Analyses were performed using SPSS 16.0 for windows.

Results

Radiological Features

Tumors were divided into 3 groups according to their imaging features on MRI. The most important radiological group included 18 giant tumors with multiple solid and cystic components extending to more than 1 cerebral lobe. The second group consisted of 11 smaller lesions with a deep cyst and a superficial solid component; 6 were located in the frontal lobe and 5 in the parietal lobe. Seven had no contact with the ventricles. The remaining 5 tumors were located in the midline.

Contrast enhancement was present in all solid components of the tumors regardless of the tumor radiological group. In the second group, thin and often weak contrast enhancement of the margins of the cyst was present in all cases. Peritumoral edema was present in 9 tumors, mostly in the giant tumor group (8 of 9) but also around the cystic component of 1 tumor of the second group. Calcifications were present in 5 of 11 tumors for which CT scans were available. Particular radiological subtypes were not associated with OS and the expression of neuronal markers.

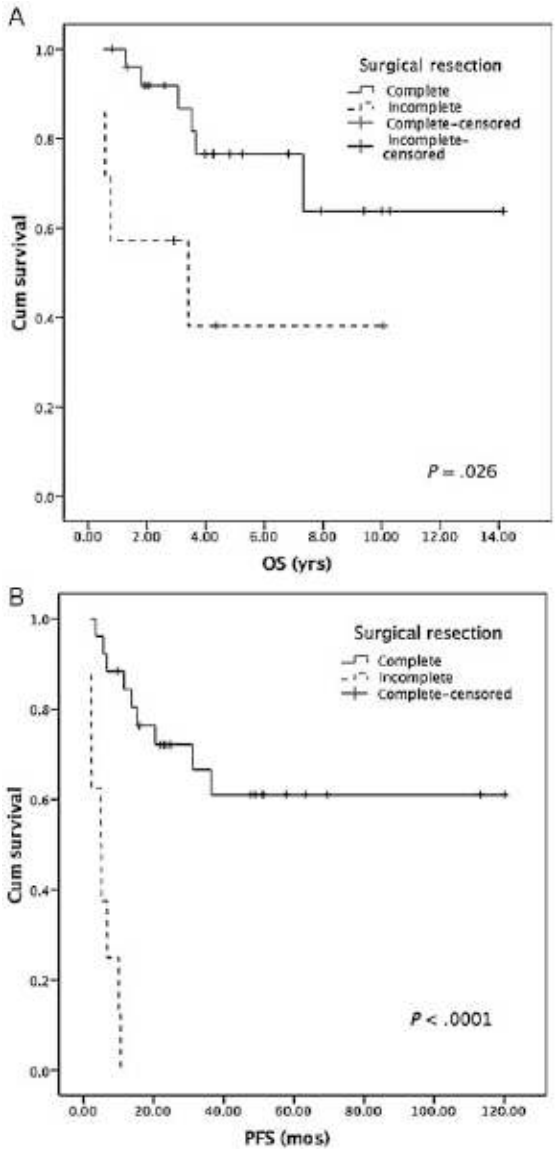

Fig. 2. The Kaplan–Meier survival curves in SE according to surgical treatment. (A) OS and (B) PFS.

Outcome

Results of clinical outcome for patients are shown in Table 1. As in previous reports, the only significant clinical variable for survival in SE was the extent of surgical resection with better OS (P ¼ .026) and PFS (P , .0001; Fig. 2). Tumor grade, location, and patients' age and sex were not significant prognostic factors in SE.

Histopathological and IHC Findings

After review, the majority of both SE (30 of 34) and IE (27 of 32) were classified as grade III (Table 1). The

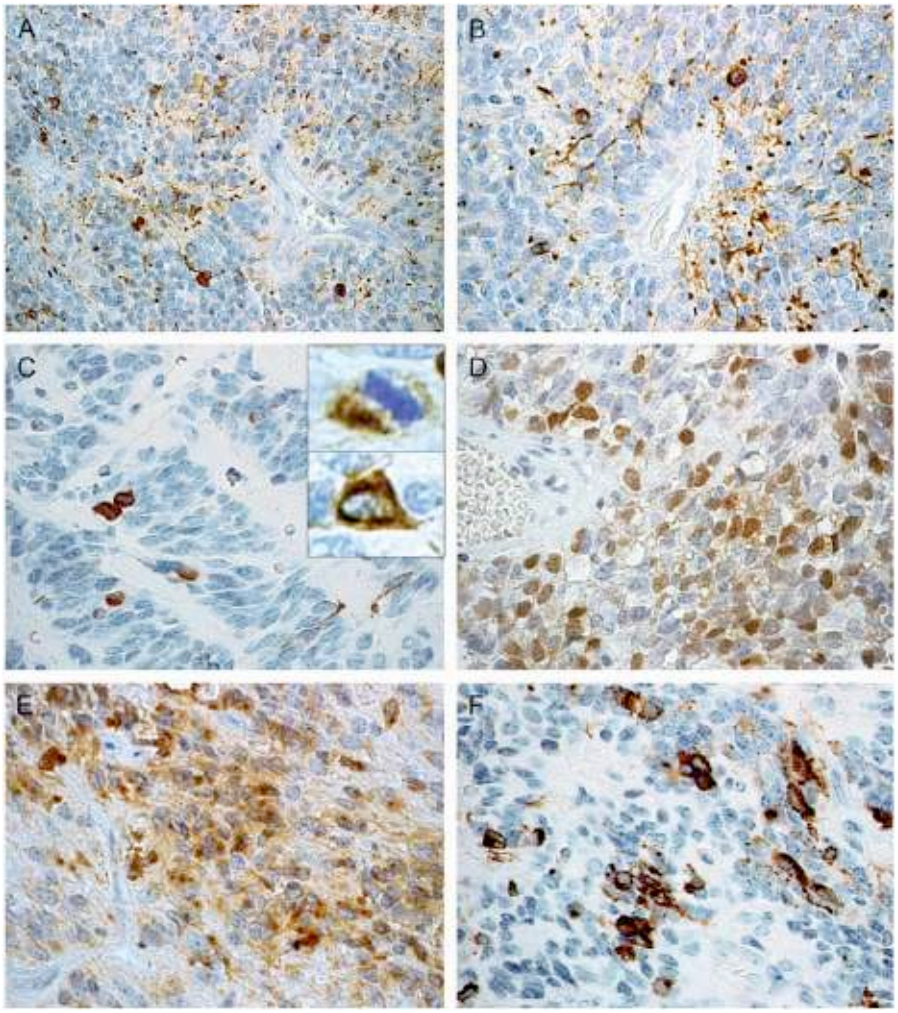

Fig. 3. Immunostaining for neuronal markers in SE. (A and B) High expression of neurofilament light polypeptide (NEFL) defined as more than 5% positive tumor cells, which often showed elongated morphology. (C) Cellular detail of NEFL staining, including a positive cell in mitosis and strong cytoplasmic pattern. (D) Immunostaining for neuronal nuclei (NEUN). (E) Immunostaining for synaptophysin. (F) Immunostaining for chromogranin A. Original magnifications: ×200 (A), ×400 (B), ×600 (C–F).

immunoexpression of NEFL, NeuN, Olig2, chromogranin A, and synaptophysin is reported in Table 1. The expression rates of NEFL and chromogranin A were statistically associated with ST location (P , .0001 for both; chi-square test), whereas the expression of Olig2 was associated with the location in posterior fossa (P ¼ .018, chi-square test). The NEFL score in the supratentorial group was established as low or negative for 24

(score 2) for 10 tumors (Fig. 3, Table 2). Immunoexpression of NEFL was associated with a better PFS at 5 years, 57.8% and 30% for the groups expressing and not expressing NEFL, respectively, but it did not reach statistical significance. Among children with tumors that expressed NEFL, survival correlated with the scoring. Indeed, an NEFL score of 2 was associated with a better OS (P ¼ .10, log-rank test) and was a significant predictor of good PFS (P ¼ .009, log-rank test; Table 2, Fig. 4). There was no association between NEFL expression and the quality of surgical resection, radiological features, or age. We found an association between the positive expression of NEFL and Olig2, chromogranin A, and synaptophysin (chi-square test, P ¼ .038, .008, and .04, respectively). The median MIB-1 index was 10% among IE and 23% among SE (P ¼ .003).

Quantitative PCR

The genes NEFL, LHX2, FOXG1, TLX1, and NPTXR were markedly overexpressed in SE compared with IE, whereas TNC and RELN were overexpressed in IE. NOTCH1 was expressed equally in IE and SE (Fig. 5).

Table 2. Clinical and IHC characteristics of childhood SEs according to NEFL expression

	NEFL High (10)	NEFL Low/negative (24)	P value
Median age at diagnosis (mos)	79.15	61.27	NS
Sex	6 M, 4 F	10 M, 14 F	NS
Gross total resection	9 (90%)	17 (71%)	NS
Subtotal resection	1 (10%)	7 (29%)	NS
Radiotherapy	4 (40%)	4 (17%)	NS
Chemotherapy	2 (20%)	10 (42%)	NS
Progression-free survival (3 yrs)	90%	28%	.007
Overall survival (5 yrs)	90%	60%	.14
NeuN expression	6 (60%)	12 (50%)	NS
Synaptophysin expression	3 (30%)	3 (12%)	.041
Chromogranin expression	6 (60%)	4 (17%)	.008
Olig2 expression	9 (90%)	14 (58%)	.038
MIB-1[a] index (,30%; .30%)	6 (66.7%)/3 (33.3%)	16 (80%)/4 (20%)	NS

Abbreviations: NEFL, neurofilament light polypeptide 70; M, male; F, female; NS, not significant; NeuN, neuronal nuclei; Olig2, oligodendrocyte transcription factor 2.
[a] The MIB-1 proliferation index was performed for 29 of 34 patients.

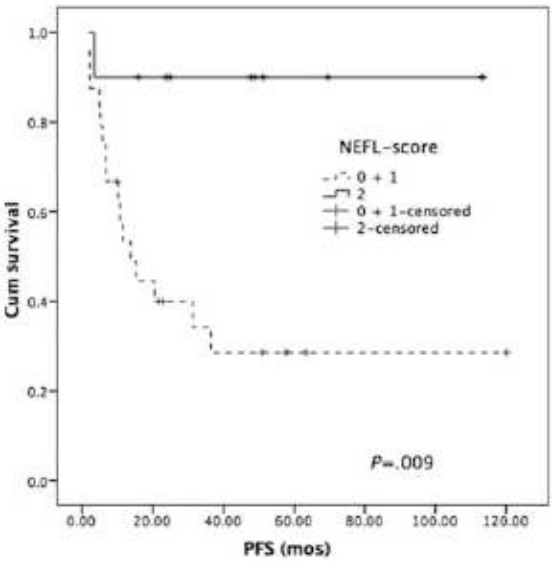

Fig. 4. PFS in months for SE, according to neurofilament light polypeptide (NEFL) immunostaining—strong vs weak/no staining.

Discussion

Our study shows that childhood SEs often exhibit neuronal differentiation in the form of immunoexpression of neuronal markers such as NEFL and chromogranin. Except for 1 recent study, which reported 6 SE and IE in children with immunophenotypic neuronal differentiation,[16] little is known about neuronal differentiation in ependymomas, and to the best of our knowledge no previous data from a large pediatric cohort are actually available. Neuronal differentiation within typical ependymoma implies that such tumors may histogenetically originate from a glioneuronal progenitor rather than from a committed glial progenitor.[12,17] Interestingly, tumors showing definite morphological features of neuronal differentiation have been reported and support the existence of a neuronal differentiation spectrum in ependymal tumors.[16,18,19] We excluded from this series 3 published cases of gangliogliomas with ependymal differentiation, which we consider to be a different entity. They exhibit benign behavior and display important perivascular inflammation, granular bodies, and often binucleated ganglion cells.[13] However, it could be hypothesized that these may represent terminal differentiation of neuronal precursors in ependymomas, which is an established phenomenon in medulloblastomas and other primitive neuroectodermal tumors.[20,21]

Expression of NEFL and chromogranin was strongly correlated with supratentorial location, which supports the suggestion that SE and IE are different entities, in view that there are molecular differences between ependymomas according to the location.[8,9,22] Although this hypothesis is based on a limited number of SE, our studies support this idea CGH array showing a gain of 9q33-34 is significantly more frequent in IE than in SE.[10]

Strong expression of NEFL in SE was significantly associated with a better PFS. Classically, the infratentorial location is associated with a worse outcome in most,[6,23,24] but not all, studies.[25] The predominance of neuronal markers in SE, particularly NEFL, may be related in some as yet undefined manner to the different behavior of SE and IE, as it has been shown that a proneural molecular signature is associated with better prognosis in high-grade gliomas.[26,27]

In our series, RELN was significantly overexpressed in IE compared with SE, confirming earlier studies.[8] RELN is implicated in cell-fate decision as it can induce a radial glial cell phenotype in neural stem cell progenitors via the activation of NOTCH1.[28] Similar to RELN, TNC is an extracellular matrix protein also

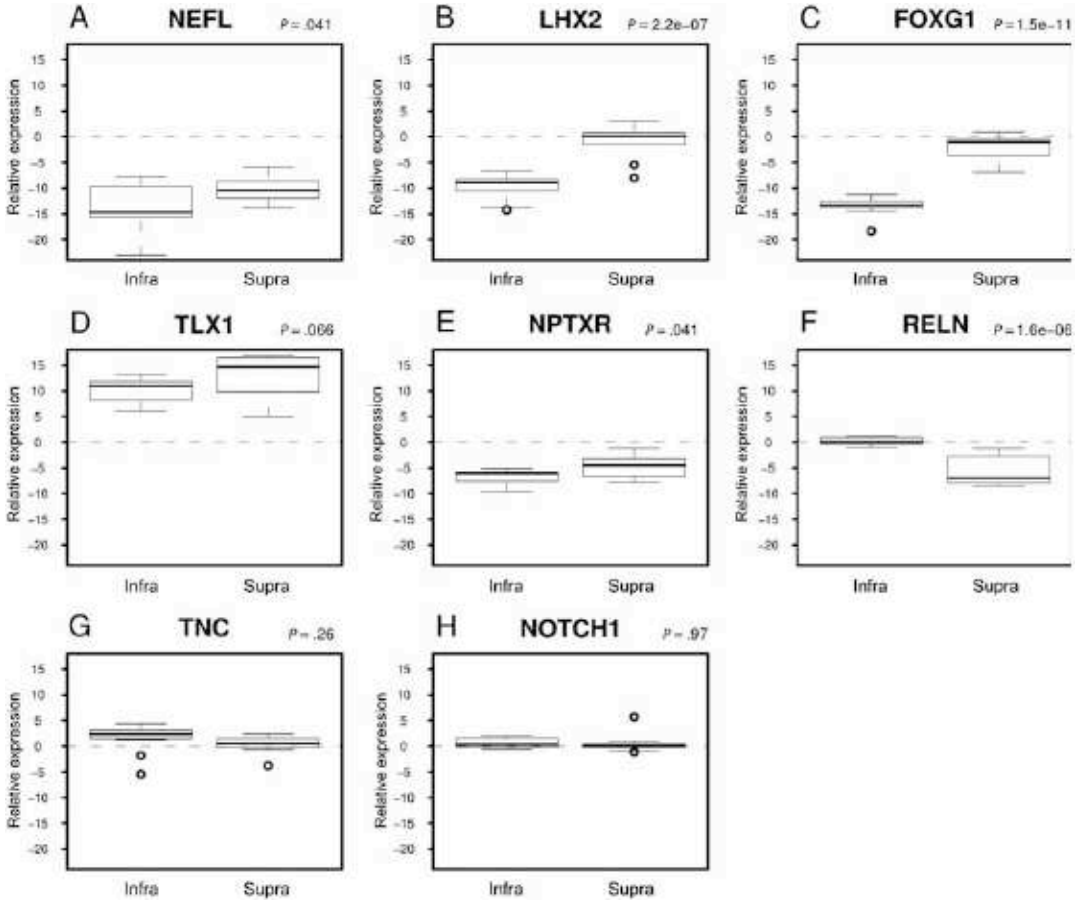

Fig. 5. qPCR confirms the overexpression of neuronal markers in SE (supra, n ¼ 10) when compared with IE (infra, n ¼ 11). (A) NEFL. (B) Lim homeobox protein 2 (LHX2). (C) Forkhead box G1B (FOXG1). (D) T-cell leukemia homeobox 1 (TXL1). (E) Neuronal pentraxin receptor (NPTXR). (F) Reelin (RLN). (G) Tenascin C (TNC). (H) NOTCH1. Normal brain was used as a reference.

implicated in radial glial cell phenotype and which has recently been described as a target gene for NOTCH in gliomas.[29] Moreover, TNC is located on the same chromosomal region as NOTCH1.[10] The TNC mRNA levels were higher in IE compared with SE, and both were higher than those in normal brain. However, NOTCH1 was not differentially expressed by SE and IE, according to the previous reports from our group.[10] This suggests that NOTCH1 expression is driven differently according to the location of the ependymoma.

TLX1 was overexpressed in SE compared with IE. TLX1 participates in early differentiation of the mammalian nervous system and sensory ganglia[30] and is a neural oncogene; some alternative transcripts of this gene have been implicated in the genesis of pediatric neural tumors such as neuroblastoma and primitive neuroectodermal tumor of the CNS.[31] The nuclear receptor TLX has also been implicated in neural stem cell self-renewal.[32] LHX2 was significantly overexpressed in SE compared with IE, and as previously suggested might be related to tumor location.[33] LHX2 is a transcriptional factor involved in brain development/neural stem cell differentiation and patterning of early telencephalon, which is overexpressed in both SE and supratentorial pilocytic astrocytoma. FOXG1 is involved in neurogenesis in the retina and the maintenance of neural stem cell phenotype, patterns early telencephalon,[34] and was also significantly overexpressed in SE when compared with IE. NPTXR is another gene of neuronal differentiation we found to be overexpressed in SE, which codes for an integral membrane protein, a neuronal synaptic receptor with higher expression in Purkinje and granule neurons of the cerebellum, also present in the hippocampus and cerebral cortex.[35]

Taken together our data and the literature show that SE and IE have different genomic, gene expression, and IHC signatures. Different oncogenic pathways may be involved depending on the location, driving to a neuronal phenotype in SE. The better prognosis of children with SEs may be partly related to their neuronal differentiation.

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Conflict of interest statement. None declared.

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Introduction to second article

Biomarkers for prognosis have not been validated in pediatric ependymomas. To the present most reports have described single center cohorts of patients treated over long periods of time and with different therapeutic strategies. The SIOP Ependymoma Biology Working Group has started to explore potential biomarkers in trial cohorts and designed a strategy for their cross-validation.

1. Confection of tissue microarray paraffin blocks from a French cohort

Our work showing that neuronal differentiation identifies a subgroup of patients with better prognosis in supratentorial childhood ependymoma (Andreiuolo et al., 2010) confirmed the possibility of finding histopathological markers of prognosis and hopefully prediction of response to treatment in this disease.

However one of the limitations of our previous study was the relatively small number of cases in the cohort studied (66 ependymomas of which 34 supratentorial), which did not allow for a robust statistical analysis along with other risk factors. In order to overcome this limitation we collected material from a larger number of patients, with well-documented clinical parameters. In collaboration with the team of neurosurgeons at Necker Hospital under the direction of Prof Christian Sainte-Rose and of Dr Pascale Varlet, neuropathologist at Sainte-Anne Hospital and in charge of the histological diagnoses, we were able to review the series of ependymoma patients treated with the BBSFOP protocol at IGR. Further cases treated under the same protocol from various centers in France were identified, and with the remarkable collaboration from neuro-oncologists and pathologists nationwide, a number of additional cases could be reviewed.

For each patient, all paraffin blocks and corresponding slides were obtained and reviewed by two neuropathologists (PV, FA) for diagnostic accuracy and tissue adequacy. Ependymomas were graded based on WHO 2007 criteria. Histopathological findings (ependymal differentiation, necrosis, endothelial proliferation, mitotic index and anaplasia) were evaluated and recorded for each tumor. Immunostains for EMA, GFAP, OLIG2 and Neurofilament Protein 70 were performed for selected cases.

Four tissue micro-array (TMA) blocks were built from paraffin-embedded material from a total of 112 patients with pediatric ependymoma at diagnosis and also included tumor material at diagnosis and at recurrence. Sonic aspirator extracts were excluded. A total of 153 tumors were represented, of which 112 at diagnosis and 41 at recurrence, comprising 17 patients with only one recurrence (n=17), 9 patients with two recurrences (n=18) and 2 patients with 3 recurrences (n=6). Three to four 600µM-cores were obtained from each tumor. Representative areas were selected whenever present: classical ependymal

differentiation (ependymal rosettes, perivascular pseudo-rosettes, and ependymal canals), anaplasia and high vascularisation zones. Normal adult and fetal brain samples were included as controls.

Distribution of pediatric ependymoma on TMA blocks from the French cohort:

Localization	Block	Tumors	Patients	cores/tumor
Infratentorial (posterior fossa)	PARIS 1	55	38	3
	PARIS 2	20	11	3
	PROVINCE	40	28	4
supratentorial	PROVINCE	8	6	4
	PARIS	30	29	4
total		153	112	

2. Establishing a European collaboration project: Study Design

With the support of the ependymoma SIOP Ependymoma Biology Working Group, we further proposed an international collaboration project between groups from different countries, with the aim of developing and validating prognostic markers in pediatric ependymoma, on tissue microarray blocks from national trial cohorts, with well documented clinical history, treatment and follow-up. Groups from three different countries initially joined this project, United Kingdom with Professor Richard Grundy (Nottingham University), Italy with Dr Maura Massimino (Istituto di Tumori, Milan) and France with Dr Jacques Grill (IGR, Villejuif). We were later joined by two German groups, the GPOH with Prof Torsten Pietsch (University of Bonn) and the Heidelberg group with Pr Stefan Pfister (DKFZ, Heidelberg).

A three-step study was conducted by this European consortium. First potential prognostic markers (YAP1, nucleolin/telomerase, metallothioneins 1-2 and 3, ASPM, TNC) were initially tested by immunohistochemistry and 1q25 by FISH in tumors from patients treated in three countries (United Kingdom n=105, Italy n=62, France n=93). In step 2, the most promising markers tested on a national scale were then assessed on tissue microarray slides for the entire three cohorts. Prognostic value of markers for OS and PFS were assessed through a Cox model stratified on country, testing simultaneously localization, grade, extent of surgery and treatment. In step 3 immunohistochemical techniques and analyses were blindly cross-validated in different labs. Finally, the prognostic model underwent an external validation procedure in two independent cohorts from the German Society of Pediatric Oncology and Hematology.

3. Markers Tested

A. YAP1

The Italian group proposed IHC for the proto-oncogene *YAP1*, highly expressed in stem cells and which among other effects activates NOTCH pathways (Camargo et al., 2007). The *YAP1* gene is located in the 11q22 region, and was previously found to be overexpressed in ependymoma from an almost exclusively pediatric cohort, and was amplified in one sample (Modena et al., 2006). Unfortunately this marker was difficult to analyze, score and to correlate with outcome due to the variety of patterns observed (five different patterns). Thus YAP1 was not further studied in the cohorts from UK and France.

B. 1q25 and nucleolin

For step one the group from the UK proposed nucleolin (IHC) and 1q25 (FISH). Despite previous results in single center cohorts (Ridley et al., 2008) nucleolin was not prognostic on the UK multicenter trial cohort and therefore the analysis was not extended to Italian and French cohorts. Initially, hTERT expression was assessed with IHC using the NCL-L-hTERT antibody and proved to be associated with worse prognosis (Tabori et al., 2006). Later, the use of this antibody against hTERT was questioned due to the intensity of its immunoabsorption with the nucleolin peptide. When Ridley et al found that low nucleolin expression (antibody clone 4E2 from Abcam) was independently associated with a more favorable outcome, they discarded the prognostic impact of other biomarkers such as hTERT (antibody clone 44F12 from Novocastra), ERBB2 (NCL-CB11), ERBB4 (sc- from Santa Cruz), survivin (sc-10811 from Santa Cruz) and EGFR (PharmDX from Dako) (Ridley et al., 2008).

Fish for 1q 25 showed promising results in the UK cases and the analysis was further extended to the Italian and French cohorts, confirming that 1q25 gain significantly predicts shorter time for disease progression (Kilday et al., 2012), paper presented in the Appendix 1. 2.

C. Metallothioneins 1-2 and 3, ASPM

Our group recently analyzed by CGHarray and dual-color gene expression microarray 17 pediatric ependymoma at diagnosis co-hybridized with corresponding 27 first or subsequent relapses from the same patient (Peyre et al., 2010), Appendix number 1. As treatment and location had only limited influence on specific gene expression changes at relapse, we established a common signature for relapse. Eighty-seven genes showed an absolute fold change ≥2 in at least 50% of relapses and were defined as the gene expression signature of

ependymoma recurrence. The most frequently upregulated genes are involved in the kinetochore (ASPM, KIF11) or in neural development (CD133, Wnt and NOTCH pathways). Metallothionein (MT) genes including isoforms 1, 2 and 3 were downregulated in up to 80% of the recurrences. Quantitative PCR for ASPM, KIF11 and MT3 plus immunohistochemistry for ASPM and MT3 confirmed the microarray results. Immunohistochemistry on an independent series of 24 tumor pairs at diagnosis and at relapse confirmed the decrease of MT3 expression at recurrence in 17/24 tumor pairs ($p = 0.002$). Conversely, ASPM expression was more frequently positive at relapse (87.5% vs 37.5%, $p = 0.03$).
Due to their implication in mechanisms of ependymoma recurrence (MTs 1-2 and 3, downregulated, ASPM upregulated), we hypothesized that these markers could be also related to prognosis in this disease.

D. Methallothioneins

MTs are a family of 4 similar small proteins clustered in chromosome 16q13. These proteins contain 61-68 amino acids with 20 cystein residues in conserved positions that allow binding with Cu2+ and Zn2+ which is important in physiological homeostasis phenomena and binding Cd2+ and Hg2+, which seems mainly implicated in pathological detoxification. MTs are ubiquitously expressed in most cells and tissues (Pedersen et al., 2009).
MTs 1 and 2 are the major isoforms expressed in most tissues and are believed to protect cells from damage caused by oxidative stress Mt1-2 are overexpressed in several types of human cancer, in some related to poor prognosis: melanoma, pancreatic carcinoma, breast carcinoma, and are downregulated in gastric, colorectal, hepatocellular, thyroid carcinoma (Pedersen et al., 2009). In one study, among low-grade ependymomas. MT1-2 were significantly more frequently negative in those that recurred (Korshunov et al., 1999).
MT3 was originally described exclusively in the normal brain as GIF (growth inhibitory factor), and later identified in non-neoplastic kidney, and in several types of cancer, such as kidney, breast, pancreas, intestine, bladder and prostate (Garrett et al., 1999a; Sens et al., 2001). The expression of MT3 during development seems to be associated with regulation of normal neuronal differentiation; its overexpression indicates poor prognosis in bladder and breast carcinoma (Sens et al., 2001; Zhou et al., 2006). It has been shown to be downregulated in oesophageal squamous cell carcinoma and gastric carcinoma (Deng et al., 2003; Smith et al., 2005).

E. Abnormal Spindle-Like Microcephaly-Associated (ASPM)

ASPM is a centrosomal/spindle pole protein containing 3477 aminoacids, and its gene located in 1q31. During embryonic development ASPM is expressed in the primitive neuroepithelium of the brain, where it is important for symmetric proliferative divisions (Fish

et al., 2006). Mutations of *ASPM* gene are the main cause of primary autosomal recessive microcephaly (Bond et al., 2002). Although very low levels of this protein are present in adult normal brain it is expressed in other tissues: breast, lung, pancreas, uterus, colon, ovary, testis and overexpressed in cancer of the ovary and uterus and in medulloblastoma (Kouprina et al., 2005; Vulcani-Freitas et al., 2011). In primary cell cultures from patients with ovarian carcinoma higher expression of ASPM was found in higher-grade lesions and was not associated with mitotic index (Bruning-Richardson et al., 2011). High levels of ASPM measured by PCR were associated with vascular invasion, early recurrence, and poor prognosis in hepatocellular carcinoma (Lin et al., 2008) and worse survival in adult glioblastoma (Horvath et al., 2006). In adult glioma, ASPM is overexpressed in higher grade and in relapsed lesions (Bikeye et al., 2010; Hagemann et al., 2008).

4. Immunohistochemistry

Anti ASPM affinity purified rabbit polyclonal antibodies were purchased from Bethyl Laboratories Inc (Montgomery, Texas) (reference IHC-00058). Anti MT1-2 mouse monoclonal antibodies were obtained from Zymed (clone E9, reference 18-0133), their epitope is raised against the last 5 to 7 aminoacids of the N-terminus of the B domain, and recognizes both MT1 and MT2 but not MT3. Anti Metallothionein 3 (MT3) affinity purified rabbit polyclonal antibodies were a generous gift from Dr Donald Sens; their preparation and use on formalin-fixed, paraffin embedded material have been described previously (Garrett et al., 1999b).

Paraffin sections were cut at 4µm, deparaffinised, exposed to 30 minute treatment in a steamer at 98° C in citrate pH 7.3 buffers for ASPM and MT1-2and pH 6.0 for MT3 and then treated with a peroxidase blocking agent (reference S2001, DAKO). Antibody incubation was performed overnight at 4°C for ASPM (1:100) and 60 minutes hour at room temperature for MT1-2 (1:300) and MT3 (1:1000). Antibody binding was visualized with the peroxidase-based anti-rabbit EnVision Kit (reference K4003, DAKO) for MT3 and ASPM antibodies and with Histostain Plus Kit (reference 85-9043 Zymed) for MT1-2. Diaminobenzidine tetra hydrochloride (DAB, DAKO) was used as the chromogen. Sections were counterstained with Mayer's hematoxylin. Ki67 immunostains were also performed using mouse anti-human antibodies (clone MIB1, DAKO ref M7240) in the automated Benchmark (Ventana) system after antigen retrieval carried in acid pH.

Immunohistochemical staining for MT1-2 and MT3 was scored semiquantitatively, based on staining intensity and cell number, as follows: 0, no staining; 1, weak staining (independently of the number of positive cells) or staining in less than 10% of cells (independently of the staining intensity); 2, moderate to strong staining in more than 10% of cells. Scoring was performed as of observed in the most positive areas. MTs generally

stained both nucleus and cytoplasm (figure1). Staining for ASPM was analyzed at high power view (x1000), and scoring was performed as follows: 0, no staining; 1, staining in scarce cells, 2, staining in numerous cells. Following staining patterns were observed for ASPM: cytoplasmic, nuclear, presence of paranuclear “dots” or marked cells in mitosis. Ki67 stainings were evaluated at 200 cells in most positive areas, as a percentage of positive cells/ all cells analyzed.

Figure 1: MT1-2 IHC in ependymoma A) Tumor cells were negative, blood vessels showed cytoplasmic and nuclear staining in some cases B) Mostly nuclear and weak staining C) and D) Strong nuclear and cytoplasmic staining

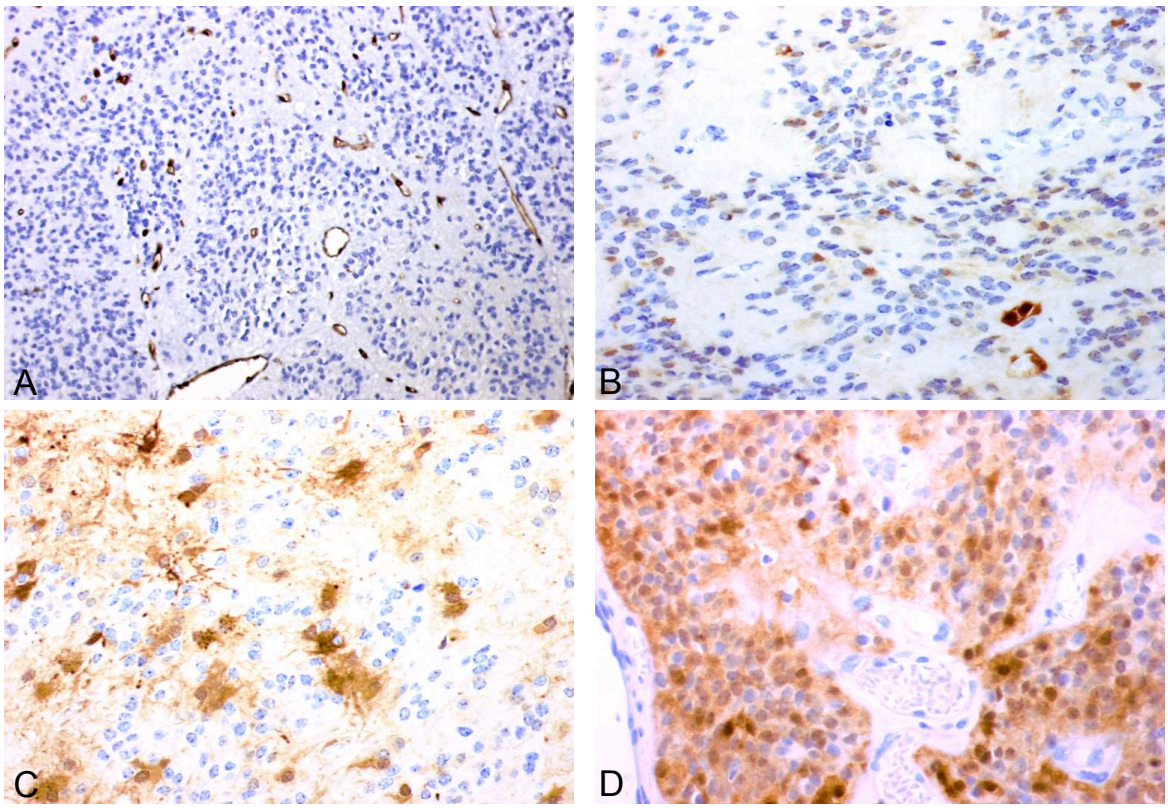


Figure 2: MT3 staining in ependymoma

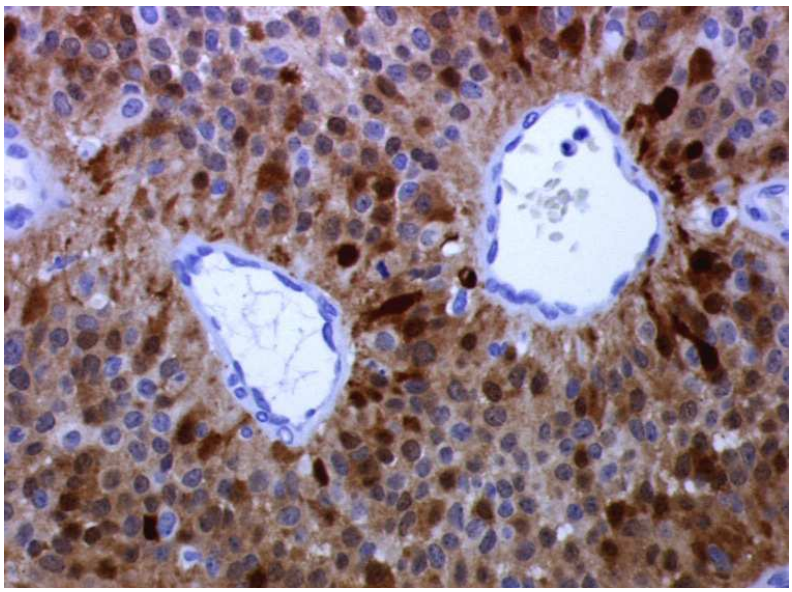
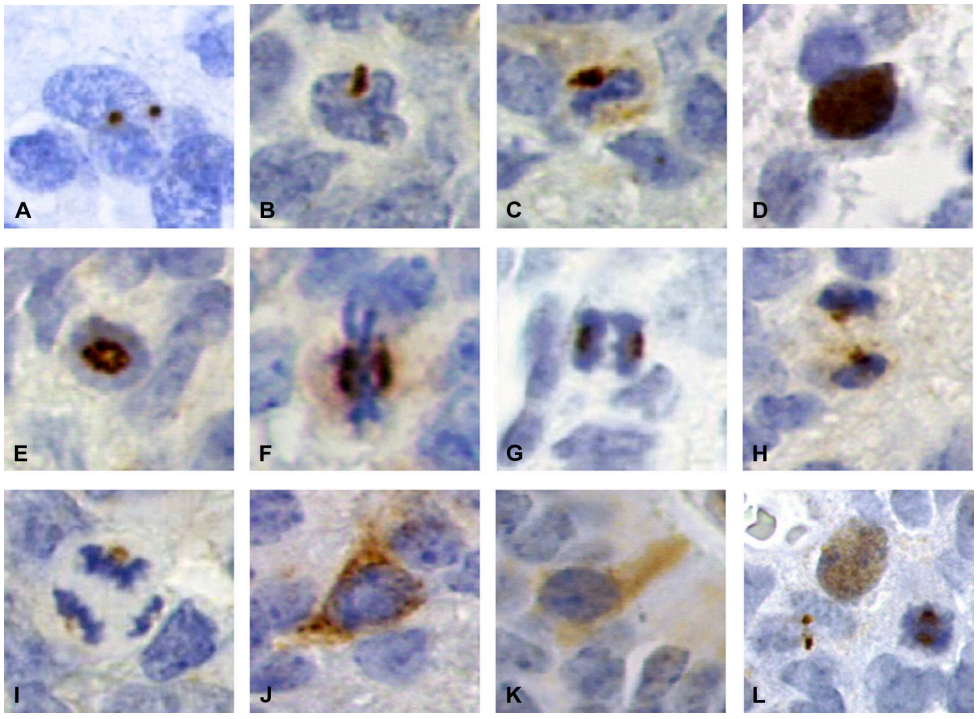


Figure 3: ASPM staining patterns in ependymoma: ASPM is detected in the mitotic spindle in every phase of the mitosis, as well as in the cytoplasm of cells not in mitosis.



The following markers were tested first in the patients of the French cohort: TNC, Nucleolin, MT3, MT1/2, Ki67 and ASPM.

The table below describes the results of these markers for overall survival:

Results	Numbers	2y-survival	5y-survival	HR	p value
TNC negative	22	95%	88%	1	p=0.03
TNC weak	26	84%	62%	2.77	
TNC strong	54	85%	47%	4.16	
Nucleolin weak	4	100%	38%	1	p=0.68
Nucleolin moderate	17	100%	43%	1.40	
Nucleolin strong	81	84%	65%	0.99	
MT1/2 negative	16	86%	60%	1	p=0.58
MT1/2 weak	54	85%	68%	0.85	
MT1/2 strong	32	91%	44%	1.23	
ASPM negative	57	83%	73%	1	p=0.44
ASPM positive	45	93%	46%	1.28	
Ki67 <10%	39	91%	68%	1	p=0.11
Ki67 ≥ 10%	62	86%	56%	1.73	
MT3 negative	13	92%	84%	1	p=0.22
MT3 weak	47	86%	55%	2.53	
MT3 strong	42	87%	55%	2.39	

In the French cohort, as seen for overall survival, TNC was also the only marker significantly associated with a worse event free survival (Figure 5). The initial cohort analyzed in the following figures is slightly larger than the one in the final article on TNC because for the multivariate analysis in the there were some data missing for some of these patients.

Figure 4: TNC and Overall survival in the French cohort (p=0.03)

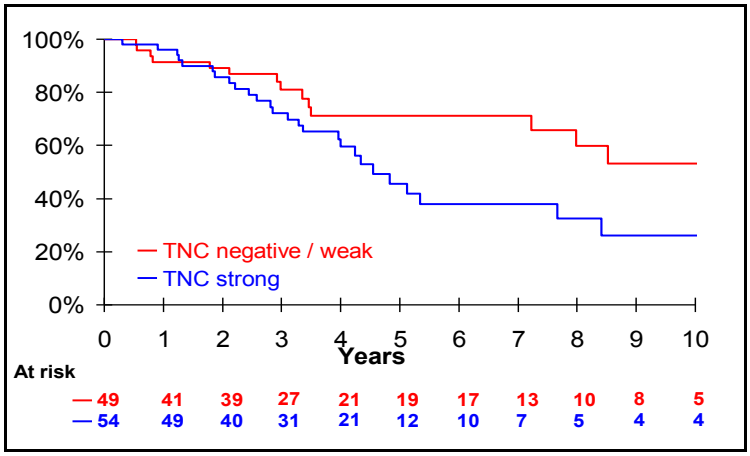
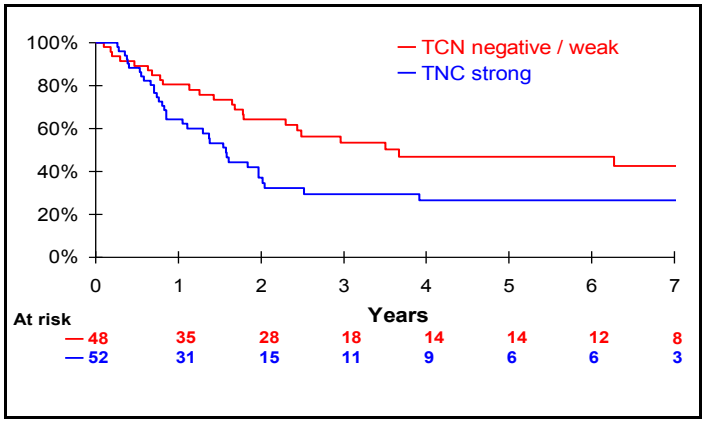


Figure 5: TNC and Event-Free Survival in the French cohort (p=0.03)

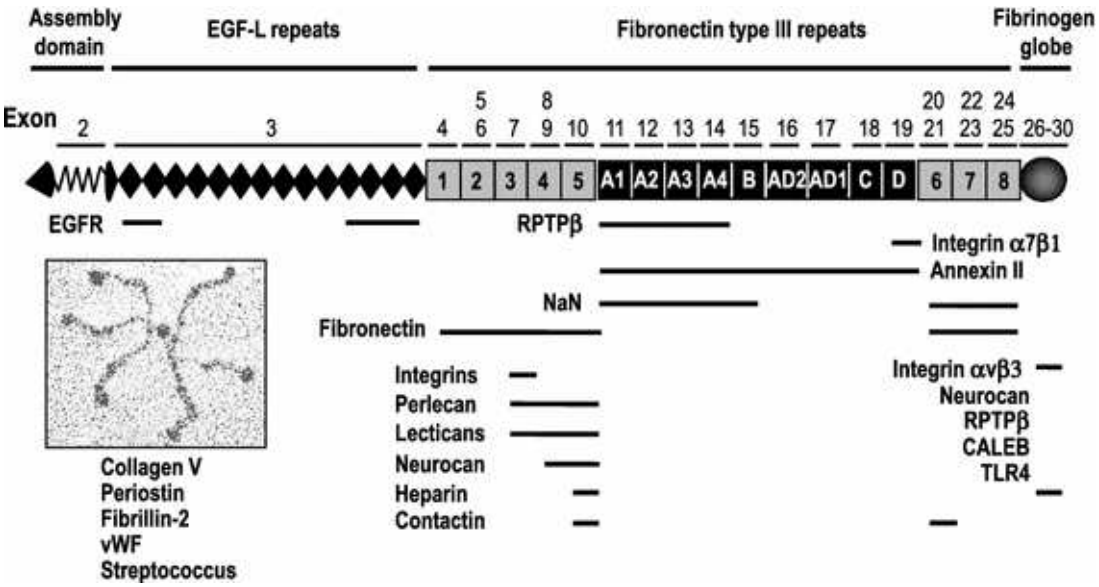


5. TNC

A previous study from our group has reported frequent gains at 9q33, the genomic region of NOTCH1 and tenascin-c (TNC), and the overexpression of TNC in pediatric ependymoma. (Puget et al., 2009).

TNC is a large hexameric extracellular glycoprotein, with little or no expression detected in healthy adult tissues. It is transiently re-expressed upon tissue injury and down-regulated after tissue repair is complete. TNC is a multimodular protein presenting four distinct domains: an assembly domain, a series of epidermal growth factor-like repeats, a series of fibronectin type III-like repeats, some of which can be alternatively spliced, and a C-terminal fibrinogen-like globe (Figure 6). Each of these domains can interact with a different subset of binding partners, including cell surface receptors and other extracellular components (Midwood et al., 2011).

Figure 6: Tenascin C structure and main binding partners (From Midwood et al., 2011)



TNC is involved in the generation of neural stem-cell niches, modulates matrix-cell interactions and in several types of cancer has been associated with increased vascularity, decreased survival and short time to relapse (Orend and Chiquet-Ehrismann, 2006). It has been recently shown to be a direct NOTCH target in gliomas. Evidence also supports its key role in the maintenance of a metastatic "niche" that would allow for the survival of disseminated tumor cells by activating NOTCH and WNT pathways (Oskarsson et al., 2011).
Although theoretically 512 different human spliced variants of TNC (with different combinations of products from exons 11 to 19) could be produced, and depend on several factors including stage of development, tissue type, cell type, cell line, extracellular pH, stage of cell cycle, growth factors and pathological conditions, isoforms seem to be less numerous and relatively tissue-specific (Guttery et al., 2010b). Higher molecular weight isoforms are overexpressed in different types of cancer such as breast carcinoma, gliomas, gastrointestinal, bladder and lung among others (Guttery et al., 2010b). The 14.16 isoform is known to be associated with invasive breast cancer and an isoform containing repeat C (exon 15) was described in human glioblastoma and not found in one case of ependymoma (Carnemolla et al., 1999; Guttery et al., 2010a).

In ependymomas, specifically, TNC expression by IHC has been shown previously in smaller retrospective series to be associated with higher grade (Korshunov et al., 2000) and poorer prognosis (Korshunov et al., 2000; Zamecnik et al., 2004). In a recent report in which two prognostic groups of posterior fossa ependymoma were identified, the group with poor prognosis was more frequently positive for TNC (94% vs. 11%, $p<0.0001$) (Witt et al., 2011).

6. Introduction to the article on TNC in pediatric ependymoma

The article that follows describes our findings concerning TNC expression in pediatric ependymoma in the context of the collaboration study described above.
As TNC positivity on IHC showed an impact on prognosis in the French cohort it was further studied in the patients from the UK and Italy (total of 250 patients in the three cohorts together). Prognostic value of markers for OS and PFS were assessed through a Cox model stratified on country, testing simultaneously localization, grade, extent of surgery and treatment.

Median age of the population was 34 months. Median follow-up was 7.8 years. Factors associated with a higher risk of death were strong expression of TNC (HR=1.77 [1.20-2.62], p=0.004), incomplete resection (HR=1.77 [1.20-2.62], p=0.004) and higher grade (HR=1.81 [1.18-2.78], p=0.007). Factors associated with a higher risk of recurrence were incomplete resection (HR=1.91 [1.35-2.70], $p<0.001$) and strong TNC expression in children below the age of three (HR=2.00 [1.24-3.23], p=0.005). Positivity for TNC immunostaining was associated with location, 70% of patients with infratentorial tumors showed strong

expression compared to 18% of those with supratentorial tumors ($p<0.001$). The technique for TNC immunostaining and the scoring system developed were reliably reproducible when performed by different labs.

The prognostic model defined in the first three cohorts was further tested in two other independent cohorts from Germany, one from the GPOH (courtesy of Torsten Pietsch, Stefan Rutkowski and André van Buren) and one from Heidelberg previously described and published in (Witt et al., 2011). The former series had a relatively short follow-up, few events and thought the model was still valid in this cohort; it did not reach statistical significance, except for EFS in the youngest children. This finding further emphasizes the impact of TNC immunopositivity for survival in the youngest children. In the latter series, the impact of TNC immunopositivity was even more important.

The mRNA expression of different TNC isoforms was correlated with the expression of total TNC, except for the excess of A2 and 14.16 variants, showing that TNC might be involved in invasiveness in this disease.

In conclusion, the expression of TNC is strongly and independently associated with a higher risk of death in pediatric ependymoma. Its expression easily detected and scored by immunohistochemistry, could be used to stratify patients in clinical trials and will be tested prospectively in the next generation of ependymoma SIOP trials.

Article submitted

Tenascin-C is an independent prognostic marker in pediatric intracranial ependymomas

Felipe Andreiuolo[1,12], Audrey Mauguen [2], John-Paul Kilday [3], Piergiorgio Modena [4], Torsten Pietsch [5], André O. von Bueren [6], Hendrik Witt [7], Andrey Korshunov [8], Stefan Pfister [7], Caroline Domerg[2], Carmela Dantas-Barbosa [1], David S. Guttery[9], Jacqueline A. Shaw [9], Lindsay Primrose[9], Pascale Varlet [10], Felice Giangaspero [11], Leila Chimelli [12], Stefan Rutkowski [6], Didier Frappaz [13], Maura Massimino [14], Richard G. Grundy [3], Jacques Grill [1,15]on behalf of the SIOP Ependymoma Biology Working Group.

[1] Université Paris-Sud, Institut Gustave Roussy, CNRS UMR 8203 "Vectorologie et Thérapeutiques Anticancéreuses", Villejuif, France.
[2] Département de Biostatistiques et d'Epidemiologie, Institut Gustave Roussy, Villejuif, France.
[3]The Children's Brain Tumor Research Centre, University of Nottingham, United Kingdom.
[4]Centro di Riferimento Oncologico, National Cancer Institute, Aviano, Italia.
[5]Institute of Neuropathology, University of Bonn Medical Center, Germany.
[6] Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Germany.
[7] Division of Molecular Genetics, German Cancer Research Center (DKFZ) and Department of Pediatric Oncology, Heidelberg University Hospital, Heidelberg, Germany.
[8]Clinical Cooperation Unit Neuropathology, German Cancer Research Center (DKFZ), Heidelberg, Germany.
[9]Department of Cancer Studies and Molecular Medicine, University of Leicester, United Kingdom.
[10]Département de Neuropathologie, Hôpital Sainte-Anne, Paris, France.
[11] Department of Anatomic Pathology, La Sapienza University, Roma, and IRCCS Neuromed, Pozzilli, (Is), Italy.
[12]Department of Pathology, University Hospital, Federal University of Rio de Janeiro, Brazil.
[13]Institut d'Hématologie-Oncologie Pédiatrique, Lyon, France
[14] Pediatric Unit, Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico, Istituto Nazionale Tumori, Milano, Italy.
[15] Département de Cancérologie de l'Enfant et de l'Adolescent, Institut Gustave Roussy, Villejuif, France.

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Corresponding Author
Jacques Grill, MD-PhD
Département de Cancérologie de l'Enfant et de l'Adolescent
Institut Gustave Roussy - 114, rue Edouard Vaillant - 94805 VILLEJUIF, France
TEL: +33142116209
FAX: +33142115275
email: grill@igr.fr

Running Head: Tenascin-C in Ependymomas

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ABSTRACT

PURPOSE: Despite multimodal therapy, mortality rates in pediatric intracranial ependymomas remain as high as 45%. Apart from the extent of resection, prognostic markers have not been validated in children. Histopathological grading has been controversial with respect to its reproducibility and clinical significance.

PATIENTS AND METHODS: Potential prognostic markers identified nationally and in the literature (metallothionein1/2 and 3, YAP1, nucleolin, ASPM, Ki67 and tenascin-C) were tested and validated internally on the TMA from 3 independent trial cohorts (UK: n=105, FRA: n=93, I: n=62). External validation was performed on two independent cohorts (GPOH: n=137 and Heidelberg: n=196).

RESULTS: Factors associated with a higher risk of death were strong expression of TNC (HR=1.77 [95% CI: 1.20-2.62], p=0.004), incomplete resection (HR=1.77 [95% CI : 1.20-2.52], p=0.004) and higher grade (HR=1.81 [95% CI : 1.18-2.78], p=0.007). Factors associated with a higher risk of recurrence were incomplete resection (HR=1.91 [95% CI: 1.35-2.70], p<0.001) and strong tenascin-C expression in children below the age of three (HR=2.00 [1.24-3.23], p=0.005). TNC staining was associated with location, 70% of patients with infratentorial tumours had a strong expression compared to 18% of those with supratentorial tumours (p<0.001). The mRNA expression of different TNC isoforms was correlated with the expression of total TNC, except for the excess of A2 and 14.16 variants.

CONCLUSION: Tenascin-C is a robust prognostic biomarker of pediatric intracranial ependymoma that could be used to stratify therapy.

INTRODUCTION

Ependymoma is the third most common brain tumor in children. Many cases are diagnosed before the age of 5, frequently in the posterior fossa. This disease comprises several entities, each with its own molecular pathogenesis strongly influenced by age and location (1-6). Conversely, these tumors share common biological and phenotypic characteristics, e.g. Notch-1 pathway activation (5) or cell of origin (4). Ependymomas represent currently a considerable therapeutic challenge, being incurable in almost half of the cases despite multimodal therapy. Some children can be cured without recourse to radiation therapy (7-9) while others will experience recurrence despite the use of optimal radiation therapy (10). The extent of resection has been regularly found as the most important prognostic factor (7,10). Several prognostic markers have been identified in single reports but none of them was validated for treatment stratification. Grading according to the current WHO classification proved to be controversial (11).

A three-step study was therefore conducted by a European consortium of British, Italian, French and German national groups. Biomarkers identified in one of the national cohorts (step one) were validated subsequently in two other national cohorts (step two). For this validated biomarker, reproducibility of staining and scoring was tested (step three). Finally, external validation was obtained in two independent cohorts (one from the German Society of Pediatric Oncology and Hematology (GPOH) and one from the German Cancer Research Center in Heidelberg).

PATIENTS AND METHODS

Patients

The discovery population consisted of 260 patients included in French (n=93), UK (n=105) and Italian (n=62) trials (Table S1). All tumors were centrally reviewed and WHO graded. Median age at diagnosis was 33.5 months (range, 4 months to 16.7 years). Young children were treated mostly with post-operative chemotherapy only as published previously (7,8,12). Older children received mostly post-operative radiation therapy with or without chemotherapy (13-15). The first external validation cohort included patients from the GPOH HIT 2000 trial (Table S2). Details of the second external validation cohort from the Heidelberg group have been published previously (2) (Table S3).

Immunohistochemistry and FISH

Potential prognostic markers (metallothioneins 1-2 and 3, YAP1, nucleolin, ASPM, KI-67, TNC) were initially tested by immunohistochemistry in tumors from patients treated in national prospective trials. Biological analyses were performed on paraffin embedded tissue microarray (TMA) blocks of pediatric ependymoma patients.Two to six core biopsies from each tumor were included in the TMA. . Each national reference centre defined its most promising immunohistochemical marker(s) (nucleolin, YAP1 and TNC), which was then tested on TMA slides in the two other cohorts by the same laboratory and with the same techniques for staining and scoring. Beta catenin and CD3immunostains were further performed in the French cohort.

Description of immunohistochemistry protocols is given in supplementary data (Table S4).

TNC staining in ependymoma stained the extracellular matrix, and was generally not observed in individual cells (neither in the nucleus nor in the cytoplasm) (Figure 1A). Two main patterns were seen, either perivascular only or more diffuse. TNC was more frequently

and strongly expressed in hypercellular and proliferative nodules. TNC staining was sometimes focal, i.e. only a single core of a given tumor being positive. Immunohistochemical staining for TNC was scored based on staining intensity, as follows: 0, no staining; 1, weak staining; 2, moderate to strong staining (Figure 1B). Scoring was performed as observed in most positive areas. For further analyses, moderate to strong staining was compared to absent to weak staining.

Reproducibility of staining and scoring for TNC was tested in the UK cohort by two independent observers (FA and JPK).

TNC expression was assessed on whole paraffin-embedded tumor slides from patients from an external cohort (GPOH HIT 2000 trial) with the same antibody and staining procedure and using the same scoring system. Subsequently, the analysis was carried on TMA slides from the Heidelberg cohort with a different primary antibody.

FISH analysis for 1q25 loss was performed as previously described on a subset of the discovery population, only from the French and UK cohorts (16).

Analysis of the expression of TNC isoforms

This analysis was carried out in 11 supratentorial and 10 posterior fossa snap frozen fresh ependymomas, from which DNA was prepared as described in (1).

Inventoried assays were available for the Tenascin-C invariant exon 17/18 boundary (Applied Biosystems Taqman Assay, Hs01115654_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Applied Biosystems Taqman Assay, Hs99999909_m1). Primers and probes were developed in house for TNC isoforms and assays were performed as previously described (17).

Statistical analysis

The main endpoint was overall survival, defined as the time between the date of diagnosis to the date of death whatever the cause. Survivors were censored at the date of their last follow-up. The event free-survival was defined as the time from the date of diagnosis until the date of first event, manifesting as recurrence or progression and death. Survivors without recurrence or progression were censored at their date of last-follow-up. The cut-off date of this analysis was January 1st, 2009.

To assess impact of studied factors on survival, hazards ratios were computed through Cox models stratified by cohort. Variables with a significant threshold below 0.25 were introduced in the multivariable model. The final model was defined as the multivariable model in which all of the variables had a p-value below 0.05.

In the event of the missing data in the multivariable model, missing values for TNC were imputed. The imputation was based on the relation between TNC and age, localization and treatment received. These three variables were used to predict the probability of having a high level of TNC using a logistic regression model. Patients with missing TNC got a value corresponding to high TNC if the predicted probability was equal or superior to 0.5 and got a value corresponding to low TNC if the predicted probability was below 0.5. A sensitivity analysis was performed excluding all patients with missing values to check the impact of imputation procedures. Patients without all clinical data available were excluded from multivariable analysis.

A bootstrap analysis was performed on the original data set in order to determine the stability of the model. At each of the 1000 steps, a sampling with replacement was performed based on our population to build a new population of 203 patients. The concordance of the prognostic models was calculated through the Harrell's c-index. The c-index was between 0.5 (discrimination by chance) to 1 (perfect discrimination).

RESULTS

Patients description

The three discovery cohorts were different with respect to age (Italian patients being older, p=0.05), grade (more grade III tumors in the French patients, p<0.001), extent of resection (more complete resection in the French and Italian patients, p=0.01), and treatment type (more Italian patients receiving radiotherapy as part of the first line treatment, p<0.01)(Table S1). Median follow-up was 8.0 years (range, 1 day to 19.0 years), slightly shorter for UK patients (p=0.03).

Median overall survival (OS) was 7.7 years (95% CI: 6.4-10.1 years) (Figure S1). OS did not differ between the three cohorts. OS was 77% (95% CI: 72-82) at 3 and 62% (95% CI : 55-68) at 5 years.

The median event-free survival (EFS) was 2.2 years (95% CI: 1.8-2.6). EFS did not differ between the three cohorts. EFS was 41% (95% CI: 35-47) at 3 and33% (95% C : 27-39) at 5 years.

Association between variables

The treatment is dependent on patient age with limited use of radiotherapy for patients under 36 months and these two variables were therefore associated (p<0.001). TNC staining score was associated with age: 71% of patients under 36 months have a strong expression of TNC versus 40% of patients above 36 months (p<0.001) (Figure S2). Consequently, TNC was also associated with the treatment received (less patients with strong expression in the group treated with radiotherapy, p<0.001).TNC staining was associated with location: 70% of patients with infratentorial tumour have a strong expression of TNC versus 18% of patients with supratentorial tumour (p<0.001).

TNC staining was positively correlated with MT3 staining (p=0.004) but neither with any other biomarker (MT1/2, ASPM, Ki67, Nucleolin, YAP1, beta catenin, CD3) nor grade (data from the French cohort only). TNC staining was not correlated with 1q25 gain in 144 patients from the French and UK cohort for whom both biomarkers could be assessed.

Tumor location was associated with age: 28% of patients under 36 months had a supratentorial tumour versus 72% of patients above 36 months and more (p<0.001).

Finally, treatment directed by age was associated with the location, patients with infratentorial tumours having less radiotherapy treatment alone than patients with supratentorial tumours (17% versus 37%) and more chemotherapy alone (63% versus 34%, p<0.001).

Prognostic factors for overall survival

Table 1 describes the prognostic analyses performed for overall survival in 260 patients (242 in the multivariable model). In the multivariable analyses, WHO grade, extent of surgery and TNC were the three factors associated with the risk of death: patients with grade III ependymoma had a higher risk of death than patients with grade II ependymoma (HR=1.81[95% CI:1.18-2.78], p=0.007), patients with incomplete surgery had a higher risk of death than patients with incomplete surgery (HR=1.77[95% CI: 1.20-2.52], p=0.004) and patients with strong expression of TNC had a higher risk of death than patients with negative or weak expression (HR=1.77[95% CI: 1.20-2.62], p=0.004).

Since the respective weight of each factor described above was similar, patients were grouped according to their number of risk factors. 5-year overall survival rates were 83% [59-94%] in the absence of risk factor, 77% [67-84%], 51% [41-62%] and 40% [23-59%] in face of one, two or three risk factors, respectively (Figure 2).

The results did not differ in the sensitivity analysis (Table S5).

Among the three variables selected in our final model, only the extent of surgery was selected in more than 75% of the times during the 1000 bootstrap steps. TNC and grade were selected in 63% and 51% of cases, respectively. The concordance of the final model, including grade, extent of surgery and TNC on the 242 patients was 0.630 (95% CI : 0.573 to 0.687).

A secondary multivariable analysis restricted to 144 patients from the French and UK cohorts showed that TNC staining and extent of resection were the only risk factors associated with overall survival even when the 1q25 status was incorporated in the model.

Prognostic factors for event-free survival

In univariate analyses, age (p=0.004), extent of surgery (p<0.001), type of therapy (p<0.001) and TNC expression (p=0.003) were the four factors associated with the risk of event (Table S6). In multivariable analysis, only extent of resection remained significantly associated to prognosis (p<0.001). An interaction was found between age and TNC expression, suggesting the prognostic ability of TNC expression differed according to age: TNC was significantly associated with event-free survival especially in patients < 36 months (p=0.006) (Table S7).

Bootstrap analysis selected extent of surgery and TNC expression in 73% and 75% of the 1000 bootstrap steps, respectively, confirming their association with the event-free survival. Age was selected only in 15% of the cases; however, it is important in the model as it is a confusion factor for TNC and must be included when the interaction term remains. The concordance of the final model, including age, extent of surgery, TNC and interaction term on the 242 patients was 0.609 (95% CI: 0.567 to 0.651).

A multivariable analysis restricted to 144 patients from the French and UK cohorts showed that extent of resection (HR=2.39, 95% CI: 1.51-3.79,p=0.0002), 1q25 gain (HR=2.52, 95% CI : 1.52-4.18, p=0.0003) and TNC positive staining (HR=1.77, 95% CI : 1.10-2.87, p=0.02) were significantly associated with progression-free survival.

Reproducibility of TNC scoring

The kappa index, measuring the concordance between the two independent scorers in three classes (negative, weak and strong), was 0.78 (95% CI: 0.67-0.90). No patient classified as having strong expression of TNC by one of the two scorers was classified having a negative expression of TNC by the second scorer, or conversely (Table S8). As prognostic factor, TNC was used as negative/weak versus moderate/strong. When considering these two categories, the kappa index became 0.90 (95% CI: 0.82-1.00), showing a high reproducibility of the scoring (Table S9).

External validation of the prognostic marker TNC

We first analysed a cohort of German patients treated in the GPOH HIT 2000 trial. Compared to the discovery cohort, these 136 patients were older and treated with irradiation as a first line treatment in 80% of cases. Histology was centrally reviewed (TP). Their median follow-up was also shorter: 2.9 years (range, 9 days to 8.3 years). OS was 82% (95% CI:73-89) at 3 and 74% (95% CI, 63 to 83%) at 5 years, significantly better than in the discovery cohort. EFS was 66% (95% CI, 56 to 74%) at 3 and 61% (95% CI, 51 to 71%) at 5 years, with significantly fewer events than in the discovery cohort.

In this new cohort, none of the known risk factors were associated with overall survival. The HR of strong TNC staining for overall survival was 0.99 [0.38 - 2.55], compared to 1.77 in the discovery cohort. Both extent of tumor resection (HR=4.10 [95% CI :2.07-8.14], $p<0.001$) and TNC staining (HR=3.82 [05% CI: 0.50-29.30], $p=0.19$) were associated with EFS in patients under 36 months. The prognostic model established in the discovery cohort remained valid, particularly for the event-free survival, with similar c-indexes on this validation cohort.

A second validation cohort from Heidelberg (2) was used to validate the prognostic impact of strong TNC expression on immunohistochemistry. Positive TNC staining was associated with significantly poorer PFS and OS (Figure 3 and 4).

TNC isoforms in pediatric ependymomas

Significant expression of the different isoforms of TNC was detected, which correlated with the total TNC mRNA expression. The ratio of isoform/TNC was higher for AD2 and 14.16 isoforms (Figure 5). Since TNC was overexpressed in infratentorial tumors by immunohistochemistry, we analysed whether the relative expression of the isoforms differed according to location. While TNC expression was 3 times higher in infratentorial tumors compared to supratentorial tumors (median 32.29 [range 18.47 - 46.12] versus 10.05 [range 7.20 - 12.91], respectively), the different isoforms were similarily distributed in both locations.

DISCUSSION

This report presents TNC as the first immunohistochemical marker of pediatric intracranial ependymoma prognosis that could be validated in international trial cohorts. Puget et al have previously shown the overexpression of the *TNC* gene located in the 9q33-34 chromosomal region that is specifically gained in recurrent ependymomas (5). Here, we demonstrate that tumors with high TNC expression on immunohistochemistry had a worse outcome irrespective of tumor grade or extent of resection. TNC overexpression has already been associated with shorter time to progression or survival in various cancers (18-20) and in particular in adult gliomas (21,22). In ependymomas, specifically, TNC overexpression on IHC has been shown previously in single center series to be associated with higher grade (23)

and poorer prognosis in low-grade tumors (23,24). In the recent report from Witt et al (2), where two prognostic groups of posterior fossa ependymoma were identified, the group with poor prognosis was more frequently positive for TNC (94% vs. 11%, $p<0.0001$).

Weaker association with survival observed in the GPOH cohort could be explained by a shorter follow-up resulting in a relatively low number of events available for prognostic calculation. In addition, significant differences in patients selection could also influence the outcome of this trial cohort with respect to TNC IHC results: more grade III tumors were included (82% vs 61% in the discovery cohorts), radiotherapy was systematically applied to the majority of these patients at diagnosis and these children were significantly older (median age 51 vs. 33 months in the discovery cohorts). It seems in fact that TNC overexpression may be more relevant as a prognostic indicator in young children, in whom TNC overexpression was more frequent. Radiation therapy may possibly weaken the impact of TNC overexpression on progression-free survival.

The prognostic role of TNC expression by IHC was independent from other known risk factors (25). The controversy of the impact of tumor grade on prognosis cannot solely be explained by the absence of reproducibility of the WHO grading scheme (11). From the literature available, it seems that the impact of grade was more frequently observed in trials using a therapeutic strategy based on irradiation (26-30).

TNC immunostaining was generally extracellular, sometimes restricted to the perivascular area but more often diffuse throughout the tumor. This is consistent with previous reports (23,24). Due to the distribution of immunopostive areas for TNC, the scoring needs to consider a representative sampling of tumor areas.

Several mechanisms by which TNC expression may influence survival in tumors have been proposed: inhibition of T cells migration (31), invasion through epithelial to mesenchymal transition enforced by Wnt (32), PKC signaling (33) or FAK phosphorylation (34),

maintenance of a stem cell niche (35). Although TNC was more frequently and strongly expressed in hypercellular and proliferative nodules, TNC expression was not associated with higher proliferation (Ki67 index). Wnt pathway activation (betacatenin nucleo-positive cells) was not seen in TNC positive areas contrary to what has been described in colon or breast carcinomas (32). Lymphoïd cells immobilization (CD3 positive lymphocytes) was not observed in the TNC positive areas as shown in glioblastoma (31).

Since certain isoforms of TNC have been associated with higher proliferation and invasion in various cancers (17,36), we analysed the expression of the different splice variants. The ratio variant/total TNC was higher for the A2 and 14.16 isoforms known to be associated with invasive breast cancer.

The approach used herein to test and validate immunohistochemichal markers on homogenously treated trial cohorts prove to be useful to discard biomarkers such as nucleolin (13,37) previously identified in single center studies with more mixed cohorts.

This method will also be useful to test simultaneously more than one biomarker as shown here with 1q25 loss.

In conclusion, TNC is a robust biomarker for the risk of recurrence and death in pediatric intracranial ependymomas, especially in young children. Its expression, easily detected and scored by immunohistochemistry, could be used to stratify patients in clinical trials and will be tested prospectively in the next generation of ependymoma SIOP trials.

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Table 1: Prognostic analysis for overall survival (based on 260 patients; 242 patients in the multivariate model)

	Univariate			Multivariable**		
Characteristic	**HR**	**CI95%**	**p-value**	**HR**	**CI95%**	**p-value**
Age*						
< 36 months	1*	-	0.02	*1**		*0.13*
≥ 36 months	0.64	[0.44 – 0.93]		*0.73*	*[0.48-1.10]*	
Localization (8 MD)						
Infratentorial	1*	-	0.05	*1**		*0.10*
Supratentorial	0.63	[0.40 – 0.99]		*0.65*	*[0.38-1.10]*	
Grade (8 MD)						
Grade II	1*	-	0.07	**1***	**-**	**0.007**
Grade III	1.48	[0.97 – 2.24]		**1.81**	**[1.18 – 2.78]**	
Extent of surgery (1 MD)						
Complete	1*	-	0.004	**1***	**-**	**0.004**
Incomplete	1.74	[1.20 – 2.52]		**1.77**	**[1.20 – 2.52]**	
Treatment received (2 MD)						
Radiotherapy	1*	-	0.11	*1**		*0.74*
Chemotherapy	1.83	[1.06-3.18]		*1.24*	*[0.69-2.23]*	
Chemotherapy + radiotherapy	1.97	[1.04-3.72]		*1.05*	*[0.50-2.23]*	
None (surgery only)	1.05	[0.30 – 3.65]		*0.74*	*[0.20-2.69]*	
Tenascin C (imputation)						
Negative-weak	1*	-	0.008	**1***	**-**	**0.004**
Strong	1.66	[1.14– 2.41]		**1.77**	**[1.20 - 2.62]**	
Nucleolin (25 MD)						
< 50 %	1*	-	0.91	-	-	-
≥ 50 %	1.03	[0.63 – 1.68]		-	-	-

MD: missing data

* Age could not enter the multivariate model due to its strong correlation with treatment (-0.70); only tested in final model as treatment not included.

** Final model includes grade, extent of surgery and TNC; italic results are results for variables added one by one to the final model.

Figures

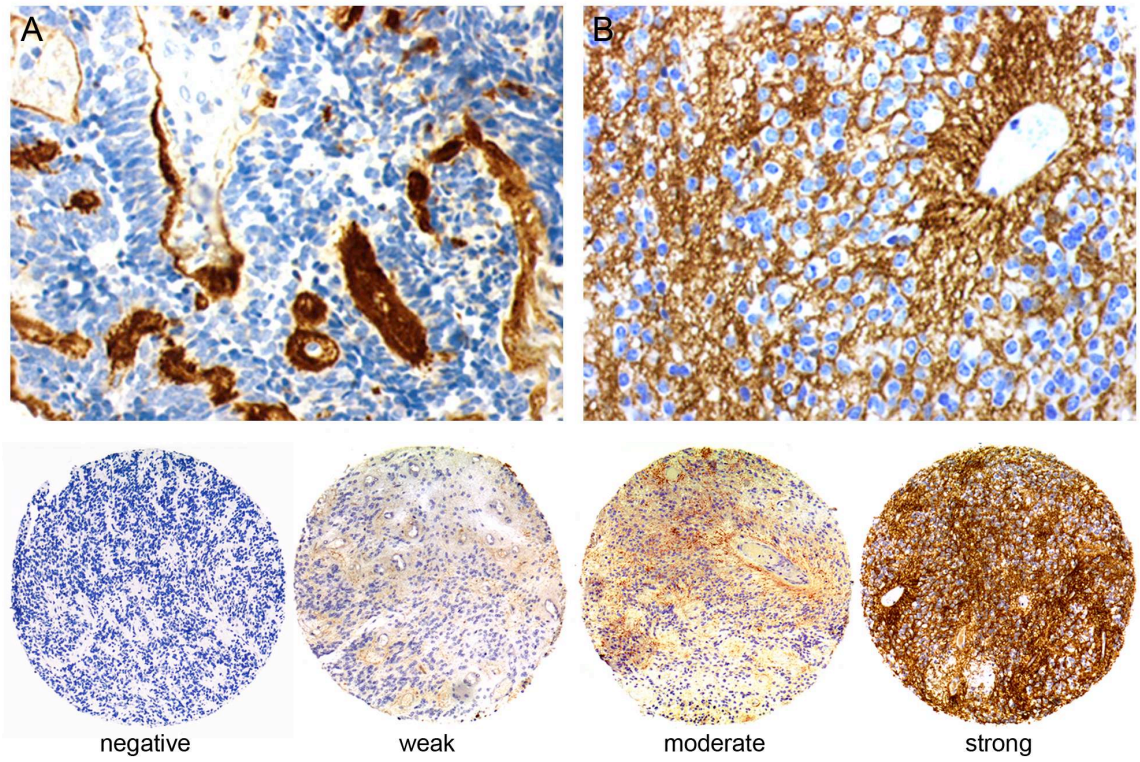


Figure 1: TNC staining in pediatric ependymoma
Upper panel: qualitative aspects of TNC staining
A) Perivascular staining (less frequent)
B) Perivascular and intercellular staining (most common)
Lower panel: TNC scoring
Most positive areas were analysed and scored for intensity of staining as shown. Only moderate and strong staining were considered as overexpression.

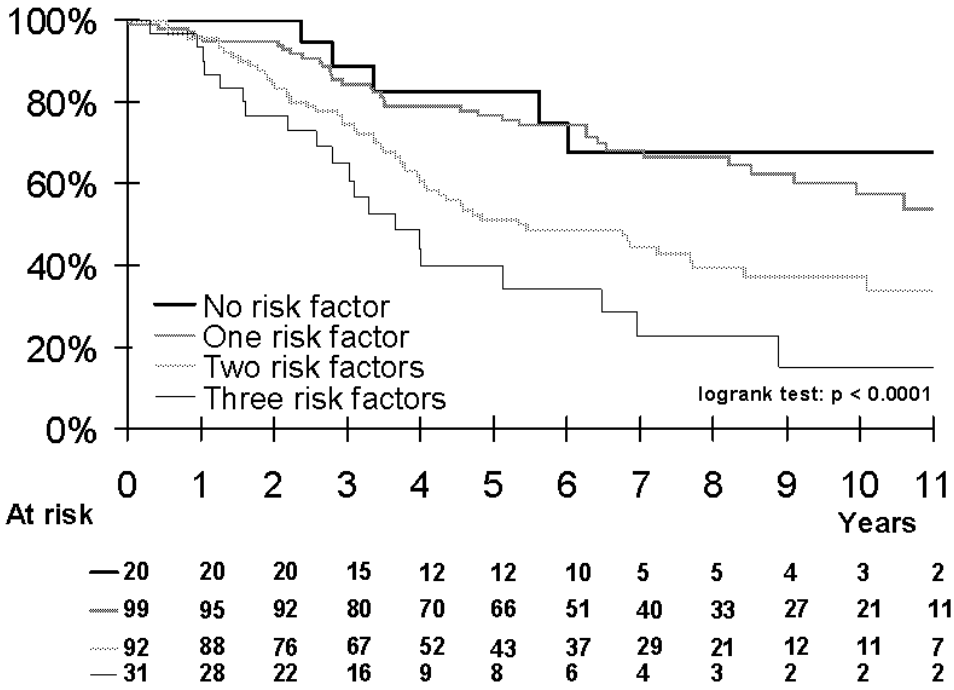


Figure 2: overall survival according to the number of risk factors.

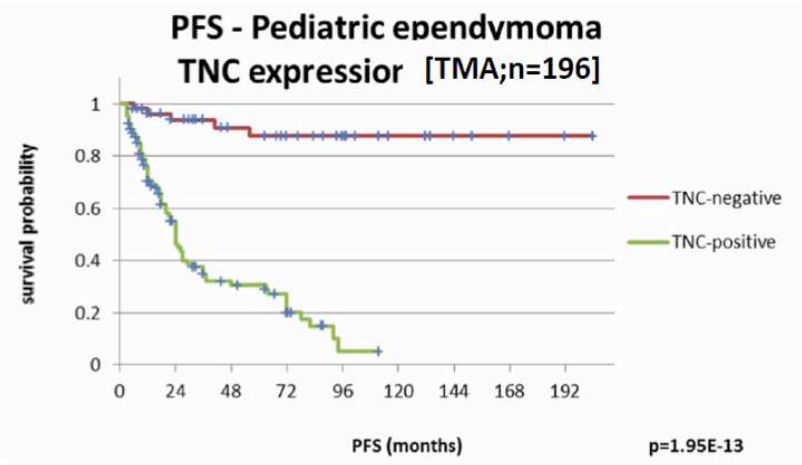


Figure 3: Progression-free survival in children with ependymoma in the Heidelberg cohort.

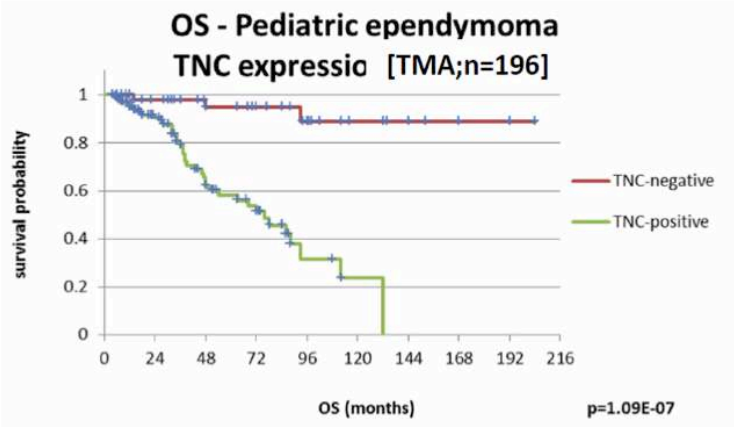


Figure 4: Overall survival in children with ependymoma in the Heidelberg cohort

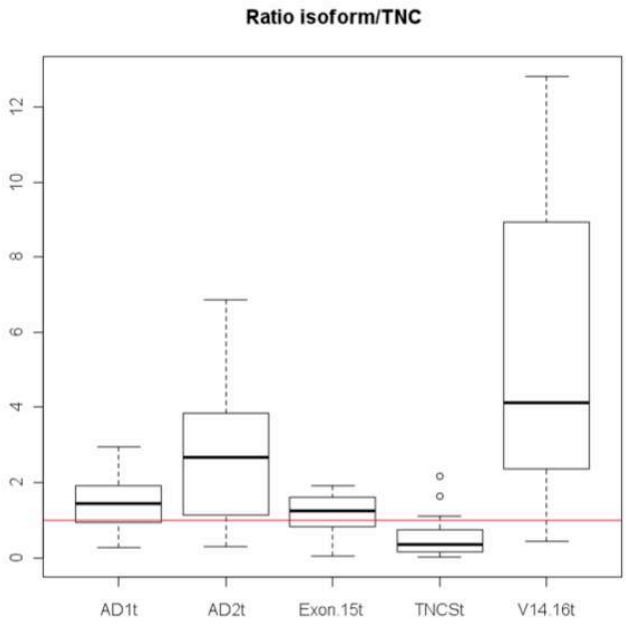


Figure 5: Ratio of the different isoforms of tenascin-C in ependymomas.

Distribution of TNC isoforms compared to normal brain (set as one in the presentation) measured by nested PCR, most of the splice variants are overexpressed in ependymomas, especially variant V14.16 and AD2.

Supplementary Data

Figure S1 : Survival of the patients in the discovery cohort

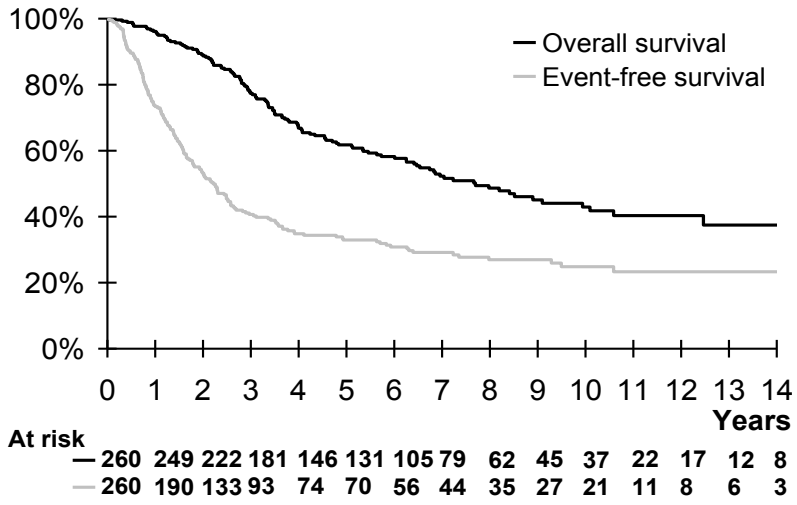
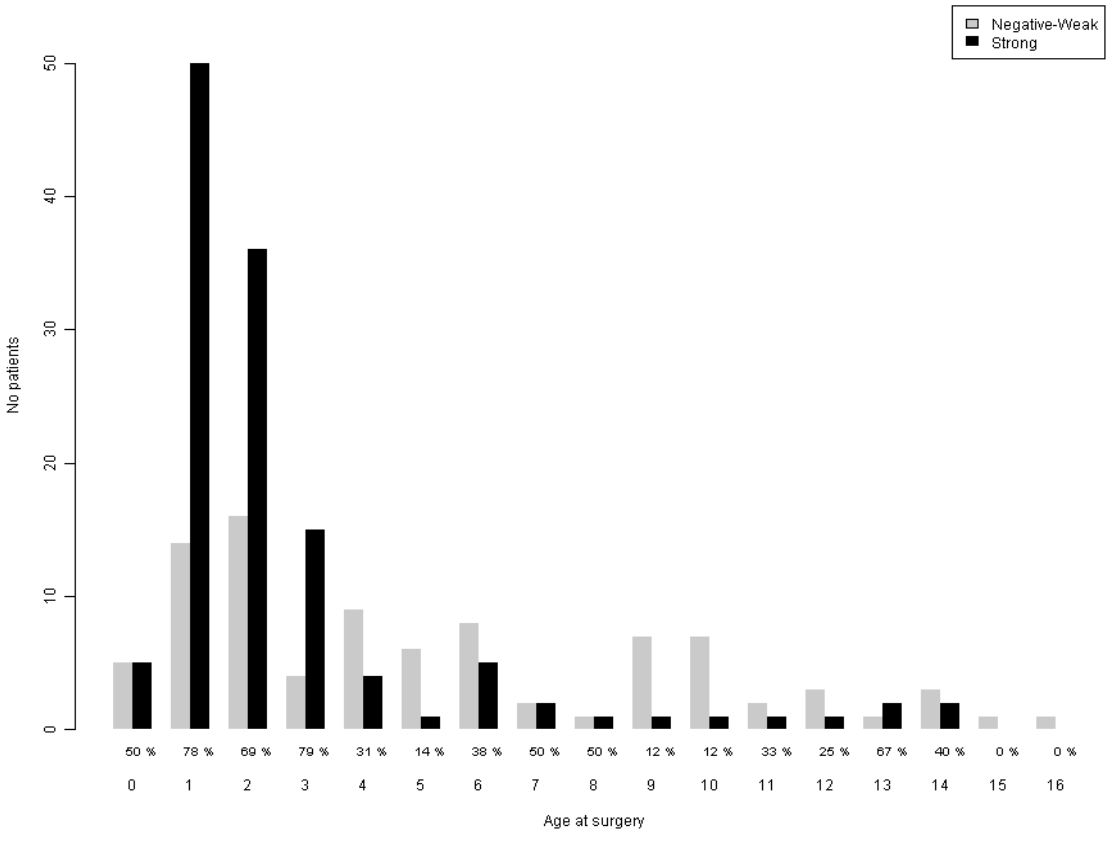


Figure S2: Age distribution of strong TNC staining



legend: Y-axis indicates the number of samples at each age group, upper line of the X-axis indicates the percentage of tumor samples with strong TNC staining according to age.

Table S2: Characteristics of patients by study in the discovery population

	France		UK		Italy		All		p-value
	N	*%*	**N**	*%*	**N**	*%*	**N**	*%*	
All	93	*100*	105	*100*	62	*100*	260	*100*	
SEX	48	*52*	65	*62*	36	*58*	149	*57*	
Male									0.34
Female	45	*48*	40	*38*	26	*42*	111	*43*	
Age	56	*60*	58	*55*	25	*40*	139	*53*	
<36 months									0.05
≥ 36 months	37	*40*	47	*45*	37	*60*	121	*47*	
Localisation of tumor	0	-	8	-	0	-	8	-	
Missing									0.57
PF	66	*71*	73	*75*	42	*68*	181	*72*	
ST	27	*29*	24	*25*	20	*32*	71	*28*	
Grade	0	-	8	-	0	-	8	-	
Missing									<0.001
Grade II	16	*17*	51	*53*	32	*52*	99	*39*	
Grade III	77	*83*	46	*47*	30	*48*	153	*61*	
Extent of surgery	1	-	0	-	0	-	1	-	
Missing									0.01
Complete	59	*64*	51	*49*	43	*69*	153	*59*	
Incomplete	33	*36*	54	*51*	19	*31*	106	*41*	
Treatment	2	-	0	-	0	-	2	-	
Missing									<0.01
None	1	*1*	0	*0*	8	*13*	9	*3*	
CT alone	70	*77*	60	*57*	16	*26*	146	*57*	
CT + RT	2	*2*	23	*22*	21	*34*	46	*18*	
RT alone	18	*20*	22	*21*	17	*27*	57	*22*	
Recurrence at cut-off date									
Number of events	65	*70*	67	*64*	46	*74*	178	*68*	
Status at cut-off date	44	*47*	59	*56*	30	*48*	133	*51*	
Number of deaths	49	*53*	46	*44*	32	*52*	127	*49*	

Overall, 260 patients are included in the analysis: 93 patients from France, 105 patients from UK and 62 patients from Italy. The median age at diagnosis is 33.5 months (range, 4 months to 16.7 years). p-values correspond to the tests of the differences between the three cohorts. (Status is defined by the occurrence of death).

Table S3: Characteristics of patients in the GPOH cohort

	Germany	
	N	***%***
All	137	*100*
SEX		
Male	84	*61*
Female	53	*39*
Age		
<36 months	48	*35*
≥ 36 months	89	*65*
Localisation of tumor		
PF	88	*64*
ST	49	*36*
Grade		
Grade II	24	*18*
Grade III	113	*82*
Extent of surgery		
Missing	6	-
Complete	82	*63*
Incomplete	49	*37*
Treatment		
Missing	2	-
None	1	*1*
CT alone	13	*10*
CT + RT	108	*80*
RT alone	13	*10*
Recurrence		
Number of events	48	*35*
Status	116	*85*
Number of deaths	21	*15*
Tenascin C		
Missing	2	-
Negative-weak	56	*41*
Strong	79	*59*

Table S3: characteristics of patients in the Heidelberg cohort

	N	%
All	196	100
Gender		
Male	122	62%
Female	74	38%
Age		
<36 months	53	27%
≥ 36 months	143	73%
Localisation of tumour		
PF	129	66%
ST	67	34%
Grade		
2	46	23%
3	150	77%
Extent of surgery		
Missing	/	/
Complete	95	48%
Incomplete	101	52%
Treatment		
Missing	/	/
None	/	/
CT alone	/	/
CT + RT	/	/
RT alone	/	/
Recurrence		
0	87	44%
1	109	56%
Status/Mortality		
0	153	78%
1	43	22%
Tenascin C		
Missing	/	
Negative	54	28%
Positive	142	72%

Table S4: Immunohistochemistry- Methods

Primary antibody	Clone, Manufacturer's reference	Type of antibody (anti-human)	Dilution	Antigen retrieval	Primary antibody incubation	Revelation system	Comments/References
TNC	E-9, Santa Cruz, ref. sc-25328	Mouse monoclonal	1:50	Citrate pH 6.0, 30 minutes	1 hour, room temperature	Vectastain ELITE (Vector Laboratories, ref. PK 6200)	Sections rinsed with Tris-buffered Saline. Positive controls included borders of infiltrative glioma and squamous cell carcinoma, as in Puget et al, J Clin Oncol. 10:1884-92
MT1-2	E9, Zymed, ref 18-0133	Mouse monoclonal	1:300	Citrate pH 7.3, 30 minutes	1 hour, room temperature	Histostain Plus (Zymed, ref. 85-9043)	
MT3	Non-commercial, kindly provided by Dr D. Sens, University of North Dakota	Rabbit polyclonal	1:1000	Citrate pH 6.0 30 minutes	1 hour, room temperature	EnVision (DAKO, ref. K4003)	Peyre et al., PLoS One. 24;5(9):e12932 Garrett et al Toxicol Lett.;105:207-14.
ASPM	Polyclonal, Bethyl Laboratories Inc., ref. IHC-00058	Rabbit polyclonal	1:100	Citrate pH 7.3, 30 minutes	Overnight, 4°c	EnVision (DAKO, ref. K4003)	Peyre et al., PLoS One.: 24;5(9):e12932.
KI 67	MIB1, DAKO ref M7240	Mouse monoclonal	1:50	Acid PH	Short protocol	Ventana automated (Benchmark)	
H-TERT/ NUCLEOLIN	4E2, Abcam, ref. Ab13541	Rabbit monoclonal	1:2000	Citrate pH 6.0, 1 minute	Overnight, 4°c	EnVision (DAKO, ref. K4003)	Ridley at al., Neuro Oncol.10: 675–689.
YAP1	Polyclonal, Cell Signaling ref. 4912	Rabbit polyclonal	1:50	Citrate pH 6.0, 15 minutes	1 hour, room temperature	Ultra Vision (LabVision Co, ref TL-125-HL0)	

Endogenous peroxidase was blocked for all antibodies. Negative controls omitting primary antibodies were performed systematically.

Table S5: Results of the sensitivity analysis for overall survival (n=203)

	Multivariate		
Characteristic	**HR**	**CI95%**	**p-value**
Grade (8 MD)			
Grade II	1*	-	0.03
Grade III	1.70	[1.04 – 2.77]	
Extent of surgery (1 MD)			
Complete	1*	-	0.008
Incomplete	1.78	[1.16 – 2.74]	
Tenascin C (39 MD)			
Negative-weak	1*	-	0.001
Strong	2.10	[1.34 – 3.31]	

The multivariable analysis was repeated in the population excluding the patients with missing data (MD) (n=203). Results were similar than the analysis where TNC results were imputed.

Table S6: Univariate analysis for event-free survival (based on 260 patients)

	Univariate		
Characteristic	**HR**	**CI95%**	**p-value**
Age			
< 36 months	1*	-	0.004
≥ 36 months	0.63	[0.46 – 0.86]	
Localisation (8 MD)			
Infratentorial	1*	-	0.17
Supratentorial	0.79	[0.56 – 1.11]	
Grade (8 MD)			
Grade II	1*	-	0.36
Grade III	1.17	[0.84 – 1.64]	
Extent of surgery (1 MD)			
Complete	1*	-	<0.001
Incomplete	2.09	[1.54 – 2.84]	
Treatment received (2 MD)			
Radiotherapy	1*	-	<0.001
Chemotherapy	2.31	[1.48-3.62]	
Chemotherapy + radiotherapy	2.57	[1.53-4.33]	
None (surgery only)	1.36	[0.51 – 3.60]	
Tenascin C (imputation)			
Negative-weak	1*	-	0.003
Strong	1.59	[1.18 - 2.15]	
Nucleolin (25 MD)			
< 50 %	1*	-	0.08
≥ 50 %	1.46	[0.95 – 2.25]	

MD: missing data

Table S7: Multivariable prognostic analysis for event-free survival (242 patients)

Characteristic	Multivariate*		
	HR	**CI95%**	**p-value**
Localisation (8 MD)			
Infratentorial	1*	-	0.99
Supratentorial	1.00	[0.66 – 1.51]	
Grade (8 MD)			
Grade II	1*	-	0.10
Grade III	1.34	[0.95 – 1.89]	
Extent of surgery (1 MD)			
Complete	**1***	**-**	**<0.001**
Incomplete	**2.04**	**[1.48 – 2.80]**	
Tenascin C (imputation) **when age < 36 months**			
Negative-weak	**1***	**-**	**0.006**
Strong	**1.92**	**[1.20 – 3.06]**	
Tenascin C (imputation) **when age ≥ 36 months**			
Negative-weak	**1***	**-**	**0.98**
Strong	**0.99**	**[0.61 – 1.62]**	

MD: missing data
* Final model includes age, extent of surgery, Tenascin C and an interaction term between age and Tenascin C. Results showed are adjusted on age. Results on treatment received are not shown because treatment received could not enter the multivariate model due to its strong correlation with age (-0.67); but it was non significant in a variable selection excluding age.
** Final model includes age, extent of surgery and Tenascin C; italic results are results for variables added one by one to the final model.
*** Not tested in the final model due to its strong correlation with age (-0.67); but non significant in a variable selection excluding age.

Table S8: Reproducibility of TNC scoring (in three classes)

	Scoring UK			
Scoring France	**Negative**	**Weak**	**Strong**	**Total**
Negative	12	4	0	16
Weak	3	14	3	20
Strong	0	1	57	58
Total	15	19	60	94

This assessment was performed on 94 patients of the UK cohort by two independent observers (FA & JPK) using the same grading to score TNC staining.

Table S9: Reproducibility of TNC scoring (in two classes)

	Scoring UK		Total
Scoring France	**Negative/Weak**	**Strong**	
Negative/weak	33	3	36
Strong	1	57	58
Total	34	60	94

7. Tenascin C and NOTCH: Further evidence of functional association in ependymoma short-term cell cultures.

A recent article has shown that TNC is a NOTCH target in glioblastoma cell lines, being transactivated by the NOTCH effector RBJk protein which binds to TNC promoter (Sivasankaran et al., 2009). On the other hand TNC also seems to enhance NOTCH activity, as in a model of human breast cancer cell lines "metastatic" to the lungs in mice it prevents NOTCH inhibition by JAK2 and STAT5 by relieving MSI1 from repression (Oskarsson et al., 2011). Interestingly in this article TNC depletion in these cells did not inhibit brain metastases, which could be explained by the action of other molecules of the extracellular matrix favoring the metastatic microenvironment in the brain, or simply by other pathways involved in this mechanism. TNC also activated WNT pathways in these cell lines, as previously described in colon cancer (Beiter et al., 2005). Of note we performed Bcatenin immunostains in our ependymoma cases (n=112) and only two cases out of showed some nuclear accumulation of Bcatenin, supporting the notion that TNC does not act through WNT signaling in ependymoma.

As mentioned above a previous study from our group showed frequent gains at 9q33, the genomic region of NOTCH 1 and TNC in pediatric ependymoma (Puget et al., 2009).

To elucidate the interaction between NOTCH and TNC in ependymoma we conducted analyses on TNC expression in short term ependymoma cell cultures focusing on the effect of *NOTCH1* inhibition by siRNAs and using anti-NOTCH drugs gamma secretase inhibitor (GSI XVII).

In vitro modulation of TNC expression in ependymoma tumor cells

Primary short-term cell cultures and cell lines. Primary ependymoma short-term cultures NEM65, NEM78, and NEM94 were established by direct culture of tumor tissues in AmnioMAX C-100 Basal Medium completed with AmnioMAX C-100 Supplement (Invitrogen SARL, Cergy Pontoise, France). After removal of vessels and necrotic tissues, the tumor was mechanically dissociated and passaged serially through 18 to 22 Gauge needles to obtain a single cell suspension. The pediatric glioblastoma cell line SF188 was provided by Dr. Chris Jones (The Institute Cancer Research, Sutton, UK) and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37°C and 5% CO2.

GSI treatment: NEM65 cells were cultivated in 60 mm dishes until 80% confluence. At this time fresh medium containing 3.0 µM of DAPT or DMSO were added and cells incubated for 1, 3, 6, 12, 24 and 48 hours.

NOTCH1 inhibition using RNA interference and sensitivity to chemotherapy.

Gene silencing experiments were performed by cell transfection using JetPRIMETM (Polypus transfection, Ilkirch) and a siRNA for *NOTCH1* (sc-36095; Santa Cruz). Twenty-four hours post-transfection, cells were collected for RNA extraction.

DNA and RNA extraction.

RNA were purified using RNeasy Mini Kit according to manufacturer's instructions (Qiagen).

Real-time RT-PCR.

Total RNA was used to synthesize cDNA using random hexamers and the Mu-MLV reverse transcriptase (Applied Biosystems). Real-time RT-PCR for the genes HES1, HEY1, TNC was carried out using Taqman Gene Expression Assays on Demand (Applied Biosystems) and ABI Prism 7700 Sequence Detection System (Applied Biosystems). Expression profile in each specimen was assessed using the comparative threshold cycle (2-ddCt) method. GAPDH was used as endogenous control.

RESULTS

Both siRNA against NOTCH and gamma secretase inhibitors downregulate efficiently TNC expression in ependymoma cell lines. (Figures 7 and 8)

Figure 7: Transcriptional downregulation of TNC after *NOTCH1* siRNA transfection. The ependymoma short term culture cells NEM78 and NEM94 and the glioblastoma cell line SF188 Were transfected with *NOTCH1* siRNA and siRNA control. TNC expression level were analyzed related to siRNA control.

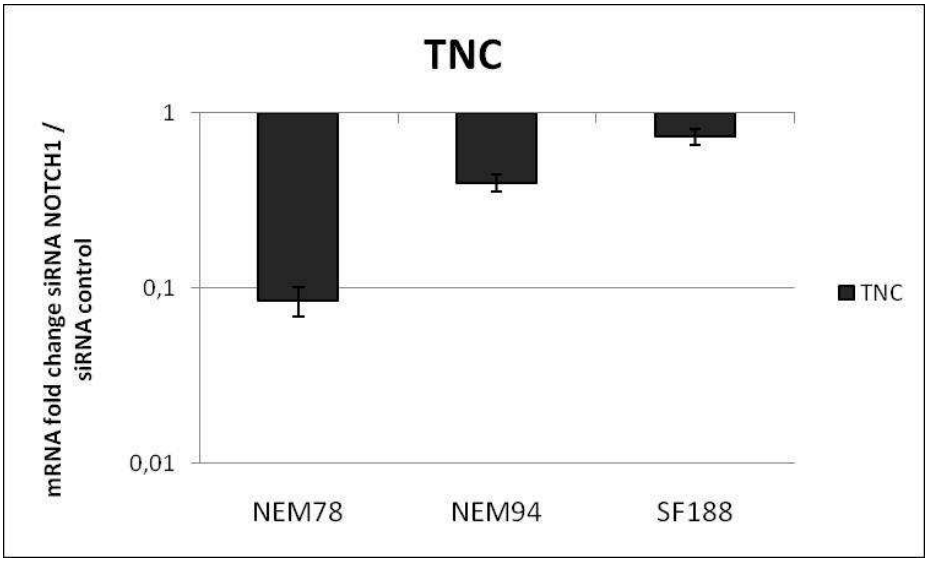
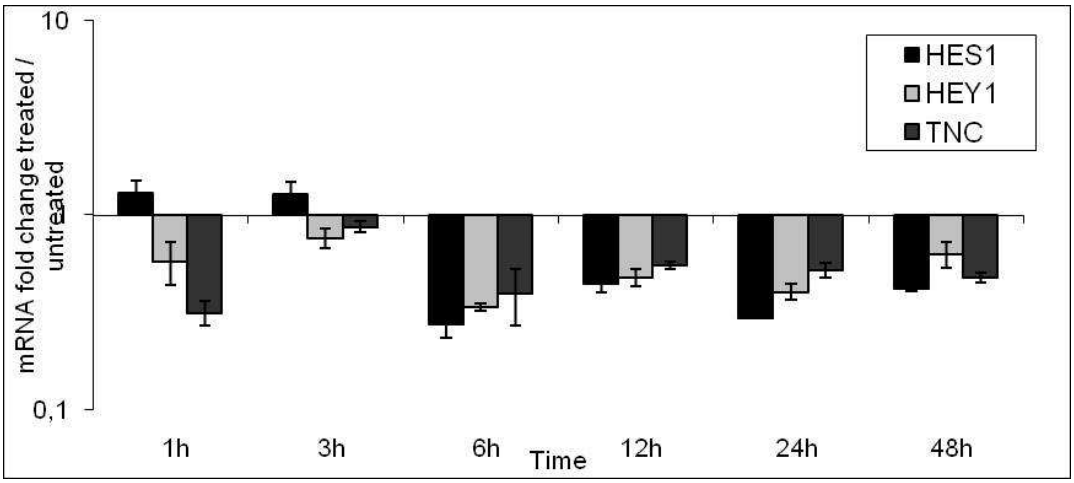


Figure 8: Transcriptional downregulation of TNC and other NOTCH1 effector genes after GSI treatment. NEM65 ependymoma short-term culture cells were treated with 3.0 µM of DAPT for the designed times. RNA extracted were subjected to real time RT-PCR for HES1, HEY1 and TNC genes. Expression is related to DMSO treated cells.



These preliminary results show that NOTCH pathway targeting may be useful to decrease TNC expression and therefore, possibly, also invasion. Further experiments are needed to further characterize the interaction of NOTCH and TNC in ependymoma.

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Appendix 1.1

Portrait of ependymoma recurrence in children: Biomarkers of tumor progression

Portrait of Ependymoma Recurrence in Children: Biomarkers of Tumor Progression Identified by Dual-Color Microarray-Based Gene Expression Analysis

Matthieu Peyre[1.], Fre´de´ric Commo[2.], Carmela Dantas-Barbosa[1.], Felipe Andreiuolo[1,4], Ste´phanie Puget[1,3], Ludovic Lacroix[4], Franc¸oise Drusch[4], Ve´ronique Scott[1], Pascale Varlet[5], Audrey Mauguen[6], Philippe Dessen[2], Vladimir Lazar[7], Gilles Vassal[1,8], Jacques Grill[1,8]*

1 Universite´ Paris-Sud, CNRS UMR 8203 ''Vectorology and Anticancer Treatments'', Gustave Roussy Institute, Villejuif, France, 2 CNRS FRE 2939, Bioinformatics Group, Gustave Roussy Institute, Villejuif, France, 3 Department of Neurosurgery, Necker Sick Children's Hospital, Universite´ Paris V Descartes, Paris, France, 4 Translational Research Laboratory, Gustave Roussy Institute, Villejuif, France, 5 Department of Neuropathology, Sainte-Anne Hospital, Paris, France, 6 Department of Biostatistics, Gustave Roussy Institute, Villejuif, France, 7 Functional Genomics Unit, Gustave Roussy Institute, Villejuif, France, 8 Department of Pediatric and Adolescent Oncology, Gustave Roussy Institute, Villejuif, France

Abstract

Background: Children with ependymoma may experience a relapse in up to 50% of cases depending on the extent of resection. Key biological events associated with recurrence are unknown.

Methodology/Principal Findings: To discover the biology behind the recurrence of ependymomas, we performed CGHarray and a dual-color gene expression microarray analysis of 17 tumors at diagnosis co-hybridized with the corresponding 27 first or subsequent relapses from the same patient. As treatment and location had only limited influence on specific gene expression changes at relapse, we established a common signature for relapse. Eighty-seven genes showed an absolute fold change $2 in at least 50% of relapses and were defined as the gene expression signature of ependymoma recurrence. The most frequently upregulated genes are involved in the kinetochore (ASPM, KIF11) or in neural development (CD133, Wnt and Notch pathways). Metallothionein (MT) genes were downregulated in up to 80% of the recurrences. Quantitative PCR for ASPM, KIF11 and MT3 plus immunohistochemistry for ASPM and MT3 confirmed the microarray results. Immunohistochemistry on an independent series of 24 tumor pairs at diagnosis and at relapse confirmed the decrease of MT3 expression at recurrence in 17/24 tumor pairs ($p = 0.002$). Conversely, ASPM expression was more frequently positive at relapse (87.5% vs 37.5%, $p = 0.03$). Loss or deletion of the MT genes cluster was never observed at relapse. Promoter sequencing after bisulfite treatment of DNA from primary tumors and recurrences as well as treatment of short-term ependymoma cells cultures with a demethylating agent showed that methylation was not involved in MT3 downregulation. However, in vitro treatment with a histone deacetylase inhibitor or zinc restored MT3 expression.

Conclusions/Significance: The most frequent molecular events associated with ependymoma recurrence were over-expression of kinetochore proteins and down-regulation of metallothioneins. Metallothionein-3 expression is epigenetically controlled and can be restored in vitro by histone deacetylase inhibitors.

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* E-mail: grill@igr.fr

. These authors contributed equally to this work.

Introduction

Ependymomas are tumours thought to derive from radial glial cells [1] and display morphological characteristics of normal ependyma [2]. They represent the third most common intracranial tumour in children and intracranial location account for more than 90% of cases [3]. The incidence is higher in young children as more than fifty percent occur before the age of 5 [4]. The overall prognosis of this tumour remains poor, especially in young children [5] with a 10-year survival between 30 and 70% [6,7]. Extent of initial surgery remains the only consensual prognostic factor across studies [6,8,9]. Recurrences are most of the time local, at least at the beginning of the natural history; distant metastases become more frequent with more effective local treatment [7]. Treatment strategy is actually based on surgery at diagnosis and at each relapse completed with local radiotherapy

[4,6,10]. The role of chemotherapy is circumscribed to children under 3 years of age to avoid or postpone radiotherapy due to its potential neuropsychological side effects [10,11]. There is actually no treatment strategy specific for tumour recurrences after radiotherapy.

Advances have been made in our understanding of the molecular mechanisms underlying the oncogenesis of ependymoma with the discovery of specific cancer stem cells [1] and the definition of gene expression profiles specific of each location [1]. In addition, specific molecular signatures associated with clinical characteristics have been identified [12–16]. However, reports on prognostic biological markers have shown little consistency or reproducibility [9,12,17–29]. The Notch pathway, however, has been implicated in three independant studies as a key regulator of ependymoma oncogenesis [1,12,30]. Nonetheless, most of these reports concerned only tumours at diagnosis. Progression of ependymoma is possibly related to multiple factors and activated pathways that cannot always be unraveled by tumour analysis at diagnosis.

Aiming at learning more about tumor progression, we hypothesized that relevant information could be obtained by comparing with high throughput technologies tumours from the same patient at diagnosis and at relapse. Dual-color microarray-based gene expression analysis with the two samples labeled with different dyes on the same array, that hybridize competitively to probes on the same spot, allows to adjust for many factors that introduce noise and errors in studies where the comparison of expression differences is made with three different arrays (one for the control and two for the samples at diagnosis and at relapse) [31,32]. Conversely, this design does not allow to have absolute expression data at diagnosis but only the changes between diagnosis and relapse, but with a higher sensitivity through the limitation of normalization problems [33].

This study revealed pathways specifically up- or down-regulated at relapse that may be used as targets for drug development in pediatric ependymomas. Downregulation of metallothionein-3, also known as neural growth inhibitory factor, was observed at relapse in more than 80% of the recurrences. Conversely, genes of Wnt and Notch pathways were upregulated at recurrence together with numerous genes of the kinetochore and mitotic spindle.

Materials and Methods

Tumour material and patient characteristics

Seventeen patients with at least two frozen samples from two different surgeries (one diagnosis and one relapse) were included in this study. Frozen samples of tumour at diagnosis and at least one relapse were obtained for each patient. All samples were snap frozen at the time of surgery. For ten patients, one relapse was available and for seven patients two to three relapses were available. The study encompassed a total of forty-four tumour samples, seventeen at diagnosis and twenty-seven at relapse. Paired tumour samples (diagnosis and relapse) from fourteen patients were obtained from the Tumour Bank at the Necker Enfants Malades Hopital, Paris, France. Two additional paired tumour samples were obtained from the Tumour Bank of the Pierre Wertheimer Hospital, Lyon, France and one from the Neurosurgery Department of the Vrije Universiteit of Amsterdam. The biological study was approved by the Internal Review Board of the Biological Ressource Center of the Necker Sick Children Hospital in Paris, by the Internal Review Board of the Neurosurgery Department of the Vrije Universiteit in Amsterdam and by the Scientific Advisory Board of the NeuroBioTec Tumor Bank in Lyon. Parents/guardians gave their written informed consent for the biological studies performed with the tumor samples.

Patients' characteristics are described in the supplementary data (Table S1). Male to female ratio was 8:9. Median age at disease onset was 3.4 years (range: 0,4–10,6 years). Tumour location was infratentorial in 11 of 17 patients. Median follow-up of the patients was 42 months (range: 19–96 months). Evaluation of the extent of resection was based on the surgeon's report and post-operative contrast enhanced imaging. External beam irradiation protocol consisted of a local irradiation with surimpression on the operating site. Total radiation doses varied from 50 to 55 Gy and conventional fractioning was used for all irradiated patients. Almost all patients who received chemotherapy were treated according to the BBSFOP protocol [10] except two patients who received fotemustin alone and etoposide alone respectively. When considering the treatment received before a given relapse, we analysed the entire therapeutic sequence between diagnosis and this relapse. Three groups of treatment were considered: surveillance only, chemotherapy only, or irradiation with or without chemotherapy.

Relapse was defined in fifteen patients as a local recurrence of the tumour. In one case, the relapses were loco-regional metastases in the same cerebral hemisphere (Patient 15). In one patient, the relapses were spinal and supra-tentorial metastases of an initially posterior fossa tumour (Patient 3). The median delay between diagnosis and recurrence was 22 months (range: 2.2 – 62.4).

Histological diagnosis and tumour grading review were performed by two independent neuropathologists (PV and FA). Subependymomas and myxopapillary ependymomas were excluded from the study. Before nucleic acid extraction, sections from frozen tumour samples were colored with hematoxylin to discard those containing necrosis or calcifications.

Nucleic acid isolation

DNA and RNA were extracted from frozen samples with the Microkit (Qiagen). On the forty-four samples studied, eighteen were previously analysed by BAC array-CGH [30]. RNA quality was assessed by 2100 BioanalyzerH (Agilent Technologies). Quality criteria included 28S/18S ratio .1.2 and RIN (RNA Integrity Number) .8.

Gene expression array

For each patient, relapses were co-hybridized against their corresponding tumour at diagnosis which served as reference. Probes from tumour tissue and from the reference tissue were differentially labeled by the incorporation of cyanine 3 (Cy3) and cyanine 5 (Cy5) (Dual Color 44K microarray, Agilent Technologies), respectively. Briefly, probes were synthesized from 500 ng of total RNA in two steps according to the manufacturer's instructions. One microgram of purified cRNA from each relapse was mixed with the same amount of diagnosis-tumour cRNA. Hybridizations were performed, in dye-swap, on whole-human-genome 44K oligonucleotide microarrays (product G4112A; Agilent). Feature extraction software provided by Agilent (version 7.2) was used to quantify the intensity of fluorescent images and to apply a Lowess Normalization to correct for artifacts caused by non-linear rates of dye incorporation as well as inconsistencies of the relative fluorescence intensity between some blue and red dyes. All data were imported into Resolver software (Rosetta Biosoftware, Kirkland, WA) for database management, quality control, computational re-combination of dye-swaps, and statistical analysis. Functional analysis was carried out through the Ingenuity Pathway Analysis (IngenuityH System, http://www.ingenuity.com). Microarray data have been posted on Array Express

(IGR_EPENDYMOMA_STUDY_MP ArrayExpress accession number: E-TABM-873, password for reviewer: 1260902888493).

Comparative Genomic Hybridization (CGH) array

DNA was hybridized to 4644K whole-genome Agilent arrays (G4426A). For each sample, 500 ng of DNA were fragmented by a double enzymatic digestion (AluI + RsaI) and checked with LabOnChip (2100 Bioanalyzer System, Agilent Technologies) before labeling and hybridization. Tumor DNA and control DNA matched for sex (Promega) were labeled by random priming with Cy5-dCTPs and Cy3-dCTPs, respectively and hybridized at 65uC for 17 h. The chips were scanned on an Agilent G256BA DNA Microarray Scanner and image analysis was done using the Feature-Extraction V9.1.3 software (Agilent Technologies). Feature-Extraction was used for the fluorescence signal acquisition from the scans. Normalization was done using the ranking-mode method, with default value for any parameter. Raw copy number ratio data were transferred to the CGH Analytics v3.4.40 software for further analysis. Raw data have been submitted to the Array Express database (IGR_EPENDYMOMA_CGH_STUDY_MP ArrayExpress accession number: E-TABM-1023, password for reviewer: 1277231149363). The ADM-2 algorithm of CGH Analytics v3.4.40 software was used to identify DNA copy number anomalies at the probe level. A low-level copy number gain was defined as a log2 ratio .0.25 and a copy number loss was defined as a log2 ratio ,20.25. A high-level gain or amplification was defined as a log2 ratio .1.5. DNA copy number anomalies were plotted by the aCGH software package v1.10.0 using the R statistical language.

Statistical analysis

According to our Gene Expression experimental design, the LogRatios represented the expression changes from diagnosis to recurrence. An initial filtering was applied to retain sequences which appeared as significantly differently expressed (p#0.01) in at least 50% of recurrences studied. This threshold of 50% was more stringent than the 20% cut-off usually used, but this choice was motivated our decision to include in the statistical analysis only probes which were highly relevant. On this probe set, a one-group t-test was carried out to define a common signature. In this context, the test considered mean (LogR) = 0 as the null hypothesis. Group comparisons (localization and treatment) were performed using a two-group t-test or an analysis of variance (in case of groups.2) to define differential signatures. For these analyses, the same initial filtering was first applied before carrying out a one-group t-test on each group, independently. This procedure allowed us to retain only probes which were significantly modified in at least one of the compared groups. Finally, the selected probe sets were pooled for the statistical analysis.

For each signature, the networks/pathways search, and functional analysis were generated trough the use of Ingenuity Pathway AnalysisH. Briefly, each signature, containing probe identifiers and LogRatio values, were uploaded into the application. Agilent probe identifiers were mapped to their corresponding gene objects in the Ingenuity Pathways Knowledge Base. These genes were then overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Identification of biological functions was based on a Fischer's exact test which calculated a p-value determining the probability that each biological function assigned to each signature is due to chance alone.

CGH-array analysis were performed by using the aCGH R package (v1.26.0), and the step down maxT multiple testing procedure of Westfall and Young. Statistical analysis consisted in comparing chromosomal regions imbalances at relapse vs diagnosis, and identifying new abnormalities in recurrences, in general and in association with location or treatment.

Quantitative Real-Time PCR (qPCR)

Approximately 1 mg of total RNA was used to synthesize cDNA using random hexamers and the Mu-MLV reverse transcriptase (Applied Biosystems). qRT-PCR for the genes MT2A, MT3, KIF11 and ASPM was carried out using Taqman Gene Expression Assays on Demand (Applied Biosystems) and ABI Prism 7700 Sequence Detector (Applera). Expression profile in each specimen was assessed by using the comparative threshold cycle (2^{-ddCt}) method. 18S Ribosomal RNA was used as and endogenous control and normal whole brain cDNA (Ambion) as a calibrator.

Methylation Assay

Investigation of methylation status of the MT3 promoter was assessed by combined bisulfate treatment of genomic DNA and sequencing after PCR amplification. One microgram of genomic DNA was treated with bisulfite, which converts the nonmethylated cytosines to thymines, using the CpGenome™ Universal DNA Modification Kit (Chemicon) according to the manufactor's instructions. PCR amplification was accomplished with primers that do not discriminate between methylated and unmethylated alleles that overlap 4 regions covering the promoter, exon 1 and intron 1 of the MT3 gene, as described [34] and also with two additional pairs of primers (sequences available under request).The PCR products were sequenced using the ABI3730 DNA analyser (Applied Biosystems). The methylation status of CpG islands was determined by direct sequencing of both strands and by estimation of the relative peak height of the PCR products. Normal human DNA and methylated DNA were used as reference control.

Primary-culture cells

In the absence of an available ependymoma cell line, we used short term cell cultures derived from 2 pediatric ependymomas operated at Necker Sick Childrens Hospital in Paris. Parents/guardians gave written informed consent for research according to the policy of the Internal Review Board of the Biological Ressource Center of Necker Sick Childrens Hospital. Right after surgery, tissues were suspended in DMEM cell culture medium and transferred to the laboratory. After mechanical dissociation, tumour cells were seeded in a 25 cm^2 flask and maintained in AminioMAX C-100 supplemented medium (Invitrogen) in a tissue culture incubator. Subcultures were processed when cells achieved 80–90% confluence. These primary culture cells were designated as EP1 and EP2. The glial nature of the cultured cells were assessed by morphology and expression of GFAP on immunocytochemistry (Figure S1).

Epigenetic regulation of metallothioneins expression in vitro

Primary cell cultures of ependymoma and DAOY medulloblastoma cell line (ATCC) were added to 60 mm dish at a density of 5610^5 cells and incubated overnight in a 5% CO_2 incubator. The following day they were treated with 5 mM of 5-Aza-desoxyCytidine (5-Aza), a demethylating agent, for 3 to 7 days (accordingly to the proliferation rate/doubling time of each cell type) or with 300 nM of TSA, a histone-deace´tylase inhibitor, for 16 hours. For

the combination 5-Aza-dC/TSA treatments, 5-Aza-dC treatment in the same conditions were performed first, followed by identical TSA treatment. Every day, new medium containing freshly prepared 5-Aza was added. At the end of the incubation period, after medium removal, cells were lysed in RTL buffer. RNA was extracted using the RNeasy mini kit (Qiagen) for analysis by qPCR of MT2A and MT3 gene expression levels.

In vitro regulation of MT3 gene by metal cations and steroids in brain tumor cells Immunohistochemistry

Anti-ASPM affinity purified rabbit polyclonal antibodies were purchased from Bethyl Laboratories Inc (Montgomery, Texas) (reference IHC-00058). Anti-MT3 affinity purified rabbit polyclonal antibody was obtained from Dr Donald Sens (Professor of Pathology, University of North Dakota, School of Medicine and Health Sciences, Grand Forks, ND); their preparation and use on formalin-fixed, paraffin embedded material have been described previously (38, 39). Sections were cut at 4 mm, deparaffinized, exposed to 30 minutes treatment in a steamer at 98uC in citrate pH 7,3 buffer for ASPM and pH 6,0 buffer for MT3 and then treated with a peroxidase blocking agent (reference S2001, DAKO, Glostrup, Denmark). Antibody incubation was performed overnight at 4uC for ASPM (1:100) and 60 minutes hour at room temperature for MT3 (1:1000). Antibody binding was visualized with the peroxidase-based anti-rabbit EnVision Kit™ (reference K4003, DAKO) for both antibodies. Diaminobenzidine tetra hydrochloride (DAB, DAKO) was used as chromogen. Sections were counterstained with Mayer's hematoxylin.

Immunohistochemical staining for MT3 was scored semiquantitatively, based on staining intensity and cell number, as follows: 0, no staining; 1, weak staining (independently of the number of positive cells) or staining in less than 10% of cells (independently of the staining intensity); 2, moderate to strong staining in more than 10% of cells. Scoring was performed as of observed in the most positive areas. MT3 generally stained both nucleus and cytoplasm (Figure S2). Staining for ASPM was analysed at high power view (x1000), and scoring was performed as follows: 0, no staining; 1, staining in scarce cells, 2, staining in numerous cells. Following staining patterns were observed for ASPM: cytoplasmic, nuclear, presence of paranuclear ''dots'' or marked cells in mitosis (Figure S3).

Tissue micro array

Tissue microarray blocks from ependymoma patients treated with the BBSFOP protocol were built [10]. For each patient, all paraffin blocks and corresponding slides were obtained and reviewed by two neuropathologists (PV, FA) for diagnostic accuracy and tissue adequacy. Sonic aspirator extracts were excluded from the study. Ependymomas were graded based on WHO 2007 criteria. Histopathological findings (ependymal differentiation, necrosis, endothelial proliferation, mitotic index, anaplasia) were evaluated and recorded for each tumour. Immunostainnings for EMA (1:1, clone E29, DAKO), GFAP (1:200, clone 6F2, DAKO), OLIG2 (1:100, RnD systems, Abingdon, UK) and Neurofilament Protein 70 (1:50, clone 2F11, DAKO) were performed for selected cases. Tumour material was available at diagnosis and at recurrence for 24 patients. There was a total of 29 tumours at recurrence including 17 patients with one recurrence, 6 patients with two recurrences (n = 12) and 1 patient with 3 recurrences (n = 3). Three to four 600 mM-cores were obtained from each tumour. Representative areas were selected whenever present: classical ependymal differentiation (ependymal rosettes, perivascular pseudo-rosettes, and ependymal channels), anaplasia and high vascularisation zones. Normal adult and fetal brain samples were included as internal controls. Frequencies of positivity of MT3 and ASPM at first recurrence were compared to frequencies at diagnosis by McNemar test for paired data, taking into account the intra-patient correlation.

Results

Copy number abnormalities with CGHarray

Considering the whole patient population, there was no statistically significant increase in copy number abnormalities from diagnosis to the relapse. The most frequent chromosomal changes between the diagnosis and the relapse were losses of the short arm of chromosome 3 and the long arm of chromosome 6; only the locus 6q25.2 (RBM16, NM_014892) being statistically significant (Figure S4). Copy number changes in 19 regions on chromosome 9 discriminated supratentorial and posterior fossa tumors (Figure S5, Table S2). There was no specific chromosomal copy number variation according to the type of treatment received, albeit loss on chromosomes 3p and 6q were more frequent after radiotherapy (Figure S6).

Gene expression profiling

We first determined the number of gene expression probe sets differentially expressed between recurrences and initial tumours. These signature volumes were found to be highly variable, ranging from 374 to 18814 probe sets (median: 6275 probes – mean: 9054 probes). The number of probes differentiating the recurrence from its corresponding initial tumour could not be statistically correlated with age at onset, location of the tumour or treatment received but was only linked to the delay between the diagnosis and the relapse. For recurrences occurring before 22 months (ie , to the median delay of recurrence), mean signature included 4799 probes versus 9058 for recurrences that appeared after 22 months ($p = 0.013$, Student t-test).

To study the molecular signature of the 27 relapses, we used a hierarchical unsupervised clustering for 41000 probes present on the arrays. Recurrences from the same patient were found to be clustered together in 6 out of 7 patients who had experienced several recurrences (Figure 1). Pearson's correlation coefficient between gene expression profiles of recurrences of the same tumour ranged between 0.4191 and 0.8303 (median: 0.5492). As illustrated in the upper lines on Figure 1, localization and adjuvant therapy were not associated with the clustering of recurrences based on their specific expression profile.

Differences in the recurrence signature according to location

This analysis allowed the identification of 197 genes differentially expressed between the two type of recurrence according to localization (Table S3). A clear difference between frequencies in gene expression according to location was observed. Figure 2A shows the genes most frequently upregulated at recurrence according to the location of the primary tumor. In PF relapses, the ribosomal proteins were the most represented (12 genes). The most abundantly upregulated genes in the relapses of ST ependymomas were involved in cytoskeleton organization (gelsolin, SEMA5A, contactin-1, sarcoglycan, villin-like, scinderin) and extracellular matrix/cellular interactions (gliomedin, EXTL1, galectin-9, desmuslin, tetranectin, versican, COL21A1, COL16A1, CXCL12). A functional analysis of each group signature revealed that the main functional networks associated with posterior fossa relapses were cell cycle, cellular assembly and organization plus DNA Replication, Recombination and Repair

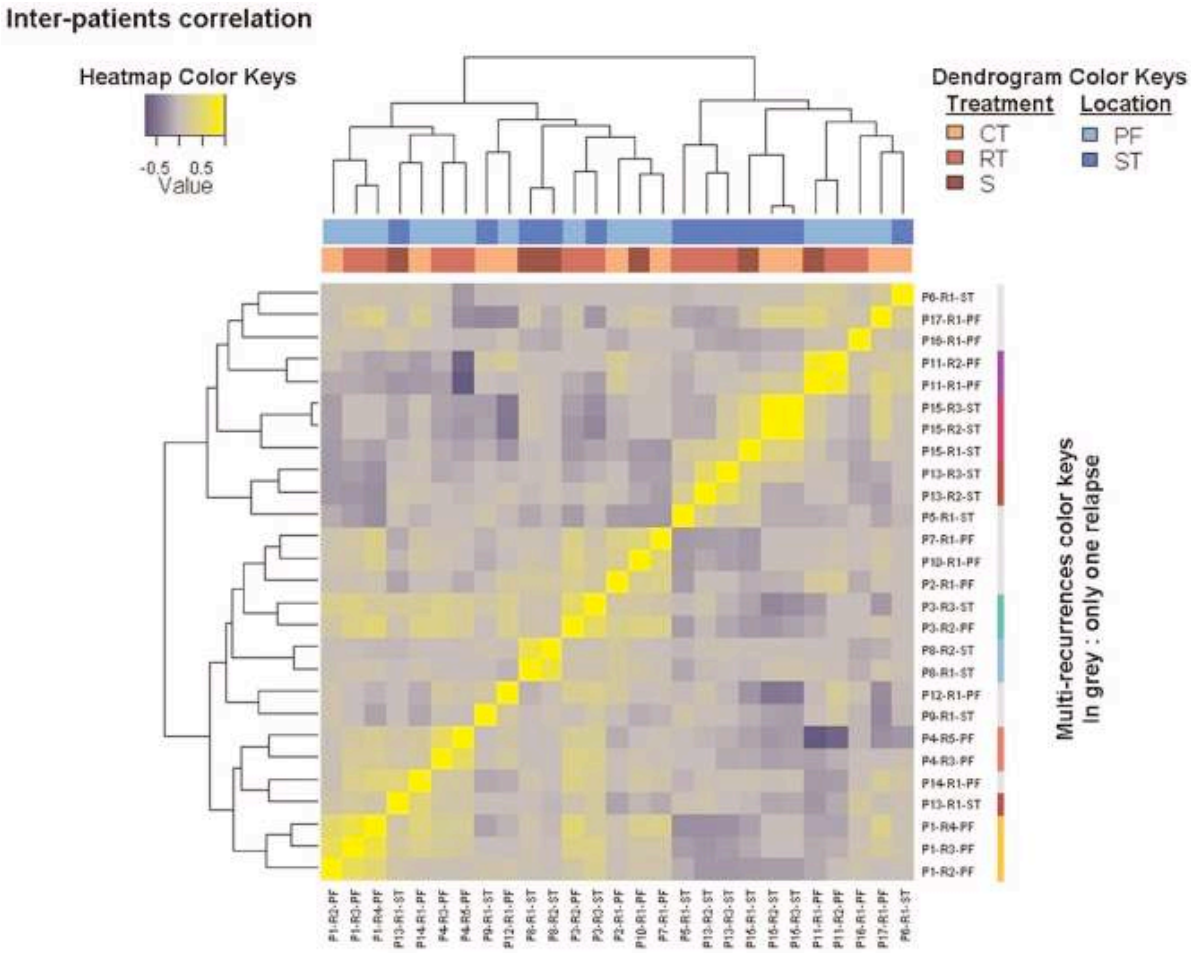

Figure 1. Correlation matrix of the gene expression signatures of the recurrences. After a low-stringent initial filtering (p#0.01 in at least 20% of experiments), a subset of 29783 probes was used to measure the correlations between relapses (Pearson correlation). As expected, and because each relapse was co-hybridized with its own reference, 6 of the 7 multi-recurrence patients clusterized together. There was however no similar evolution of profiles according to relapse locations or to the treatments received before the recurrence. PF = posterior fossa, ST = supratentorial, RT = radiotherapy, CT = chemotherapy, S = surveillance.
doi:10.1371/journal.pone.0012932.g001

(Figure 2B). On the other hand, molecular transport and cell death were evidentiated in the ST relapses.

Although tumor location was not statistically discriminant for the recurrence signature as shown above, our results indicate that progression pattern of supratentorial ependymomas may differ from the one of posterior fossa ependymoma by the overexpression of genes involved in the mesenchymal transition. Conversely, ependymoma recurrences in the posterior fossa progress more often with the overexpression of genes associated with ribosomal functions.

Differences in the recurrence signature according to treatment

Specific gene expression profiles of each group (surveillance/chemotherapy only/radiotherapy +/2 chemotherapy) (Table S4) failed to identify differentially expressed genes between the chemotherapy and the surveillance group; 58 genes appeared significantly modified in relapses after RT compared to relapses after chemotherapy or surveillance (Figure 3). Recurrences occurring after RT were characterized by downregulation of three potential tumor suppressor genes NKX2-2, YWHAE and WWOX (Student t-test, p,0.01) by at least ten fold and upregulation of HES-2, a known target of NOTCH pathway. Treatment received before recurrence had thus only limited influence on the differential gene expression signature.

Common signature of recurrences

Considering the limited gene expression signature differences (number of probes and genes) we could identify as influenced by the location of the tumor or by the type of treatment received before the recurrences, we decided to analyse all recurrences together in order to find common genes associated with progression. Considering the poor correlation between the different relapses of the same patient (median Pearson's correlation coefficient = 0.5492), we decided to include in the analysis all the relapses of patients with multiple relapses.

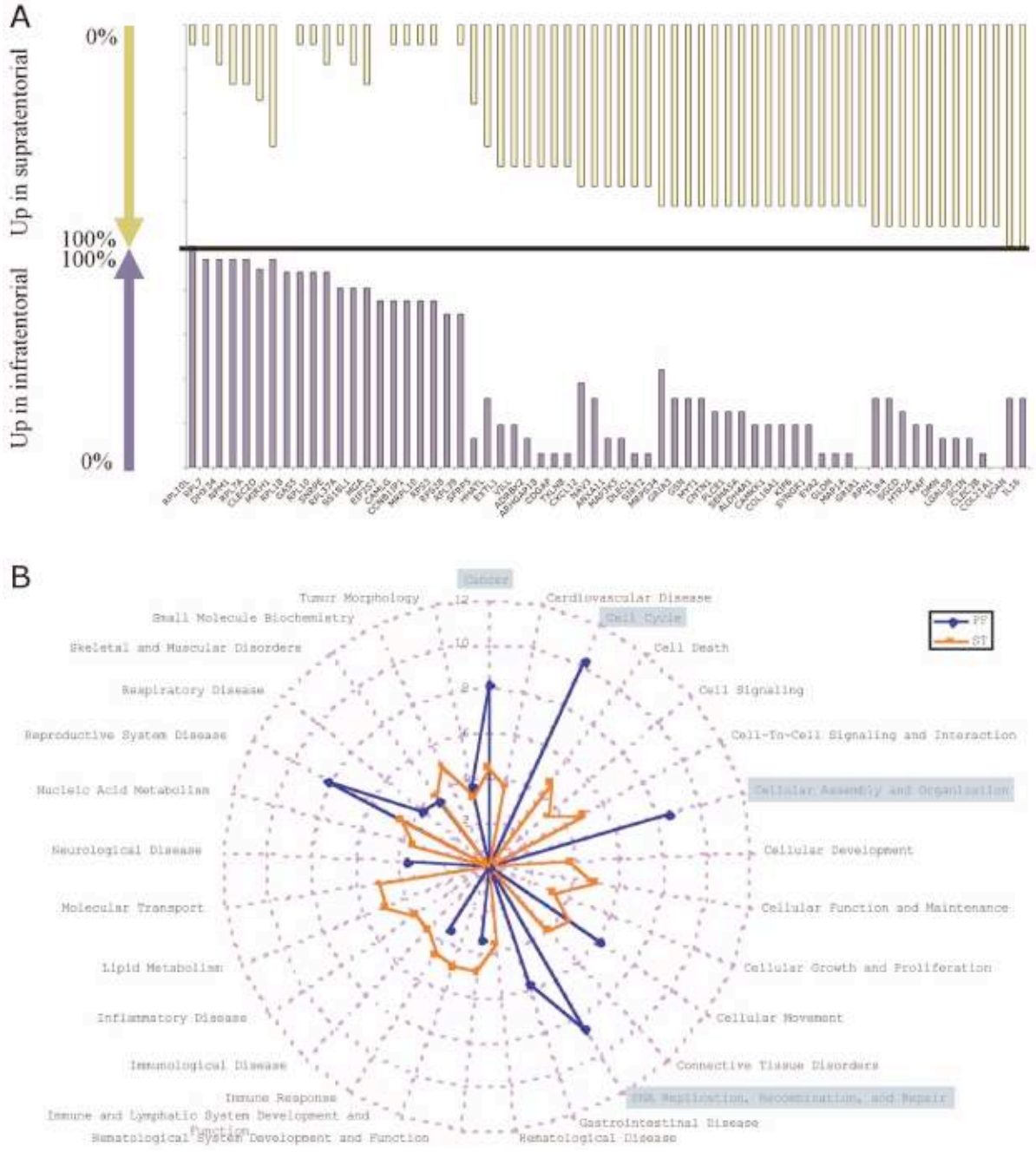

Figure 2. Comparison of functions associated with location of relapses. A Gene most frequently upregulated at relapse for supratentorial (upper yellow panel) are represented with the genes most frequently upregulated in infratentorial tumors (lower blue panel). Bars indicated the percentage of tumors in each location with upregulation of the specific gene. B The -Log$_{10}$(p-values) of the most discriminatory functions in each group are represented. The p-value for a given function was calculated using the right-tailed Fisher Exact Test by considering 1) the number of uploaded functional analysis molecules that participate in that function, and 2) the total number of molecules that are known to be associated with that function in Ingenuity's knowledge base.
doi:10.1371/journal.pone.0012932.g002

To identify the specific genes associated with tumour progression, we chose to consider probes with a significant LogRatio (Relapse/diagnosis, p-value#0.01) in at least 50% of the samples. This filter selected 7384 of the 41 000 initial probes. A one-group t-test was then carried out on this subset of probes by considering LogRatio = 0 as the null hypothesis. The 298 probes identified

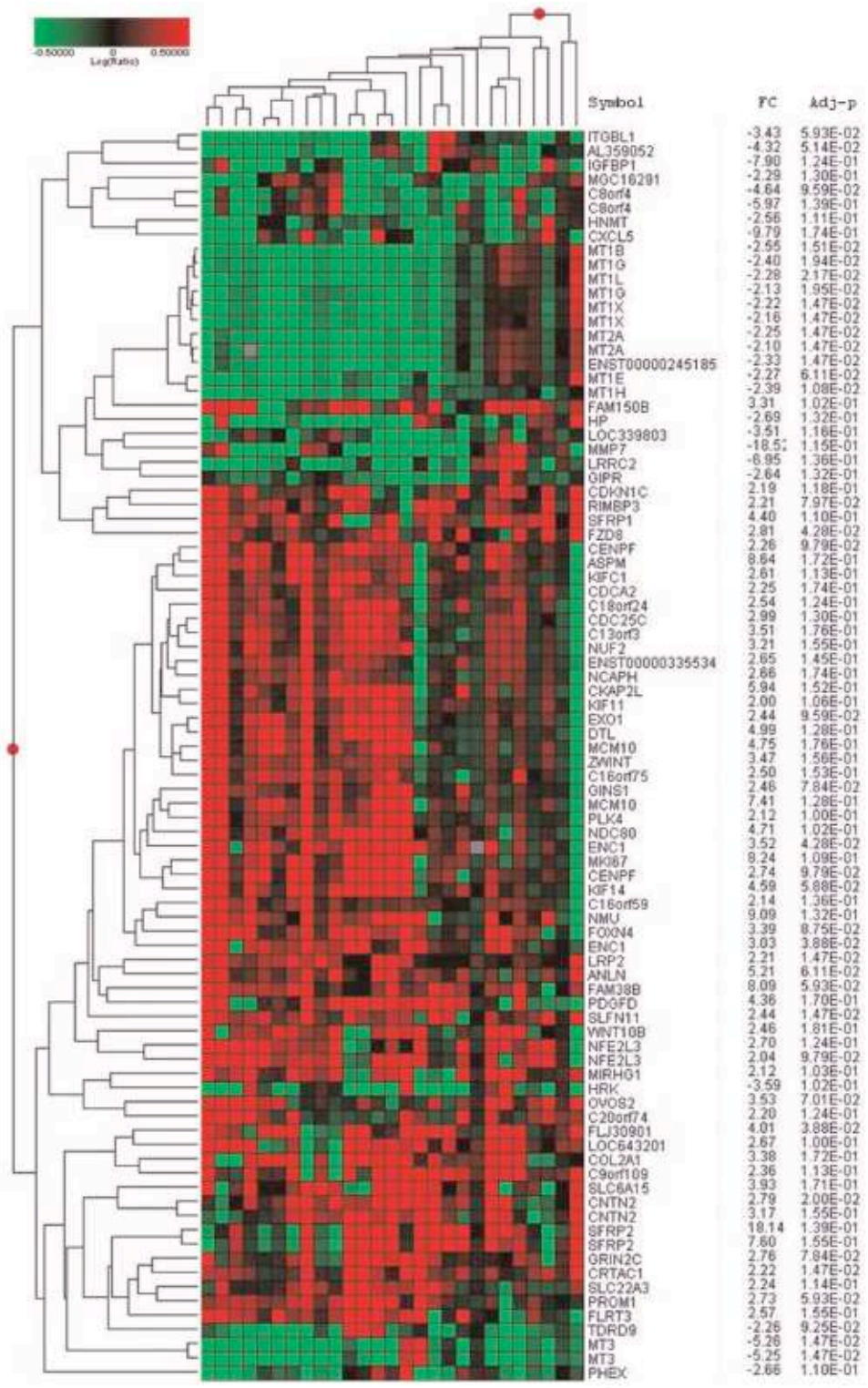

Figure 3. Supervised hierarchical clustering of differentially expressed genes in ependymoma relapse compared to diagnosis.
Heatmap showing the 87 genes signature of the genes statistically up- or down-regulated in more than 50% of relapses with a fold change .2. Notice the homogeneity of the underexpression of the metallothioneins.
doi:10.1371/journal.pone.0012932.g003

were then analyzed in the IngenuityH database: 240 sequences were mapped, ie related to known genes, 165 were network eligible and 146 were pathway-eligible. This subset of 146 genes was defined as the common signature of ependymoma recurrences (Table S5). A reduced 87-genes signature of specific genes associated with tumour progression is represented in Figure 4 and corresponds to the genes differentially expressed with a fold change $2 in at least 50% of the recurrences. This signature is characterized by the activation of the Wnt pathway with overexpression of the following genes SFRP1, SFRP2, FZD2, FZD8, WNT10B besides the upregulation of the stem cell marker CD133 (PROM1) and the proliferation antigen identified by the monoclonal antibody Ki-67 (MKI67). Two other groups of genes were very homogenously differentially expressed in relapses. The first one corresponds to proteins of the kinetochore (KIF14, KIF11, KIF1C, KIF2C, PRC1, BUB1B, ZWINT, ASPM, KNTC2, CENPF), all significantly upregulated. The second one is the group of metallothioneins (MT1L, MT1G, MT1E, MT1X, MT1B, MT2A, MT3) found to be downregulated in 65 to 85% of relapses depending on the MT. MT3, also known as neural growth inhibitory factor, was the most frequently downregulated gene among metallothioneins. The expression of the proliferation marker Ki67 was inversely correlated with MT3 at relapse (Pearson's correlation, r = 20.51, p,0.0001).

Several genes involved in the immune system were found to be downregulated in the common signature of recurrence: CXCL5, CX3CL1, TRAF3IP2, ITGBL1, SERPING1, IFT20, ENTPD3, HP and HPR. Conversely, TIA1, a RNA-binding protein with nucleolytic activity against cytotoxic lymphocytes, was significantly overexpressed (adj. P value = 0.005).

Validation of microarray data by qPCR and immunohistochemistry

Three genes expressed differentially in relapses compared to diagnosis were chosen for further analysis. qPCR analysis were performed for the genes KIF11, ASPM and MT3 in the tumours previously analyzed by gene-expression microarray. The heat-map on Figure 4A illustrates the correlation between microarray and qPCR results. These analyses confirmed the progressive upregulation of the genes KIF11 and ASPM at recurrence. For the MT3 gene, the results showed a low expression level at diagnosis that tended to become even lower throughout relapse. Progressive down-regulation of the MT3 gene expression during progression was thus confirmed for 12 of the 17 (70.5%) patients. Among the 5 patients whose MT3 gene expression was stable or increased during progression, 3 had expression levels below the one of normal brain (Figure 4B). To verify the microarray data at the protein level, immunohistochemistry for ASPM and MT3 was performed on 7 patients among the 17 studied in microarray. The same trend of decreasing MT3 and increasing ASPM staining was confirmed (Figure 5).

Immunodetection of MT3 and ASPM expression in a independant cohort of pediatric ependymomas

To confirm the changes in expression of MT3 and ASPM in an independent cohort, we studied the expression of these two genes on a TMA of childhood ependymomas composed of 24 tumours at diagnosis with at least one relapse. Among those 24 patients, 23 had a posterior fossa tumour and 1 a supratentorial tumour. Seventeen patients (70.8%) displayed a weaker expression of MT3 at relapse compared to diagnosis (Table 1), 13 of which becoming negative for MT3 during progression. Two patients were negative at both diagnosis and relapse. Four had a stable positive expression of MT3 over all samples, and only one patient had a stronger expression of MT3 at recurrence compared to diagnosis. ASPM staining was stronger at relapse compared to diagnosis in 12 patients (50%), being even negative at diagnosis in ten of then (Table 2). Among the other 12 patients, ASPM staining was identical at diagnosis and relapse, either negative (3 patients) or positive (9 patients). Frequency of positivity was significantly different at diagnosis comparing to relapse for both markers (MT3: p = 0.002 and ASPM: p = 0.03, McNemar test).

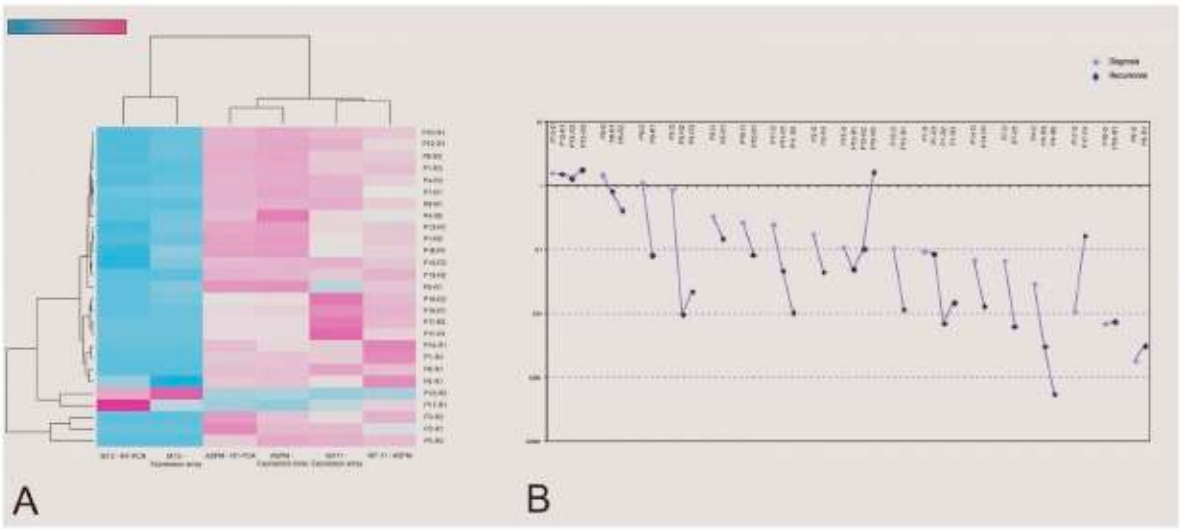

Figure 4. Confirmation analyses (internal validation of gene expression). A qPCR Heatmap showing expression of 3 candidate genes (MT3, KIF11, ASPM) in gene expression array and RT-PCR side to side. Pearson correlation coefficients between the two analyses are indicated. B Evolution of MT3 expression throughout progression. RT-PCR levels are given as Log scale compared to normal brain.
doi:10.1371/journal.pone.0012932.g004

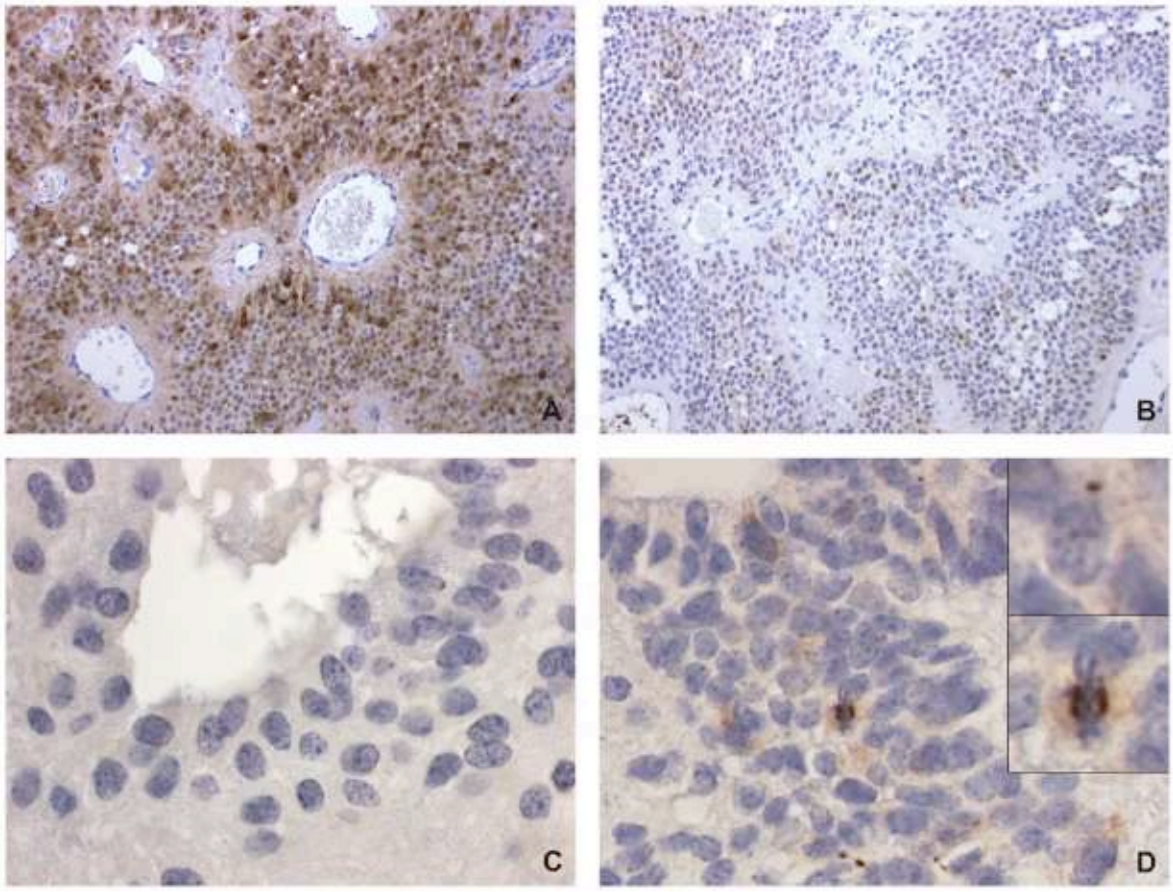

Figure 5. MT3 and ASPM immunostains differ at diagnosis and relapse. (A) Strong nuclear and cytoplasmic staining for MT3 at diagnosis. (B) At relapse the same patient shown at A displayed only weak MT3 staining. Another example of paired tumours, for which ASPM immunostaining was negative at diagnosis (C) and positive at relapse (D). Please also note paranuclear dots and a marked cell in mitosis (D, inserts), two patterns typically observed in ASPM immunostains, together with weak cytoplasmic staining.
doi:10.1371/journal.pone.0012932.g005

Mechanism of regulation of metallothionein in ependymomas

Metallothioneins being the most homogeneously downregulated genes at relapse, we decided to investigate the possible mechanisms of their repression at the genetic and epigenetic levels.

Considering that all MT genes are clustered on chromosome 16q13, we first verified a possible deletion of this chromosome region. CGH array analysis did not show a loss for this chromosome region at relapse (Figure S5). To rule out the possibility of a small genomic deletion missed by CGHarray analysis we carried out quantitative PCR analysis for the MT2A gene. Amplification products could be obtained in all 37 samples tested with a CT corresponding to the one of normal DNA reference (Figure S7).

The absence of DNA deletion prompted us to investigate the regulation of gene expression at transcriptional level. We first verified whether genes known to interact with the MT gene promoters were differentially expressed at relapse. None of the transcriptional activators (MTF1, USF1, NF1, STAT3, IL6) was found to be down-regulated at relapse compared to diagnosis. None of the transcriptional repressors (SIN3A, SIN3B, MTA1, HDAC1) was found to be upregulated.

Since differential expression of regulatory factors could not explain MT3 downregulation, we investigated whether epigenetic factors and especially CpG islands methylation, were implicated in MT3 down-regulation at relapse. If methylation would be the cause of MT3 downregulation, more methylated CpG islands should be observed at relapse. We observed limited to no methylation of the 74 CpG islands in regulatory regions and intron 1 of MT3 and no increasing methylation at relapse (Figure 6A). None of the few methylated sites was correlated with gene expression measured by qPCR (Figure 6B). To confirm this data, ependymoma primary cells (EP1 and EP2) were treated with the demethylating agent 5-Aza-DeoxyCytidine (5-Aza) followed by MT3 and MT2Agene expression analysis by qPCR (Figure 6C). DAOY, a medulloblastoma cell line was used for comparison. The 5-Aza treatment alone induced a small increase in MT2A expression level in all cells tested (1.8, 2.2 and 5.7 fold for EP1, EP2 and DAOY respectively). The 5-Aza treatment alone did not increase MT3 expression in the ependymoma cells, while a 2 log increase in expression was found for DAOY. This data confirms the CpG islands methylation results, since the EP1 and EP2 exhibit very few methylated sites on the contrary to DAOY cells which harbor a hypermethylated pattern on MT3 promoter region (data not shown).

Table 1. Metallothionein 3 immunohistochemical expression in ependymomas at diagnosis and relapse.

Evolution	Pattern	Patient	D	R1	R2	R3
Decreased	++/-	A	++	-		
	++/-	B	++	-		
	++/-	C	++	-		
	++/-	D	++	-		
	++/-	E	++	-	-	
	++/-	F	++	++	-	-
	++/-	G	++	+	+	-
	+/-	H	+	-		
	+/-	I	+	-		
	+/-	J	+	-		
	+/-	K	+	-		
	+/-	L	+	-		
	+/-	M	+	+	-	
	++/+	N	++	+		
	++/+	O	++	+		
	++/+	P	++	+		
	++/+	Q	++	++	+	
Stable	++/++	R	++	++		
	++/++	S	++	++		
	++/++	T	++	++	++	
	+/+	U	+	+		
	-/-	V	-	-		
	-/-	W	-	-	-	
Increased	+/++	X	+	+	++	

D = tumor at diagnosis, R1 = first relapse, R2 = second relapse, R3 = third relapse, ++ = medium to strong staining, + = weak staining, – = negative.
doi:10.1371/journal.pone.0012932.t001

Table 2. ASPM immunohistochemical expression in ependymomas at diagnosis and relapse.

Evolution	Pattern	Patient	D	R1	R2	R3
Increased	-/+	A	-	+		
	-/+	D	-	+		
	-/+	S	-	+		
	-/+	F	-	+		
	-/+	H	-	+		
	-/+	E	-	-	+	
	-/+	M	-	-	+	
	-/+	Q	-	-	+	
	-/+	F	-	-	+	+
	-/+	G	-	-	-	+
	+/++	U	+	++		
	+/++	V	+	++		
Stable	-/-	B	-	-		
	-/-	C	-	-		
	-/-	I	-	-	-	
	+/+	L	+	+		
	+/+	N	+	+		
	+/+	O	+	+		
	+/+	P	+	+		
	+/+	K	+	+		
	+/+	R	+	+		
	+/+	S	+	+		
	+/+	W	+	+	+	
	+/+	T	+	+	+	

D = tumor at diagnosis, R1 = first relapse, R2 = second relapse, R3 = third relapse, ++ = medium to strong staining, + = weak staining, – = negative.
doi:10.1371/journal.pone.0012932.t002

To test if epigenetic inactivation of MT genes could be due to histone acetylation, cells were also treated with trichostatin A (TSA), a deacethylating agent, alone or in combination with 5-Aza (Figure 6D). On the other hand, TSA proved effective in increasing dramatically MT3 expression in all tested cells: 135 fold for EP1, 198 fold for EP2, and 73 fold in the DAOY. MT2A expression level did not change significantly in the ependymoma primary and in DAOY cells after TSA treatment. Combination of 5-Aza and TSA was more effective than either agent alone in increasing MT3 levels in EP2 and in DAOY cell lines. MT2A levels in EP cells were significantly increased after a treatment combining 5-Aza and TSA compared to either treatment alone.

Epigenetic modulation of EP cells in vitro confirmed that MT3 expression was not regulated by promoter methylation but more likely by histone acetylation status. On the contrary, MT2A was not regulated only by histone acetylation status but also by methylation.

The MT3 promoter presents many regulatory elements, such us MRE (Metal Responsive Element) that allows MT induction by metal cations through MTF1 transcription factor and GRE (Glucocorticoid Responsive Element) for glucocorticoids induction. To check chromatin accessibility, we treated ependymoma primary cells EP1 and EP2 and DAOY cell line with $ZnSO_4$ and dexamethasone (Figure 6D). Both agents were able to induce MT2A expression in all cells tested. Nevertheless MT3 was induced by $ZnSO_4$ in only one of the two EP cells and in DAOY. Dexamethasone did not induce a significant upregulation of MT3 in any of the three cell lines tested. These results suggest that the MRE in the promoter of MT3 is not always accessible in EP cells in vitro.

Discussion

Despite several molecular studies, the oncogenesis of ependymoma remains elusive. Specific molecular events occurring during progression have only been seldom reported [30,35]. This work focused on recurrence-specific gene expression signature variations. We choose a dual color microarray system in order to maximize the likelihood to discover significant changes in gene expression. The tumour of the patient at diagnosis was used as the reference and marked with Cy5 and the tumour of the same patient was marked with Cy3 and co-hybridized competitively. Consequently, only real changes in gene expression occurring at relapse were detected. As ependymoma's cell of origin remains uncertain, we also thought that most of the gene expression studies have suffered from the lack of specificity of the reference mRNA used. When comparing the tumour with the normal brain, most of the genes that are overexpressed correspond to cellular processes linked to proliferation while most of the genes downregulated correspond to neuronal proteins. Moreover, it has been recently assumed that glial tumours display brain-region specific expression profiles regardless of the tumour histology [1,12,14,36]. To

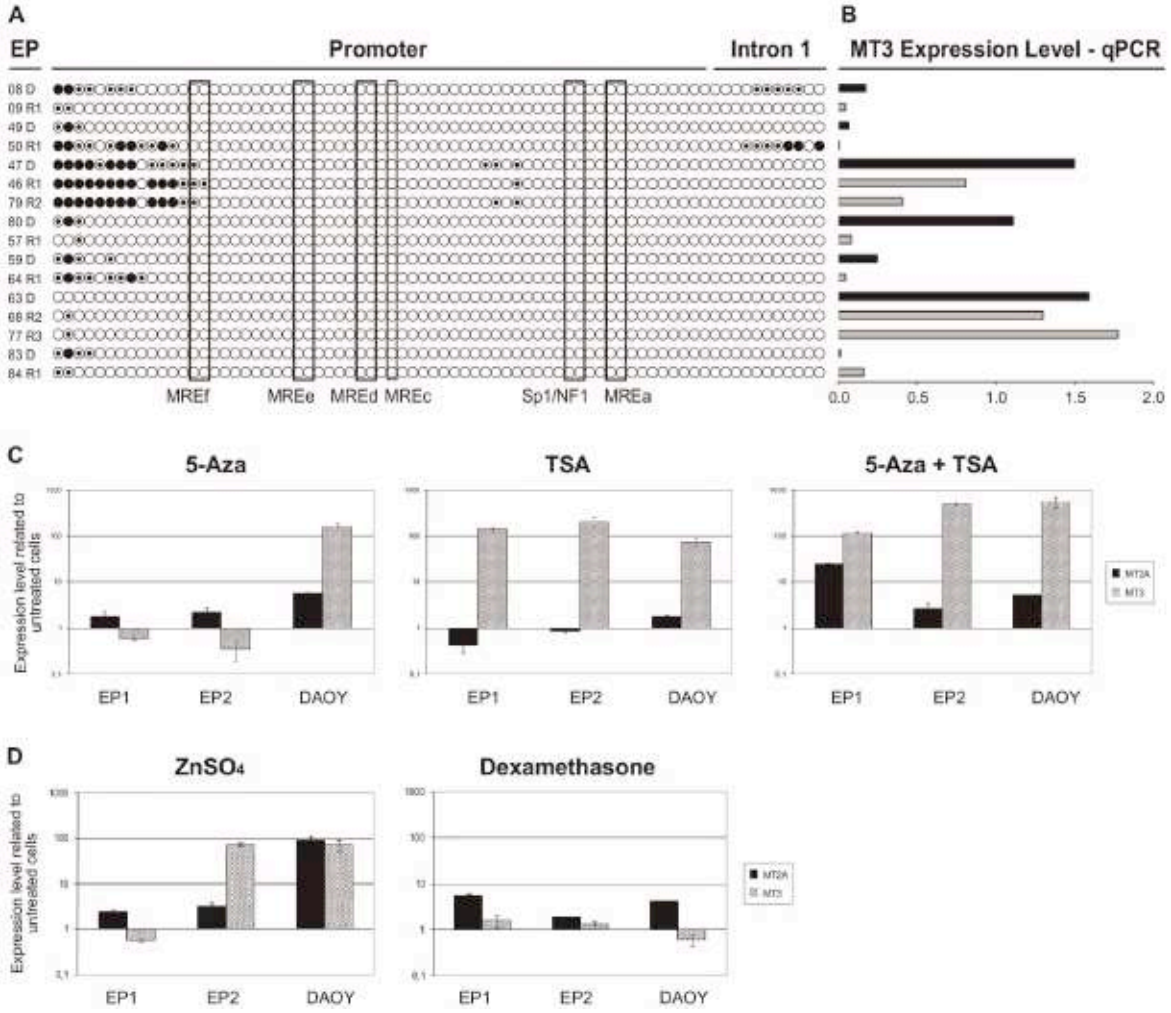

Figure 6. A: Methylation status of 74 CpG sites at MT3 promoter and intron 1. Each row of circles represents one EP sample sequenced from PCR products generated from amplification of bisulfite-treated DNA. Empty circles = unmethylated cytosines; Dotted circles = hemimethylated cytosines; Dark circles = methylated citosines. B: MT3 expression analysis by quantitative PCR in the corresponding ependymoma tumors. Samples with results under 1.0 are downregulated and those over 1.0 are upregulated compared to normal brain. The black bars correspond to sample at diagnosis, and the grey bar to relapse. Each histogram represent the corresponding sample studied for methylation. C: Epigenetic modulation of MT2A and MT3 expression on short term ependymoma cultures EP1 and EP2 and medulloblastoma cell line DAOY as control. Demethylation by 5-Aza-Deoxycytidine (left panel). Histone deacetylation inhibition by Trichostatin A (middle panel). Combined treatments (right panel). D: Treatment with zinc sulfate restores the expression of MT3. MT2A and MT3 expression level after 24 hours of 200 mM of ZnSO4 (left panel) and 5 microM of dexamethasone (right panel) treatments in the ependymoma primary culture cells EP1 and EP2 and in the medulloblastoma cell line DAOY.
doi:10.1371/journal.pone.0012932.g006

analyse properly gene expression changes compared to the control, one should therefore use the normal brain control from the same location as the tumor. The brain region-specific expression pattern should remain stable over time between diagnosis and local recurrence. It is thus not surprising that none of the genes described as location–specific at diagnosis appeared in our recurrence signature.

Microarray studies conducted to discover molecular pathways linked with tumour progression and papers comparing metastases to initial tumours have already been performed [37–41]. The use of paired samples alleviate the bias associated with interindividual variation; for example, comparison of expression profile of tumours prior to and following systemic chemotherapy allowed the identification of differentially expressed genes correlated with chemoresistance in ovarian carcinomas. [37] In addition, paired samples by reducing variability increase strikingly the statistical power of the study [42].

Microarray analysis of recurrence-specific expression changes demonstrates the existence of a common signature for recurrence in ependymoma. This signature pinpoints pathways already described in other ependymoma studies focusing on tumours at diagnosis such as the Wnt and the Notch pathways [12,14,30]. The common signature at relapse also unveils several genes already described in the death-from-cancer signature [43]

including MKI67, KNTC2 (HEC1) and BUB1B. These last two genes belong to a broader group of molecules overexpressed at relapse in ependymoma and playing a role in spindle formation. Several kinetochore molecules were found in our signature and have already been described as prognostic markers in other tumours: KNTC2 in lung cancer and kinesin KIF14 in breast and lung cancer [44–46]. The spindle molecule ASPM has been shown to be involved in the malignant progression of gliomas possibly through expansion of a cancer stem cell compartment [47]. Beside their prognostic value, these molecules may also represent new therapeutic targets. Classic spindle poisons target tubulin and have not demonstrated their efficacy in ependymoma. But new chemotherapies, known as kinesin spindle inhibitors, are actually under evaluation. Among them, monastrol, a kinesin Eg5 (KIF11) inhibitor, has already demonstrated its efficiency in glioma in vitro [48–50] and is currently under clinical development.

Apart from upregulation of genes associated with proliferation, the key event associated with recurrence in our common signature was down-regulation of metallothioneins, especially MT3. The metallothioneins are small proteins that posses about 60 amino acids, with a high level of cysteines that confers to then the ability to bind divalent metals. Metallothioneins function as metals reservoirs, maintaining metal homeostasis and contributing to heavy metals detoxification, phenomenon that can lead to chemoresistance in some cancers, [51–53] and scavenging free radicals [54]. In mammals there are four groups of MT proteins: MT1, MT2, MT3 and MT4, that are coded by a family of genes clustered on chromosome 16q13. MT2 protein coded by MT2A gene accounts for 80% of the MTs proteins. The MT1 and MT2 are ubiquitously expressed. MT3 was first detected in the brain of patients with Alzheimer's disease, identified as a factor inhibiting neuronal growth in culture and called neural GIF (growth inhibitory factor) [55]. MT3 is expressed predominantly within the CNS and has been found both in neurons and in astrocytes [56]. MT3 is expressed at a lower level in other tissues such as kidney [57,58]. MT4 is specifically expressed in the stratified squamous epithelium [59]. A number of studies have shown enhanced synthesis of MTs in proliferating tissues suggesting its crucial role in normal and neoplasic cell growth [60] but their precise role in carcinogenesis is still unclear, once they can also act as oncosupressor [61]. In several carcinomas indeed, metallothioneins are downregulated compared to the tissue of origin [34,62–65]. In ependymoma, the expression of MT 1-2 has been studied at diagnosis by immunohistochemistry; MT1-2 positivity was statistically more frequent in low grade ependymomas and was associated with a better survival [66]. To the best of our knowledge, our study is the first one focusing on MT3 and displaying immunostains for MT3 in brain tumors. Of note, in our controls consisting of normal adult and fetal brain we observed prominent immunostaining for MT3 in astrocytes but no staining in neurons (Figure S2). Since no genomic loss was observed on the chromosome 16q13 region in our ependymoma samples, this downregulation was more likely to be linked to transcriptional inactivation.

Former descriptions of MT3 inhibition by promoter methylation [34] prompted us to perform a methylation assay on the MT3 gene in ependymomas. No significant hypermethylation was observed, even if we consider exclusively the intron 1, reported to be the region abnormally hypermethylated associated with low MT3 expression in gastric carcinoma cells [44]. The inability of 5-Aza to restore MT3 expression in EP cells confirmed these data.

Since all metallothioneins were homogeneously downregulated at recurrence compared to diagnosis, we hypothesized that chromatin changes in the 16q13 region of the MT genes cluster could explain their repression. Histone deacetylases can regulate expression of tumor suppressor genes and activities of transcriptional factors involved in both cancer initiation and progression through alteration of either DNA or the structural components of chromatin. We therefore used the prototypic histone deacetylase inhibitor TSA to modulate MT expression. While MT3 expression was restored by TSA treatment, this was not the case for MT2A, shown above to be also dependant on methylation. The TSA effect could be explained either by inhibition of HDAC1, a known repressor of MT genes or by opening of the chromatin structure and upregulation of MTF1[67]. Although the main regulatory event for MT3 seems to be associated with histone acetylation, the synergistic effect observed in EP2 by combining TSA and 5-Aza suggest that other methylated genes or histones maybe indirectly involved in the regulation of MT3. Histone deacetylase inhibitors may therefore be interesting drugs in ependymomas.

While the expression of MT3 and MT4 are constitutive and tissue-specific, MT1 and MT2 expressions are more ubiquitous and highly inducible by a variety of developmental and environmental signals, such as metals, oxidative stress, cytokines, glucocorticoids hormones and irradiation [68]. In this work, we show the possibility to induce MT3 expression with zinc in brain tumor (EP and medulloblastoma) cells. However, metal-responsive element in the promoter of MT3 are not accessible in all EP cells. Indeed, MT3 has been considered for a long time as a non metal-inducible gene in normal astrocytes and neurons cultures [69,70], but recently Wei and co-workers showed MT3 induction after zinc treatment in prostate cancer cells [71]. Due to poor penetration of zinc into the brain, modulating MT3 expression in ependymomas with this cation would need proper formulations.

Several genes involved in the immune system were found to be downregulated in the common signature of recurrence (Figure 2B, Table S5); some of them being already reported by Donson et al, as associated with the absence of recurrence [72]. Our data are thus consistant with the hypothesis of these authors suggesting a role for the immune system to prevent recurrence in ependymoma.

Analysis of gene expression profiles specific of each location pointed out overexpression of genes related to the epithelial-mesenchymal transition in supratentorial locations [72]. The overexpression of genes involved in cytoskeleton organization as well as those involved in cel/cell and cell/matrix interactions could explain the higher invasive capacities of these tumors at the time of relapse. Contactin 1 (CNTN1), for example, has already been proposed as a key factor in glioma dissemination and its expression tends to be increased in several brain tumours [73]. In addition, contactin 1 has recognized interactions with developmental control genes belonging to the Notch pathway [74]. With respect to the recurrences of posterior fossa ependymomas, the upregulation of ribosomal proteins is consistent with increased proliferation usually seen in these tumors at recurrence, depicted for example by increased Ki67 labeling; in medulloblastomas as well, the overexpression of ribosomal proteins has been shown to be the hallmark of aggressive tumors[75].

The analysis of genes specifically downregulated at relapse after radiotherapy identified NKX2-2, a transcription factor involved in glioma histogenesis [76]. Its repression is associated with the blockade of oligodendrocyte differentiation [77] and the oncogenic phenotype of cancer [76].

Conclusion

Our data suggests that the gene expression profile of ependymoma shows limited but significant changes upon relapses. This gene expression profile is only minimally influenced by the treatments

used. However, the changes in expression profile at recurrence were linked to some extent with the location of the initial tumor. Despite interindividual variations, ependymoma relapses display a common gene expression signature that is marked by the upregulation of kinetochore proteins and downregulation of metallothioneins. The therapeutic strategies targeting kinesin proteins or those aiming at restoring metallothionein expression, such as histone deacetylase inhibitors, deserve further study in these tumours.

Supporting Information

Table S1 Clinical characteristics of the patients. D = diagnosis. R = recurrence. Delay of relapse in days. BBSFOP = polychemotherapy protocol, see ref 7. RT = radiotherapy. PF = posterior fossa, ST = supratentorial, SPI = spinal. DOD = dead of disease, ADF = alive disease-free, AWD = alive with disease. Time of follow-up in months.
Found at: doi:10.1371/journal.pone.0012932.s001 (0.03 MB XLS)

Table S2 Chromosome 9 imbalances differentiating posterior fossa and supratentorial ependymomas. Corresponding genes are indicated in the second column.
Found at: doi:10.1371/journal.pone.0012932.s002 (0.18 MB XLS)

Table S3 List of genes differentially regulated according to location of the initial tumor. The site of the relapse was not identical to the initial site in only one case (Pt3).
Found at: doi:10.1371/journal.pone.0012932.s003 (0.16 MB XLS)

Table S4 List of genes differentially regulated according to the treatment(s) received. All treatments received between diagnosis and relapse are considered. Patients having received chemotherapy and radiotherapy between diagnosis and the relapse considered are included in the radiotherapy group for the analysis.
Found at: doi:10.1371/journal.pone.0012932.s004 (0.21 MB XLS)

Table S5 List of genes differentially expressed at recurrence.
Found at: doi:10.1371/journal.pone.0012932.s005 (0.07 MB XLS)

Figure S1 Ependymoma short-term cultures. Cells were cultured after mechanical dissociation of fresh tumor material kept in DMEM. Low passages (5th to 15th) were used for the experiments.
Found at: doi:10.1371/journal.pone.0012932.s006 (1.32 MB DOC)

Figure S2 Metallothionein 3 (MT3) staining of normal brain. MT3 is detected in the astrocytes but not in the neurons (panel A) nor in the oligodendrocytes (panel B).
Found at: doi:10.1371/journal.pone.0012932.s007 (3.97 MB TIF)

Figure S3 ASPM staining of ependymomas. ASPM is detected in the mitotic spindle in every phase of the mitosis, as well as in the cytoplasm of cells not in mitosis.
Found at: doi:10.1371/journal.pone.0012932.s008 (10.17 MB TIF)

Figure S4 Comparison of CGHarray profiles at diagnosis and at relapse. D = diagnosis; R = relapse.
Found at: doi:10.1371/journal.pone.0012932.s009 (0.22 MB TIF)

Figure S5 Chromosomal imbalances that are distinct in supratentorial and posterior fossa tumors. ST = supratentorial; PF = posterior fossa.
Found at: doi:10.1371/journal.pone.0012932.s010 (0.20 MB TIF)

Figure S6 Chromosomal changes at relapse according to treatment received. CT = chemotherapy; RT = radiotherapy; Surv = surveillance.
Found at: doi:10.1371/journal.pone.0012932.s011 (0.16 MB TIF)

Figure S7 Quantitative PCR of MT2A gene in ependymoma samples.
Found at: doi:10.1371/journal.pone.0012932.s012 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: MP FC CDB PD VL GV JG. Performed the experiments: MP FC CDB FA LL FD VS PV. Analyzed the data: MP FC CDB FA LL PV AM PD JG. Contributed reagents/materials/analysis tools: FC FA SP PV PD VL GV. Wrote the paper: MP FC CDB FA JG.

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Appendix 1.2

Copy number gain of 1q25 predicts poor progression-free survival for pediatric intracranial ependymomas and enables patient risk stratification

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Clinical Cancer Research

Copy Number Gain of 1q25 Predicts Poor Progression-Free Survival for Pediatric Intracranial Ependymomas and Enables Patient Risk Strati cation: A Prospective European Clinical Trial Cohort Analysis on Behalf of the Children's Cancer Leukaemia Group (CCLG), Societe Francaise d'Oncologie Pe diatrique (SFOP), and International Society for Pediatric Oncology (SIOP)

John-Paul Kilday[1], Biswaroop Mitra[1], Caroline Domerg[3], Jennifer Ward[1], Felipe Andreiuolo[5], Teresa Osteso-Ibanez[1], Audrey Mauguen[3], Pascale Varlet[6], Marie-Cecile Le Deley[2], James Lowe[1,2], David W. Ellison[7], Richard J. Gilbertson[8], Beth Coyle[1], Jacques Grill[4,5], and Richard G. Grundy[1]

Abstract

Purpose: The high incidence of recurrence and unpredictable clinical outcome for pediatric ependymoma reflect the imprecision of current therapeutic staging and need for novel risk stratification markers. We therefore evaluated 1q25 gain across three age- and treatment-defined European clinical trial cohorts of pediatric intracranial ependymoma.

Experimental Design: Frequency of 1q gain was assessed across 48 ependymomas (42 primary, 6 recurrent) using Affymetrix 500K single-nucleotide polymorphism arrays. Gain of 1q25 was then evaluated by interphase FISH across 189 tumors treated on the Children's Cancer Leukaemia Group/International Society for Pediatric Oncology (SIOP) CNS9204 (n ¼ 60) and BBSFOP (n ¼ 65) adjuvant chemotherapy trials, or with primary postoperative radiotherapy (SIOP CNS9904/RT, n ¼ 64). Results were correlated with clinical, histologic, and survival data.

Results: Gain of 1q was the most frequent imbalance in primary (7/42, 17%) and recurrent ependymomas (2/6, 33%). Gain of 1q25 was an independent predictor of tumor progression across the pooled trial cohort [HR ¼ 2.55; 95% confidence interval (CI): 1.56–4.16; P ¼ 0.0002] and both CNS9204 (HR ¼ 4.03; 95% CI: 1.88–8.63) and BBSFOP (HR ¼ 3.10; 95% CI: 1.22–7.86) groups. The only clinical variable associated with adverse outcome was incomplete tumor resection. Integrating tumor resectability with 1q25 status enabled stratification of cases into disease progression risk groups for all three trial cohorts.

Conclusions: This is the first study to validate a prognostic genomic marker for childhood ependymoma across independent trial groups. 1q25 gain predicts disease progression and can contribute to patient risk stratification. We advocate the prospective evaluation of 1q25 gain as an adverse marker in future international clinical trials. Clin Cancer Res; 18(7); 2001–11. Ó2012 AACR.

Authors' Af liations: [1]Children's Brain Tumour Research Centre, University of Nottingham; [2]Department of Neuropathology, Nottingham University Hospital, Queens Medical Centre, Nottingham, United Kingdom; Departments of [3]Biostatistics and Epidemiology and [4]Pediatric and Adolescent Oncology; [5]CNRS UMR 8203 "Vectorology and Anticancer Treatment", Gustave Roussy Institute, Universite Paris-Sud, Villejuif; [6]Department of Neuropathology, Sainte-Anne Hospital, Paris, France; and Departmentsof[7]Pathology and[8]Developmental Neurobiology,StJudeChildren's Research Hospital, Memphis, Tennessee

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Richard G. Grundy, Children's Brain Tumour Research Centre, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, United Kingdom. Phone: 44-0-115-823-0620; Fax: 44-0-115-823-0696; E-mail: Richard.Grundy@nottingham.ac.uk

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Introduction

Improvements in the risk stratification and treatment of several cancers have been achieved in the postgenomic era through an appreciation of tumor-specific molecular abnormalities. Although our understanding of ependymoma biology has advanced in recent years with respect to tumor initiation and heterogeneity (1, 2), the development of novel prognostic classifications and targeted therapies is still required to enhance patient outcome for this tumor group, particularly in children.

Ependymomas represent the third most common pediatric tumor of the central nervous system (3). Although able to arise at any age, the majority occurs in children aged below 5 years (4). Significant differences are now apparent

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Translational Relevance

Because current clinicopathologic classification criteria for pediatric intracranial ependymoma are inconsistent, the introduction of novel prognostic markers for therapeutic stratification is an important requirement of future clinical trials. In this study of age- and treatment-defined trial cohorts, 1q25 gain was identified as an independent and reproducible marker of intracranial ependymoma progression in all patients, particularly in the younger children treated according to European primary postoperative chemotherapy protocols. Furthermore, incorporating degree of surgical resection with tumor 1q25 status enabled patient stratification according to disease progression risk groups across all 3 trial cohorts, irrespective of patient age or adjuvant therapy administered. We therefore advocate the prospective evaluation of 1q25 gain as an adverse risk marker in future international trials.

in the clinical and biologic characteristics of childhood versus adult ependymomas (5). Presently, prognostication for pediatric ependymoma is based solely on clinical parameters. Of these, the extent of primary tumor resection remains the most consistently reported correlate of outcome (4). European treatment schedules have hitherto been stratified by age. Trials of adjuvant chemotherapy for young children were initiated because of concerns of radiation-induced neuropsychologic and cognitive damage to the immature central nervous system (6–8), whereas postoperative radiotherapy has been reserved for older children (8). Despite these measures, the prognosis for pediatric intracranial ependymomas remains poor when compared with other childhood malignancies, with local tumor recurrence a frequently reported event, even after complete tumor excision (5). After 5 years, progression-free survival (PFS) rates range from 23% to 74% (3, 9, 10), whereas mortality is reported in up to 40% of affected children (3).

The need to incorporate novel biomarkers into future prognostic stratifications for childhood intracranial ependymoma is therefore apparent. However, although several candidates have been proposed, markers showing reproducible results in sizeable groups of young ependymoma patients are lacking (5). Indeed, several purported biologic prognostic markers in ependymoma have been shown to lose this capacity when assessed across clinical trial cohorts (11), highlighting the importance of analyzing standardized therapeutic groups.

Copy number gain of chromosome 1q has been reported as a frequent genetic aberration in both primary and recurrent childhood intracranial ependymomas (5). Retrospective analyses of cohorts comprising children and adults have identified gain of either the entire long arm or the 1q25 amplicon as adverse prognostic markers in intracranial ependymoma (12–14), although little evidence exists for such a role exclusive to a pediatric setting (15).

In this study, we established the frequency of 1q gain in pediatric ependymoma, identifying 1q21–25 among the most common subregions of gain. We then evaluated 1q25 gain as a robust prognostic marker in pediatric intracranial ependymoma by carrying out interphase FISH (iFISH) across 189 primary tumors, incorporating 3 European clinical trial cohorts. To our knowledge, this is the first study to assess the reproducibility of a genomic marker in both comparable (CNS9204 and BBSFOP) and contrasting (CNS9904) therapeutic trial groups of pediatric intracranial ependymoma patients.

Methods

Patients and clinical specimens

Forty-eight snap-frozen ependymomas (42 primary, 6 first recurrent) from 42 patients were obtained from Children's Cancer and Leukaemia Group (CCLG) registered centers in the United Kingdom for analysis using Affymetrix 500K single-nucleotide polymorphism (SNP) arrays. Constitutional blood samples from 38 of 42 (90%) patients contributing tumors were analyzed as controls. From the tumor cohort, a subset of 18 formalin-fixed paraffin-embedded (FFPE) intracranial ependymomas were used to validate microarray 1q gain results by iFISH (see below). Fifteen of these samples were also included in the clinical trial cohort analysis.

A total of 172 FFPE primary intracranial ependymomas from trial patients were analyzed by iFISH on tissue microarrays (TMA). Patients were enrolled in either the CNS9204 (ref. 6; n ¼ 60), BBSFOP (ref. 7; n ¼ 65) or CNS9904 clinical trials (n ¼ 47) and were diagnosed between 1989 and 2007. An overview of each trial is provided (Supplementary Methods). To supplement the CNS9904 cohort, 17 primary tumors (9 supratentorial, 8 posterior fossa) from therapeutically matched, nontrial patients were also examined. These children were aged between 5 and 14 years and had intracranial ependymomas treated only with cranial irradiation (54 Gy) following primary surgery.

Patient clinical information was obtained from respective trial centers. For all cases, central pathologic review was done according to WHO criteria (DWE, FA, PV; ref. 16). Cases with differing pathologic diagnoses at review were resolved by consensus opinion following discussion between responsible neuropathologists. The degree of surgical resection was evaluated by central review of postoperative imaging according to International Society for Pediatric Oncology (SIOP) guidelines (17). The study obtained CCLG, Soci et e Franc¸aise d'Oncologie P ediatrique (SFOP), SIOP, and Multiple Centre Research Ethics Committee (MREC) approval. Consent for tumor tissue use was taken in accordance with national tumor banking procedures (uk:05/MRE/04/70).

Nucleic acid isolation

DNA was extracted from 10 mg of frozen tumor tissue and peripheral blood mononuclear cells as described previously (18). Before tissue extraction, hematoxylin/eosin stained

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smears from each specimen underwent pathologic review to confirm tumor presence and viability.

500K SNP array analysis

SNP microarray profiles for tumor and constitutional DNA were generated using the Affymetrix GeneChip Human Mapping 500K assay, with data analysis and visualization done as described previously (Supplementary Methods; refs. 1, 18, 19, 20). Chromosomal arms and cytobands were defined as gained or lost if more than 80% of encompassed probes showed copy number gain or loss, as defined previously (18). The microarray data generated during this study has been deposited in GEO with an accession number GSE32101.

Ependymoma TMA construction

TMAs were constructed from blocks of FFPE tumor material. Viable and representative tumor areas were identified by a neuropathologist using hematoxylin/eosin stained sections from each block before TMA incorporation (JL, DE, FA, and PV). Typically for each tumor sample, 3 to 4 Â 0.6 mm cores of 4 mm thickness were included, incorporating the different representative areas defined.

Interphase fluorescence in situ hybridization

Dual color iFISH was carried out as described previously (21), using a commercial 1q25 (spectrum green) and 1p36 (spectrum orange) probe (Vysis). A commercial probe was chosen in view of the need for a prognostic biologic marker to be robust and widely available for multicentre application. The evaluation criteria and scoring system adopted was based on that used by several preceding analyses (Supplementary Methods; refs. 13, 14, 22).

Statistics

Statistical analysis was carried out in SPSS (version 17.0, SPSS) and in SAS, Version 9.1.2 (SAS Institute Inc.). A detailed definition of analyses used is provided (Supplementary methods; refs. 23).

Results

500K SNP array analysis

Clinical characteristics of the SNP array ependymoma cohort are summarized in Table 1, with results from survival analysis shown in Table 2 (Comprehensive clinicopathologic data and chromosome arm imbalance results for each tumor sample are provided in Supplementary Table S1). The median age of the primary tumor cohort was 6.8 years (range: 1–20.9 years) with a male:female ratio of 1.2:1. Children with posterior fossa ependymomas were significantly younger than those with spinal tumors (ANOVA with Tukey HSD test; P ¼ 0.009, eta 0.2), whereas the age difference between patients with posterior fossa and supratentorial tumors was not significant. The median follow-up period for all 42 patients was 9.6 years (range: 0.5–21 years). Disease progression occurred in half of the cohort with a median time to progression of 1.5 years (range: 0.3–8.8 years), whereas 12 patients died with a median survival time of 3.0 years (range: 1–9.6 years). Incomplete resection was the only clinicopathologic variable to confer an adverse prognosis, associated independently with a worse PFS [HR ¼ 3.19; 95% confidence interval (CI) ¼ 1.26–8.08; P ¼ 0.01].

In keeping with previous comparative genomic hybridization (CGH) studies of ependymoma (13–15, 24), the SNP array analysis categorized primary tumors according to their broad genomic imbalance profile. Seven tumors (17%) showed 4 or more chromosomal aberrations, 11 tumors (26%) revealed 1 to 3 imbalances, whereas 24

Table 1. Clinicopathologic characteristics and chromosome 1q gain results in the SNP array cohort

Patient data	500K SNP array cohort (42 patients)
Age	
<5 y	18 (43)
>5 y	24 (57)
Gender	
Male	23 (55)
Female	19 (45)
Five-year PFS	38 Æ 9%
Five-year OS	78 Æ 8%
Survival status	
Alive	30 (71)
Dead	12 (29)

Tumor data	Primary tumors (n ¼ 42)	First recurrent tumors (n ¼ 6)
Location		
PF	24 (57)	3 (50)
ST	12 (29)	3 (50)
Spinal	6 (14)	—
WHO grade		
III	16 (38)	2 (33)
II	23 (55)	4 (67)
I	3 (7)	—
Surgical resection		
Complete	21 (50)	1 (17)
Incomplete	21 (50)	3 (50)
Unknown		2 (33)
Adjuvant therapy		
RT	12 (28)	2 (33)
CT	15 (36)	1 (17)
Both	10 (24)	3 (50)
Nil	5 (12)	—
1q gain		
No	35 (83)	4 (67)
Yes	7 (17)	2 (33)

NOTE: The values in parenthesis are given in percentage.
Abbreviations: PF, posterior fossa/infratentorial; ST, supratentorial; RT, radiotherapy; C, chemotherapy.

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Table 2. Survival analysis of clinicopathologic factors and 1q gain in the SNP array primary cohort (n ¼ 42)

Factor (numbers)	Progression-free survival				Overall survival			
	Univariate		Multivariable		Univariate		Multivariable	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Patient age								
>5 y (n ¼ 18)	1				1			
<5 y (n ¼ 24)	1.33 (0.54–3.23)	0.54			1.29 (0.38–4.35)	0.68		
Gender								
Female (n ¼ 19)	1				1			
Male (n ¼ 23)	1.07 (0.45–2.56)	0.88			0.77 (0.25–2.40)	0.65		
Location								
ST/SP (n ¼ 18)	1				1			
PF (n ¼ 24)	0.83 (0.31–2.22)	0.71			0.93 (0.28–3.09)	0.90		
WHO grade								
I/II (n ¼ 26)	1				1			
III (n ¼ 16)	1.19 (0.49–2.86)	0.69			1.17 (0.79–3.69)	0.78		
Surgery								
CR (n ¼ 21)	1		1		1		1	
IR (n ¼ 21)	3.19 (1.26–8.08)	0.01	3.19 (1.26–8.08)	0.01	2.00 (0.63–6.31)	0.24	1.74 (0.54–5.65)	0.36
Adjuvant Radiotherapy								
No (n ¼ 20)	1				1		1	
Yes (n ¼ 22)	0.66 (0.27–1.61)	0.36			0.43 (0.13–1.42)	0.17	0.24 (0.06–1.03)	0.06
1q gain result								
No gain (n ¼ 35)	1				1		1	
Gain (n ¼ 7)	1.73 (0.66–4.51)	0.26			2.60 (0.73–9.26)	0.14	4.62 (0.99–21.20)	0.05

NOTE: Probability (P) values for univariate and multivariable survival analysis obtained by the Cox proportional hazard model (see Supplementary Methods).

Abbreviations: PF, posterior fossa; ST, supratentorial; SP, spinal; IR, incomplete resection; CR, complete resection.

tumors (57%) showed no whole chromosome or arm imbalance. Within this latter group, 15 ependymomas

all SNP probes showing a diploid copy number) and were

associated with children aged below 3 years (Fisher's exact test; P ¼ 0.04). Even when accounting for different tumor location by restricting the analysis to posterior fossa ependymomas, the number of chromosome arm imbalances between patients aged below and above 5 years remained

0.0001).

Gain of chromosome 1q was the most frequent aberration in both the primary and recurrent ependymomas [7/42 (17%) and 2/6 (33%) respectively], identified in 7 patients. Higher resolution cytoband analysis revealed 1q21–25, 1q32, and 1q42–44 to be amongst the most frequently gained subregions on this arm (11/48, 23%). Whole gains of chromosomes 9 and 18 were also relatively common, seen in 6 of 42 (14%) primary tumors. The most frequent loss was of chromosome 22q, present in 3 of 42 primary tumors (7%) and 1 recurrent case.

Distinct patterns of genomic imbalance between primary ependymomas from different CNS locations were evident (Supplementary Fig. S1). Gain of chromosome 1q was associated with posterior fossa ependymomas (Fisher exact test; P ¼ 0.03). Relatively few broad chromosomal changes were seen with supratentorial tumors. In contrast, spinal ependymomas were characterized by numerous arm and whole chromosomal aberrations when compared with intracranial tumors, particularly gain of chromosomes 17, 20p, 16, 12q, 20q, 21q, 9, and 18.

In view of its frequency across primary and recurrent ependymomas, the impact of entire chromosome 1q gain on patient survival was assessed, revealing a trend toward predicting worse overall survival (OS) in multivariable

0.05). FISH was used to validate the SNP array copy number results for 1q25 copy number gain across 18 primary intracranial ependymomas (Supplementary Fig. S2; Spearman's rank ¼ 0.79; P < 0.0001).

1q25 FISH trial cohort analysis

Clinicopathologic data and 1q25 FISH results for the 3 therapeutic trial cohorts are summarized in Table 3.

The median age of the CNS9204 cohort was 2.0 years (range: 0.3–3.1 years). The median follow-up period for all 60 patients was 8.9 years (range: 0.6–16.1 years). Disease progression occurred in 40 patients with a median time to

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Table 3. Clinicopathologic characteristics and 1q25 gain results across the 3 pediatric intracranial ependymoma therapeutic trial cohorts

Variable	CNS9204	BBSFOP	CNS9904 þ RT only
Patient number	60	65	64
Median age (range), y	2.0 (0.3–3.1)	1.9 (0.6–5.1)	7.8 (3.0–16.7)[a]
Sex			
Female	21 (35%)	30 (46%)	26 (41%)
Male	39 (65%)	35 (54%)	38 (59%)
Male:Female ratio	1.9:1	1.2:1	1.5:1
Status			
Alive–no disease progression	20 (33%)	13 (20%)	29 (45%)
Alive–disease progression	11 (18%)	17 (26%)	11 (17%)
Death from disease	29 (48%)	34 (52%)	23 (36%)
Death unrelated to disease		1 (2%)	1 (2%)
Survival			
5-year PFS Æ SE (%)	38 Æ 6	26 Æ 6[b]	47 Æ 6
5-year OS Æ SE (%)	63 Æ 6	58 Æ 6	71 Æ 6
Primary tumor location			
ST	7 (12%)	12 (18%)	28 (44%)[a]
PF	53 (88%)	53 (82%)	36 (56%)
Primary tumor WHO grade			
II	33 (55%)	11 (17%)	30 (47%)
III	27 (45%)	54 (83%)[a]	34 (53%)
Primary tumor surgical resection			
Complete	28 (47%)	43 (66%)[c]	35 (55%)
Incomplete	32 (53%)	22 (34%)	29 (45%)
Metastatic disease at presentation			
Yes	4 (7%)	2 (3%)	2 (3%)
No	58 (93%)	63 (97%)	62 (97%)
Scorable tumors (n ¼ 147)	52 (87%)	41 (63%)	54 (84%)
1q25 result			
No gain	41 (79%)	35 (85%)	41 (76%)
Gain	11 (21%)	6 (15%)	13 (24%)

NOTE: The difference in median age between cohorts was assessed by the independent samples t-test. The difference in PFS between cohorts was assessed by the log rank test. Differences in the proportion of other clinical factors between cohorts, such as patient sex, tumor location, WHO grade and resection status, were assessed by the Fisher's exact test.

Abbreviations: PF, posterior fossa/infratentorial; ST, supratentorial.

[a]$P < 0.0001$ for comparison against remaining cohorts.

[b]$P < 0.01$ for comparison against remaining cohorts.

[c]$P < 0.05$ for comparison against remaining cohorts.

progression of 1.6 years (range: 0.3–9.5 years). Death occurred in 29 patients with a median survival time of 3.3 years (range: 0.6–8.9 years). Four primary cases (7%) were metastatic at presentation.

The BBSFOP cohort was comparable with the CNS9204 group with respect to patient age (median 1.9 years, range: 0.6–5.1 years), gender, tumor location, and primary adjuvant therapy administered. However, WHO grade III ependymomas (Fisher exact test; $P < 0.0001$) and complete tumor resection ($P ¼ 0.032$) were more prevalent in the French cohort, which had a poorer PFS. The median follow-up period across the entire BBSFOP cohort was 7.7 years (range: 0.3–16.9 years). Fifty-one patients had suffered disease progression, with a median time to progression of 1.3 years (range: 0.3–9.3 years). Thirty-four patients had died of disease [median survival time 3.4 years (range: 0.3–10.1 years)]. Two primary cases (3%) were metastatic at diagnosis. No associations between clinical variables were evident within either trial group.

The nature of the adjuvant therapy administered to the CNS9904/RT cohort resulted in an older patient age when compared with the chemotherapeutic trial groups [independent t test; median age 7.8 (range: 3.0–16.7); $P < 0.0001$]. A higher proportion of supratentorial ependymomas were also observed in this cohort (Fisher exact test; $P < 0.0001$), these being predominantly of anaplastic

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histology (P ¼ 0.032). This reflected a correlation of posterior fossa tumors with young children and supratentorial tumors with older patients identified across the entire FISH cohort [independent t test; mean age posterior fossa ¼ 3.5 (SD 3.2) years vs. supratentorial ¼ 6.7 (SD 4.6) years; P < 0.0001]. The median follow-up period for all 64 patients was 10.6 years (range: 0.6–11.8 years). Tumor progression occurred in 34 patients with a median time to progression of 1.4 years (range: 0.2–7.9 years) and 23 patients had died [median survival time of 3.4 years (range 0.6–5.8 years)]. Two CNS9904/RT cases (3%) were metastatic at presentation.

FISH was successful in 147 of 189 tumors (78%; Table 3). Concordance was achieved between independent scorers (Kappa 0.94; P < 0.0001). Unsuccessful cases were the result of core loss, autofluorescence or insufficient signal generation. Gain of 1q25 was evident in 30 of 147 tumors (20%). This proportion of gain did not differ significantly between the 3 therapeutic trial groups (range: 15%–24%; Table 3). No association was identified between 1q25 copy number imbalance and variables including patient age, gender, tumor histology, resection status, or intracranial location. Of the 8 metastatic primary tumors, 1q25 gain was observed in 3 cases (2 CNS9204, 1 CNS9904) and was not present in 1 case (CNS9204), whereas 4 samples were unscorable.

The prognostic impact of 1q25 gain and putative clinical factors on PFS and OS was initially evaluated across the pooled cohort of 147 eligible cases from the CNS9204, BBSFOP, and CNS9904/RT groups. Univariate analysis by log rank (Fig. 1A; P ¼ 0.008) and Cox proportional hazards model stratified on therapeutic cohorts (Table 4; P ¼ 0.002) identified 1q25 gain as a marker of adverse PFS. This was confirmed on multivariable analysis (Table 4; HR ¼ 2.55; 95% CI ¼ 1.56–4.16; P ¼ 0.0002). The only clinical variable to be independently associated with poor outcome was incomplete surgical resection, both for PFS (Table 4; HR ¼ 2.60; 95% CI ¼ 1.64–4.11; P < 0.0001) and OS (Supplementary Table S2; HR ¼ 1.75; 95% CI ¼ 1.05–2.93; P ¼ 0.03).

After adjusting for surgical resection (Table 5), 1q25 gain was identified as an independent predictor of adverse PFS for the CNS9204 (HR ¼ 4.03; 95% CI ¼ 1.88–8.63; P ¼ 0.0003) and BBSFOP patients (HR ¼ 3.10; 95% CI ¼ 1.22–7.86; P ¼ 0.02), but not the CNS9904/RT group (HR ¼ 1.39; 95% CI ¼ 0.61–3.20; P ¼ 0.43). However, 1q25 gain was not a chemotherapy cohort-specific PFS marker (prognostic heterogeneity test of 1q25 for chemotherapy vs. radiotherapy cohorts; P ¼ 0.13). Gain of 1q25 did not translate into a significantly worse OS across the cohorts (Supplementary Table S2).

In view of the above findings, the degree of surgical resection and tumor 1q25 status were integrated to enable stratification of the 147 ependymomas into 3 distinct risk groups for disease progression (Fig. 1B–E). High-risk disease was defined by ependymomas that were incompletely resected and showed 1q25 gain (2 risk factors). Intermediate-risk tumors were those which were either incompletely resected or showed 1q25 gain (1 risk factor), whereas standard risk disease encompassed completely resected ependymomas which did not exhibit 1q25 gain (no risk factors). This risk classification system showed significant differences in PFS, across both the pooled cohort (Fig. 1B; P < 0.0001), and independent trial subgroups (Fig. 1C–E; P ¼ 0.009, 0.0002, and 0.01 for the CNS9204, BBSFOP, and CNS9904/RT groups, respectively) by the log rank test.

Discussion

The incorporation of biologic prognostic markers to enhance current risk stratification, guide therapy, and improve long-term outlook for pediatric intracranial ependymoma is an aim of future clinical trials. Several candidates have been proposed, yet recent review has shown that few have been analyzed in sufficiently large cohorts to allow informed evaluation of their prognostic efficacy in childhood (5). Independent validation of such purported pediatric ependymoma markers has often yielded contradictory results, such as those for ERBB2/ERBB4, Ki-67, and Nucleolin expression (25–27), although Tenascin-C expression has reported reproducible prognostic value across standardized cohorts (11). In addition, to date, no genomic imbalance has been assessed in pediatric ependymoma patients treated within the context of a prospective clinical trial (5).

We initially confirmed 1q gain as the most frequent chromosome arm imbalance in 36 primary childhood intracranial ependymomas and a patient-matched subset of 6 recurrent tumors by SNP array analysis, finding gain correlated with a trend toward worse OS. High-resolution analysis identified 1q21–25 among the most common subregions of gain. We explored these findings by evaluating iFISH results for 1q25 copy number increase across 147 primary pediatric intracranial ependymomas, spanning 3 European clinical trial cohorts. This identified 1q25 gain as an independent marker of tumor progression for ependymomas in 2 independent trial cohorts of young children treated with primary postoperative chemotherapy (CNS9204 and BBSFOP), but not from older children administered focal radiotherapy after surgery (CNS9904/RT). Nevertheless, incorporating tumor resectability with 1q25 status enabled patient stratification according to disease progression risk groups across all 3 trial cohorts, irrespective of patient age or adjuvant therapy administered.

We found no association between 1q25 gain, as determined by FISH, and a specific intracranial tumor location. Although this contrasted with the association of 1q gain with posterior fossa ependymomas identified from our smaller SNP array study, it was consistent with a sizeable CGH meta-analysis of 175 pediatric intracranial ependymomas that revealed gain involving 1q to be a frequent aberration in both posterior fossa and supratentorial tumors (5).

The only clinical factor adversely influencing outcome across both the array and pooled trial cohorts of this study was incomplete tumor resection, although this was not applicable to the CNS 9204 cohort when assessed independently (PFS, P ¼ 0.36, OS, P ¼ 0.44), in keeping with results

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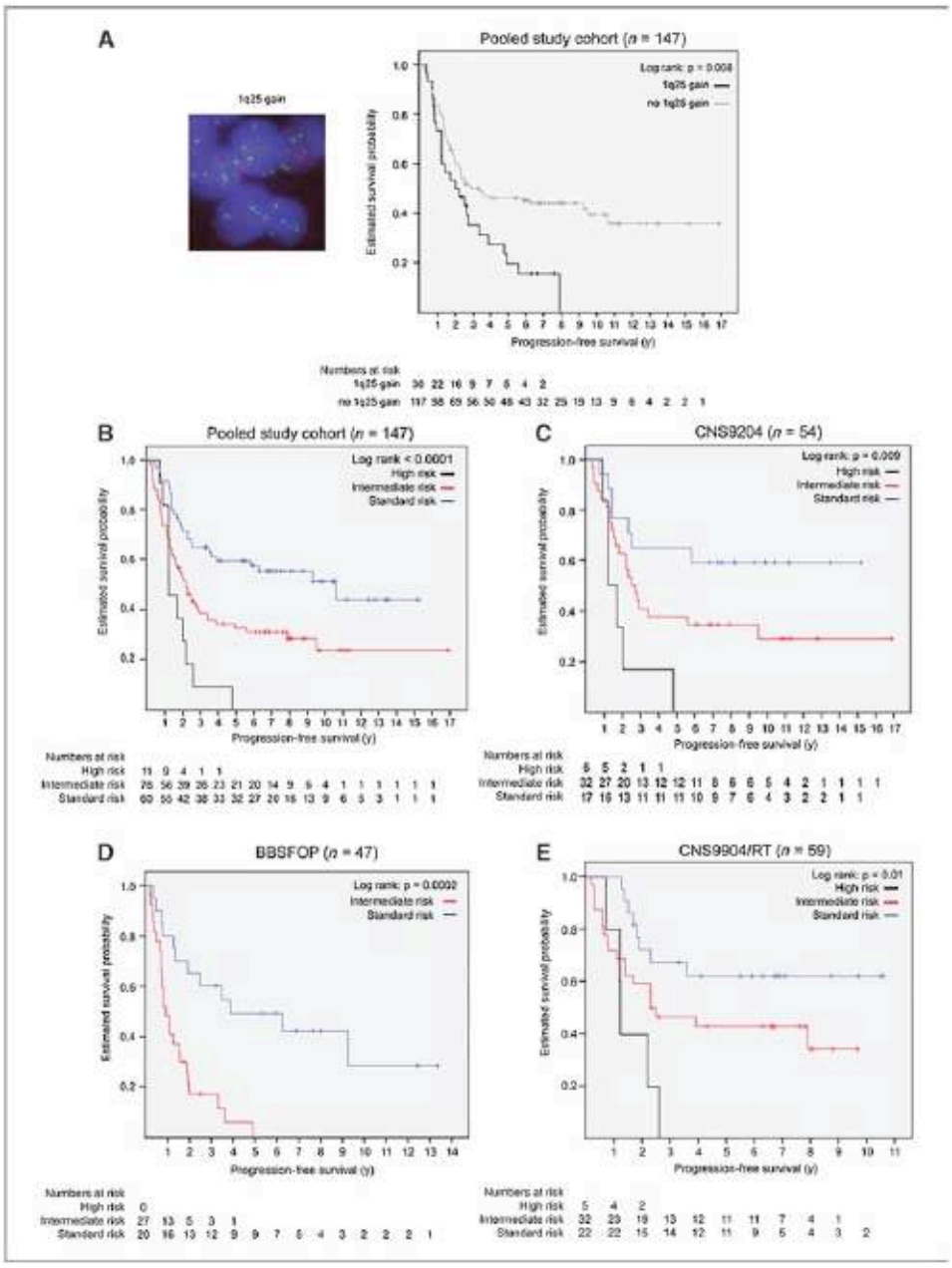

Figure 1. 1q25 signal gain is a marker of disease progression in pediatric ependymoma patients and enables risk strati cation. A, interphase FISH to a TMA ependymoma sample is shown, showing 1q25 gain (3 or more green signals per nucleus). Also shown is the Kaplan–Meier PFS curve for the pooled cohort of 147 primary intracranial ependymomas patients treated with the CNS9204, BBSFOP, and CNS9904/RT regimens, according to tumor 1q25 gain status by FISH. Gain of 1q25 was associated with a worse PFS (5-year PFS 20% vs. 46%). B to E, PFS curves for ependymoma risk strati cation groups determined by tumor resectability and 1q25 status, both in the overall study cohort (B) and the individual therapeutic trial subgroups (C–E). The black lines represent high-risk ependymomas that were both incompletely resected and showed 1q25 gain (5-year PFS of 0% in the overall, CNS9204, and CNS9904/RT cohorts, respectively). No high-risk cases were present in the BBSFOP cohort. The red lines represent intermediate-risk tumors that were either incompletely resected or showed 1q25 gain (5-year PFS of 32%, 38%, 0%, and 43% in the overall, CNS9204, BBSFOP, and CNS9904/RT cohorts, respectively). The blue lines represent standard-risk tumors which were completely resected and did not exhibit 1q25 gain (5-year PFS of 59%, 65%, 49%, and 62% in the overall, CNS9204, BBSFOP, and CNS9904/RT cohorts, respectively).

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Table 4. Pooled analysis of prognostic factors for PFS strati ed on therapeutic trial cohort for 147 iFISH-evaluable patients

	Univariate		Multivariable	
Factor	HR (95% CI)	P	HR (95% CI)	P
Location				
ST (n ¼ 37)	1		1	
PF (n ¼ 110)	1.35 (0.80–2.29)	0.25	1.21 (0.71–2.76)	0.48
WHO grade				
II (n ¼ 63)	1			
III (n ¼ 84)	0.91 (0.58–1.41)	0.67		
Sex				
Female (n ¼ 59)	1			
Male (n ¼ 88)	0.91 (0.60–1.39)	0.67		
Surgery				
CR (n ¼ 79)	1		1	
IR (n ¼ 68)	2.3 (1.47–3.59)	0.0003	2.6 (1.64–4.11)	<0.0001
1q25 result				
No gain (n ¼ 117)	1		1	
Gain (n ¼ 30)	2.16 (1.34–3.48)	0.002	2.55 (1.56–4.16)	0.0002

NOTE: The nal model includes surgery and 1q25 result. Italic values are results for other variables added one by one to the nal model.
Abbreviations: PF, posterior fossa; ST, supratentorial; IR, incomplete resection; CR, complete resection.

for the entire cohort previously published (6). Indeed, this reflects current literature, in which resection status has been reported as the most consistent adverse prognostic marker in pediatric ependymoma (4, 5, 7, 28, 29), albeit not universally (5, 6, 30–32). Histologic anaplasia did not confer a worse patient outcome, supporting a recent multiprofessional pathologic review of the CNS9204, BBSFOP, and CNS9904 trials which found the current WHO classification system subjective and lacking prognostic accuracy (33).

Results from our FISH analysis of prospective trial cohorts lends some support to the findings of 2 sizeable retrospective FISH studies of intracranial ependymomas which have previously reported 1q25 gain as a marker of reduced PFS, but also OS on mixed age cohorts (13, 14). The threshold used to define gain for a tumor in this study (15% of nuclei showing signal gain) was higher than that of the retrospective series (10% of nuclei), as the latter did not yield a satisfactory measure of agreement between independent scorers. Nevertheless, the proportion of primary ependymomas showing 1q25 gain across the 3 therapeutic cohorts (15%–24%) was comparable with the 20% to 25% reported in these preceding studies. Although FISH proved an efficient means of screening our cohorts for copy number imbalance, unsuccessful cases were observed and mostly attributed to the use of tissue fixative agents incorporating acetic acid, a practice being rectified by contributing institutions.

In contrast to the retrospective analyses, 1q25 gain was not significantly associated with worse OS across the pooled trial patients of this study, including the CNS9204 or BBSFOP cases. Demographic and therapeutic differences between retrospective and prospective studies could account for this disparity, such as the inclusion of adults in the previous analyses, or a potential beneficial effect of introducing cranial radiotherapy as standard salvage therapy post relapse in the chemotherapeutic trials (6, 7). In addition, the median patient follow-up times for the 2 retrospective studies (7.3 and 5.2 years) were shorter than that of the pooled trial cohort (9.1 years, chemotherapy cases only 8.4 years). It is therefore possible that with continued observation of the retrospective cohorts, a less significant effect on overall outcome may have been observed, particularly, as late adverse outcome events are not uncommon for this tumor type (34). Nevertheless, as more than 70% of young children who experience ependymoma recurrence despite adjuvant chemotherapy will not survive longer term (9), a biologic marker that predicts progression in these patients remains an important discovery upon which therapy can be stratified. For high-risk patients with resistant disease to conventional chemotherapy, postoperative conformal radiotherapy may be a feasible and effective alternative adjuvant therapy on the basis of results from the SJCRH RT1 trial (29). Alternatively, novel chemotherapeutic and biologic agents could be considered, including tyrosine kinase inhibitors and antiangiogenic therapy (3).

Unlike the chemotherapeutic trial results, 1q25 gain in the CNS9904/RT group was less predictive of a worse patient PFS or OS. Although this could reflect a different biologic milieu for ependymomas from older children (5), it may suggest that primary radiotherapy is an effective counteractive adjuvant measure despite the adverse effects of 1q25 gain. Postoperative focal radiotherapy was

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Table 5. Prognostic value of 1q25 gain by trial cohort and adjuvant therapy on PFS in multivariable analysis, with evaluation of prognostic stability between adjuvant therapy groups

Multivariable (n ¼ 147)strati ed on cohort			strati ed on adjuvant therapy		
Factor	HR (95 % CI)	P	Factor	HR (95 % CI)	P
Surgery			Surgery		
CR (n ¼ 79)	1		CR (n ¼ 79)	1	
IR (n ¼ 68)	2.64 (1.67–4.18)	<0.0001	IR (n ¼ 68)	1.99 (1.31–3.03)	0.001
Global effect of 1q25 result		0.0003	Global effect of 1q25 result		0.001
			Heterogeneity between adjuvant therapy modalities		0.13
1q25 result in CNS9204			1q25 result in chemotherapy patients		
No gain (n ¼ 41)	1		No gain (n ¼ 76)	1	
Gain (n ¼ 11)	4.03 (1.88–8.63)	0.0003	Gain (n ¼ 17)	2.9 (1.64–5.12)	0.0003
1q25 result in BBSFOP					
No gain (n ¼ 35)	1		1q25 result in radiotherapy patients		
Gain (n ¼ 6)	3.1 (1.22–7.86)	0.02	No gain (n ¼ 41)	1	
1q25 result in CNS9904				1.33 (0.58–3.05)	0.50
No gain (n ¼ 41)	1				
Gain (n ¼ 13)	1.39 (0.61–3.20)	0.43			

Abbreviations: IR, incomplete resection; CR, complete resection.

standardized across the entire CNS9904/RT cohort, as opposed to chemotherapy in the other trial groups. This also differed from that of the retrospective studies, in which radiotherapy was administered to certain patients, but not uniformly (13, 14). However, this explanation must be considered cautiously as our iFISH study could not conclude that the reduction in PFS associated with 1q25 gain was specific to the chemotherapeutic trial regimens when smaller sizes of the individual treatment groups. Moreover, incorporating the degree of surgical resection with tumor 1q25 status enabled a significant 3-tier stratification for disease progression risk in the CNS9904/RT cohort (Fig. 1E), suggesting a prognostic role, albeit possibly not independently, for 1q25 gain in this patient group.

Survival data from the SNP profiling cohort did not help establish or refute the prognostic value for 1q gain in patients treated with radiotherapy, despite comparable median follow-up times with the pooled trial cohort analysis. Although 1q gain was independently associated with a trend toward inferior OS (Table 2), the 3 of 7 patients with ependymomas exhibiting 1q gain who remained alive had all been treated with postoperative cranial irradiation. The differences in patient outcome observed between 1q gain from the array study and the 1q25 FISH data are again plausibly explained by the considerably smaller size of the heterogeneous SNP array cohort, together with the variable treatments given to these children compared with the administration of standardized adjuvant therapy for the trial patients. Moreover, the difference in results could suggest that regions on 1q are more sensitive and robust markers of progression in childhood ependymoma than gain of the whole arm itself, a hypothesis supported by other genomic work on ependymoma (14, 34).

The integrated clinical and biologic risk stratification for ependymoma progression reported in this study reflects the current aspiration to develop novel prognostic models for this tumor. Indeed, recent work has reported a molecular staging system for ependymoma that defined 3 cytogenetic categories, including a high-risk group (group 3) characterized by 1q gain and/or homozygous CDKN2A deletion (13). In our analysis, such a classification would also have accounted for every high-risk patient, together with 25% of intermediate-risk patients (1q gain and complete tumor resection). However, incorporating 1q25 gain and tumor resectability seemed a more robust method of stratification across the 3 therapeutic trial groups (Fig. 1) compared with 1q25 status/group 3 categorization alone (Supplementary Fig. S3). This is supported by the previous study, in which addition of the genomic classification to established clinical variables in the previous work also improved risk prediction (13).

A transcriptional and genomic profiling study of posterior fossa ependymomas, delineating 2 distinct molecular subgroups of tumors with contrasting prognosis (groups A and B; ref. 31), offers further support to the present work. Although 1q gain was a feature of the less favorable group A tumors, survival for these patients was influenced more by the degree of surgical resection than 1q gain in isolation (34). Despite this, all 8 posterior fossa tumors designated

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high risk from our analysis most likely correspond to the group A category.

In summary, 1q25 gain as determined by FISH appears an independent marker of tumor progression in European primary chemotherapeutic cohorts of pediatric intracranial ependymoma (CNS9204 and BBSFOP). Although up to 42% of young children with ependymoma can remain free of disease with prolonged chemotherapeutic regimes (6, 7), 1q25 gain can be used to delineate a high-risk group of children, accounting for approximately 20% of patients who will experience recurrence or local progression despite this therapeutic strategy. However, the relatively small size of the individual therapeutic trial groups analyzed in this study precluded a decision on whether the prognostic role of 1q25 gain was specific to this patient age and treatment group, and thereby not applicable to older children treated with postoperative radiotherapy. Such a conclusion cannot be made until prospective trials are undertaken evaluating such patient cohorts in larger numbers. Nevertheless, this study showed that tumor 1q25 status, in conjunction with the degree of surgical resection, enabled a 3-tier patient stratification system of distinct disease progression risk groups across the therapeutic trial sets, irrespective of patient age or adjuvant therapy received.

We therefore advocate the prospective evaluation of 1q25 gain as a prognostic marker in forthcoming large international clinical trials of pediatric intracranial ependymoma, both independently and integrated with tumor resectability. Upon successful validation, 1q25 gain could be incorporated into future clinical trial design to improve risk stratification for children diagnosed with this tumor.

Disclosure of Potential Con icts of Interest

P. Varlet is a consultant and is on the advisory board of Roche study. The other authors disclosed no potential conflicts of interest.

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Chapter 2:

HIGH GRADE GLIOMA

1. Definition

Pediatric high-grade glioma (HGG) is a heterogeneous group of tumors with a generally poor clinical outcome, of which, similarly to its own classification schemes, prognosis has changed little in the past four decades (Broniscer and Gajjar, 2004).

Histologically high-grade gliomas in children are diagnosed according to the WHO grading criteria in the same way as those occurring in adult patients, receiving grades III and IV (Louis et al., 2007).

According to the WHO classification glial tumors are classified as astrocytic, oligodendroglial and mixed astrocytic and oligodendroglial. Ependymomas, although presenting unequivocal glial differentiation -ependymal cells are indeed a special type of glial cells most likely derived from radial glia and express glial proteins such as S100 and GFAP, and often the epithelial marker cytokeratin (Hasselblatt and Paulus, 2003) - show particular clinical and biopathological characteristics that justify their segregation from the other tumors under the generic term HGG (Louis et al., 2007). In the pediatric population, pediatric HGG have been largely overrepresented by anaplastic astrocytoma and glioblastoma, and this has changed little even in the last fifteen years, despite the large shifts in diagnosis which have taken place in adults, among whom increases in incidences of oligodendrogliomas and oligoastrocytomas and declines in astrocytoma numbers have been observed (Davis et al., 2008).

2. Epidemiology

Gliomas are the most common pediatric CNS tumors and constitute the majority of primary supratentorial tumors (CBTRUS 2010). Childhood supratentorial high-grade gliomas (HGGs) are most often polar hemispheric lesions, and in 20%−30% of cases deep-seated and located in the midline (Pollack, 1994).

A precise estimate of the incidence of pediatric HGGs has been difficult to determine, in part because of the tendency in previous reports to misclassify various low-grade glioma (LGG) variants under the diagnostic umbrella of HGG and also due to the lack of consistently reproducible diagnostic criteria. As a consequence, given the pathological heterogeneity of the glial tumors studied, the validity of many older descriptive and clinical studies is undermined. In more recent descriptive epidemiological studies, however, the authors have begun to clarify the incidence and prevalence. Pediatric HGGs are significantly less common than are LGG and correspond to around 20% of all hemispheric gliomas (Pollack, 1994; CBTRUS 2010). In the overall context of pediatric primary CNS neoplasms, in which there is a net predominance of lesions seated in posterior fossa, however, HGGs are relatively rare and constitute approximately 6%−10% of all newly diagnosed primary intracranial neoplasms

(Halperin et al., 2004). This corresponds to an annual incidence of approximately 2 cases per million children.

3. Treatment

The role of surgery is clearly major in the treatment, with extent of resection being a strong prognostic factor in most of the studies (Wisoff et al., 1998) However, resectability may be considered as a "biological" feature since infiltration renders complete resection usually impossible. Indeed, tumors that can be resected completely are, at least radiologically, different from those that cannot be resected. The CCG-943 trial was the first to clearly demonstrate the relationship between the extent of resection and patient survival in pediatric HGG. This study reported an improved survival for children in whom some degree of resection, more substantial than a simple biopsy, had been performed (Sposto et al., 1989). A more robust conclusion emerged from the subsequent CCG-945 study, in which a 5-year PFS of around 30% was reported for children in whom a near-total resection (above 90%) was achieved, as compared to a 5-year PFS of only around 15% when a less substantial resection was performed ($p = 0.006$). The strength of association was demonstrated both in the subgroup of patients with anaplastic astrocytoma and in the GBM subgroup (Wisoff et al., 1998). Similar findings have been shown in the other trials, although complete resection has been associated with discrepant low-grade histologies following central pathology review (Chastagner et al., 2001).

Pediatric supratentorial HGGs have been treated most often according to the adult protocols combining radiation therapy and nitroso-ureas (Finlay et al., 1995). In the recent years, following the success of temozolomide in adults, this agent was extensively used and, although results were not superior when compared to historical series using nitroso-ureas (Cohen et al., 2011b), the drug became a standard backbone. Trials are testing the new drugs either as an add-on to temozolomide (as in the current HERBY study) or comparing the new protocol to temozolomide (in form of a pick-a-winner design).

In infants, however, chemotherapy-only approaches have been explored successfully and as many as fifty percent of these young children can be cured with chemotherapy after maximal surgical resection (Dufour et al., 2006). These data suggest already that HGG may be different according to age.

For diffuse intrinsic pontine gliomas (DIPG), the only efficient treatment to date has been radiation therapy and no additional treatment showed some benefit (Jansen et al., 2012) Temozolomide, in particular, has not shown any significant improvement when added to radiation therapy (Chassot et al., 2012; Cohen et al., 2011a; Jalali et al., 2010).

The development of targeted therapies has been hampered by the lack of knowledge about the biology of these tumors in children and the assumption that it would recapitulate the one seen in adults.

4. Prognosis

It is assumed that the prognosis of malignant gliomas is better in children that in adults with 25% of 5-year survivors in contemporary series (Puget et al., 2011). However, this is mainly due to the good results in infants and very young children. Indeed, the results of the combination of radiotherapy and temozolomide in glioblastoma are comparable in children and in adults with less than 20% long-term survivors (Cohen et al., 2011b; Stupp et al., 2005).

The prognosis is even worse in children with DIPG with a median survival of 9 months and most of the children dying before two years from diagnosis (Jansen et al., 2012). Although some studies have indicated possible prognostic markers, none has been validated so far.

5. Limitations of WHO classification of gliomas in children

Although this classification system has shown good correlation with outcome for adults (Louis et al., 2007) this is not always the case for pediatric patients, for whom lack of reproducibility represents a challenge.

Some earlier studies on pathology of pediatric HGG have achieved to show an impact of histology on survival. Distinct 5-year survival rates have been reported a range of 5% to 15% for children with GBM and of 20 to 40% for those with anaplastic astrocytoma (Marchese and Chang, 1990; Phuphanich et al., 1984; Sposto et al., 1989). However the impact of grading has not been shown reproducibly in children. One study suggested that HGGs other than the predominantly astrocytic anaplastic astrocytoma and glioblastoma are associated with a more favorable outcome (Finlay et al., 1995).

The largest clinical randomized study on pediatric HGG published to date was the CGC-945, which enrolled 231 patients with HGG between 18 months and 21 years of age. This study showed discordance in 41/172 single-expert and 51/172 consensus histological diagnoses and 70 tumors reclassified as low grade out of 250 HGG (Pollack et al., 2003a). The poor prognostic capacity of this classification in children has been well documented in a review of a large series of 340 pediatric supratentorial astrocytic gliomas, in which neither WHO nor revised histological categories adequately separated lesions with prognostic significance (Gilles et al., 2000).

The limitations of the WHO histological subtyping of pediatric malignant lesions have been assimilated among pathologists to such an extent that most recent series of HGG

studies in children have accepted the general term pediatric HGG, and included different histological entities within this group (Bax et al., 2010; Paugh et al., 2010; Schiffman et al., 2010).

More recently our group showed that the WHO 2007 classification scheme did not provide a prognostic stratification in a series of 96 pediatric HGG patients. In this study we found that the Saint-Anne classification (Daumas-Duport et al., 1997; Varlet et al., 2004) was closer to achieve prognostic correlation in the same cohort, notably recognizing the category of "malignant glioneuronal tumors", based on clinico-radiological and immunohistochemical grounds (Puget et al., 2011; Appendix 2.1).

In adults, molecular classification has brought more accurate prognostication (Verhaak et al., 2010) and at least three strong prognostic biomarkers have been validated: IDH1, MGMT methylation and 1p19q, this later one being also a diagnostic marker. It is therefore anticipated that it could be reproduced in children although not necessarily with the same prognostic markers.

6. Biology

Differences between children and adults

Striking differences are noted in patterns of disease progression and prevalence of anatomic location between children and adults (Broniscer et al., 2007; Qaddoumi et al., 2009). Some of the most common genetic alterations observed in adult HGG are observed at a significantly distinct frequency in childhood lesions.

Clear distinctions in the underlying biology have been recently published, leading to the conclusions that not only pediatric HGG differ from those occurring in adults, but also that the great heterogeneity within childhood tumors does not necessarily correspond well to the different morphological entities assigned to them in the WHO classification (Bax et al., 2010; Faury et al., 2007; Haque et al., 2007; Paugh et al., 2010; Qu et al., 2010; Rickert et al., 2001; Schiffman et al., 2010; Wong et al., 2006; Zarghooni et al., 2010) .

DIPG are a subset of malignant gliomas occurring almost exclusively in children for which until recently only limited amount of data regarding biology was available. These concerned frequent *TP53* mutations (Gilbertson et al., 2003; Joshi et al., 2008; Louis et al., 1993), EGFR overexpression (Gilbertson et al., 2003) and IL13R overexpression (Joshi et al., 2008). Along the development of our work several new studies have indicated specific biological features of these neoplasms that will be discussed further in the body of the thesis.

Major differences between children and adults concern oligodendroglial and mixed oligodendroglial/astrocytic tumors, which are much more prevalent in the adult population

than in children (Raghavan et al., 2003; Razack et al., 1998). Interestingly, in the adult population around 70% of tumors with oligodendroglial differentiation harbor simultaneous allelic losses on chromosomes 1p and/or 19q, which appear to have a better prognosis and response to a variety of chemotherapeutic agents (Kouwenhoven et al., 2009; McDonald et al., 2005; Ohgaki and Kleihues, 2011). The simultaneous loss of 1p and 19q is exceedingly rare in children in which the incidence of these alterations increases with age. In the anecdotal pediatric cases reported this co-deletion does not seem to be associated with response to chemotherapy (Creach et al., 2012; Pollack et al., 2003b; Raghavan et al., 2003; Suri et al., 2011).

A fundamental characteristic of diffuse gliomas in the adult population is the general tendency towards progression to higher-grade tumors. This phenomenon has been characterized on a molecular basis, includes frequent *TP53* mutations (around 60% cases), rare *EGFR* amplifications (under 10%) and even more rare *PTEN* mutations (below 5%) (Ohgaki et al., 2004) and is clearly distinct from the alterations observed in primary (de novo) glioblastomas (Maher et al., 2006). In pediatric patients supratentorial diffuse infiltrating gliomas occur much more rarely than in adults and their malignant transformation is even more rarely observed. Besides, in one study of 11 pediatric gliomas undergoing malignant transformation the acquired genetic abnormalities overlapped with those of both primary and secondary glioblastoma in adults (Broniscer et al., 2007).

Some of the low frequency genomic events that do occur in the pediatric setting may show overlap with those observed in the clinical 'secondary' pathway of adult gliomas, despite the relative rarity of malignant transformation in children (Broniscer et al., 2007). These include amplifications of *MYC*, *MYCN*, *CDK6*, *CCND2* and *KRAS*, mutations of *TP53* and deletions of *CDKN2C* (Bax et al., 2010; Maher et al., 2006; McLendon 2008; Paugh et al., 2010; Qu et al., 2010; Schiffman et al., 2010; Zarghooni et al., 2010).

Other arguments against the notion that pediatric HGG may be biologically similar to the adult secondary glioma pathway stem from the identification of frequent mutations in *IDH1/2* in secondary adult GBMs, and the lower grade lesions from which they may arise. As reported in several large studies, *IDH1/2* mutations are present in over 70% of lower grade gliomas and 85% of secondary GBMs, in contrast to less than 5% of primary (de novo) adult GBMs (Balss et al., 2008; Hartmann et al., 2009; Ichimura et al., 2009; Nobusawa et al., 2009; Parsons et al., 2008; Yan et al., 2009). In the pediatric population, most studies have rarely found mutant positive tumors. The overall frequency of *IDH1/2* hotspot mutations in childhood HGG is estimated well below 10% (Antonelli et al., 2010; Balss et al., 2008; De Carli et al., 2009; Paugh et al., 2010; Pollack et al., 2010; Schiffman et al., 2010; Setty et al., 2010; Yan et al., 2009). Interestingly, most pediatric mutated cases were reported in adolescents (De Carli et al., 2009; Pollack et al., 2010).

Chromosomal imbalances

Pediatric high-grade gliomas show more frequent gains of chromosome 1q (also frequent in other types of pediatric cancer such as ependymoma, neuroblastoma and Ewing sarcoma) than adult counterparts (19.0% versus 9.0%). They also bear more frequent losses of 4q and 16q (17.5% versus 7.4%) (Bax et al., 2010; McLendon 2008). On the other hand losses of 10q (around 15 to 20%) (Bax et al., 2010; Korshunov et al., 2005) seem less frequent in pediatric HGG as compared to adult glioblastoma samples (80%) (McLendon 2008). Conflicting results have been published regarding the frequency of chromosome 7 gains in Pediatric HGG. In one recent study based on array CHG on paraffin embedded material and containing different histological subtypes it was found in 19% (Bax et al., 2010) whereas a FISH analysis performed exclusively in non-brainstem pediatric GBM described gains of chromosome 7 in 72% cases (Korshunov et al., 2005), a similar frequency to the one reported for adults (McLendon 2008). So far the prognostic value of these alterations has not been tested prospectively. Large-scale chromosomal imbalances appear to be neither sensitive nor specific enough to fulfill the role of diagnostic markers.

EGFR

EGFR amplifications have been reported in 30 to 40% adult high-grade gliomas, its overexpression seen in 40 to 60% of which approximately 50 to 70% display the constitutively activated variant III mutation (Cappuzzo et al., 2005; Smith et al., 2001). In pediatric HGGs, EGFR was found variably overexpressed in 10% to 80% (Bredel et al., 1999; Gilbertson et al., 2003; Liang et al., 2008; Pollack et al., 2006a). Recent series of pediatric supratentorial high-grade glioma reported EGFR amplification in 8 of 74 cases (11%) and *EGFRvIII* deletions in 6 out of 35 cases (17%) (Bax et al., 2009). Some authors reported similar (Qu et al., 2010) and other groups even lower frequency of *EGFR* amplification in 3% cases (2 of 78 samples) (Paugh et al., 2010). Expression of EGFRvIII in adult glioblastoma multiforme (GBM) has been associated with poor prognosis, shorter interval to relapse and increased resistance to chemotherapy and radiotherapy (Feldkamp et al., 1999; Heimberger et al., 2005; Shinojima et al., 2003). No definite conclusions on the role of EGFR and prognosis can be drawn for the pediatric population so far.

PTEN

The potential role for *PTEN* loss in the prognosis and prediction of response to treatment of adult gliomas (the latter mostly in the context of anti-EGFR targeted therapies) has prompted different groups to assess its status in pediatric HGG. However *PTEN* mutation/homozygous deletion appear to be a rare event in pediatric HGG, including DIPG, as compared to adult tumors (Schiffman et al., 2010). Raffel et al in a series of 39 pediatric

gliomas, described that *PTEN* mutations were correlated with higher histological grade and were associated with an adverse outcome. In the same series *TP53* mutations *CDKN2A* deletions and *CDK4* amplifications had no impact on prognosis (Raffel et al., 1999). One study on 44 non brain-stem pediatric glioblastomas by FISH, showed 10q23/*PTEN* loss to be the only factor independently associated with adverse outcome. Other factors such as *EGFR* amplifications, gains of chromosome 7, 1q gains and 1p loss had no prognostic significance in this series (Korshunov et al., 2005). Another work explored immunohistochemistry on 15 pediatric HGG and showed that loss of PTEN expression was associated with worse overall survival (OS), but not with the progression-free survival (PFS) (Thorarinsdottir et al., 2008). Despite these interesting results, the definitive method for analyzing PTEN status in gliomas has not been definitely established. Few publications have shown the importance of *PTEN* inactivation through methylation (Wiencke et al., 2007) while some experts advocate the combination of immunohistochemistry with the determination of PTEN function, either by staining for downstream PIP3 effector molecules (such as pAkt or pPRAS40) or by sequencing of *PTEN* to identify loss-of-function mutations (Mellinghoff et al., 2007).

PDGF

Childhood HGG frequently shows expression of platelet-derived growth factor (PDGFR), as previously reported (Geoerger et al., 2009; Nakamura et al., 2007). Amplification of *PDGFRA* is one of the most common genomic events in pediatric HGG, and although variable frequencies have been reported from around 8 to 28% (Di Sapio 1/14 8%, Paugh 8/68 12 %, Puget 4/32 12.5 %, Wong 2/13 15%, Schiffman 2/11 19% Zarghooni 3/11 28%) (Di Sapio et al., 2002; Paugh et al., 2010; Puget et al., 2012; Schiffman et al., 2010; Wong et al., 2006; Zarghooni et al., 2010) larger series report similar findings of around 12% (Paugh et al., 2010; Puget et al., 2012) which are more common than what has been reported in adult cases (McLendon 2008; Parsons et al., 2008; Rao et al., 2010). Rare findings of *PDGFA*, *PDGFB* and *PDGFRB* gain/amplification could contribute for further activation of PDGFR signalling (Bax et al., 2010; Paugh et al., 2010; Zarghooni et al., 2010). *PDGFRA* amplification appears to occur in older children (Bax et al., 2010; Paugh et al., 2010; Qu et al., 2010) and may have some prognostic significance (Bax et al., 2010; Paugh et al., 2010), though this is not replicated at the protein level (Liang et al., 2008; Thorarinsdottir et al., 2008).

In DIPG focal gains/amplification of *PDGFRA* were reported to be even more frequent, present in about one third of cases (Paugh et al., 2011; Zarghooni et al., 2010) but a study from our cohort did not confirm these findings, in fact revealing similar frequency of amplification as described in other types of pediatric HGG (Puget et al., 2012; Appendix 2.2).

However, the distinction between gains and amplification for *PDGFRA* is not completely established in these publications, and could be the source of such discrepant results. Furthermore, these alterations have been described focally by different subclones within these tumors, which warrants a careful evaluation as they could be remain undetected or overlooked by genomic techniques departing from homogenized tissue.

Strong expression of PDGFR was seen at the protein level in 18/21 HGG and 7/11 DIPG cases. (Liang et al., 2008; Thorarinsdottir et al., 2008). Data is drawn from relatively small series, but if taken together with data from genomic studies, this pathway likely represents an important target, although the clinical associations and potential clinical utility remain to be fully elucidated.

TP53

Following the numerous reports of alterations of TP53 pathways in adult brain tumor populations, a few studies addressing TP53 status in pediatric HGG have been conducted, either by direct search for mutations, or through the surrogate nuclear accumulation of protein by immunohistochemistry. TP53 protein expression has been reported in around 40% pediatric HGG (with an elevated frequency in grade IV vs grade III tumors), and directly sequenced mutations within the DNA binding domains in about 32% cases (Antonelli et al., 2010; Badhe et al., 2004; Cheng et al., 1999; Ganigi et al., 2005; Kraus et al., 2002; Liang et al., 2008; Nakamura et al., 2007; Pollack et al., 2002; Sung et al., 2000; Sure et al., 1997; Suri et al., 2011). In the Children's Cancer Group CCG-945 trial, children who presented TP53 overexpression or mutations had a significantly worse 5-year PFS than did those who did not have these changes (17% ± 6% vs. 44% ± 6%; p = 0.0001) (Pollack et al., 2002). Abnormal TP53 expression was more common in glioblastoma than in anaplastic astrocytoma, but TP53 expression was found to be an independent predictor for each histological subgroup.

In pediatric DIPG *TP53* mutations are even more common, being present in over 50% of tumors (Louis et al., 1993). In a recent study from our group on CGHarrays and gene expression profiling of a large series of pretreatment 61 DIPG, loss of *TP53* locus was the only single chromosomal imbalance significantly associated with a poorer outcome (p=0.01) (Puget et al., 2012; Appendix 2.2).

RAS

Gene-expression profiling supported by immunohistochemichal analysis suggests that there are at least two subtypes of pediatric GBM, one associated with Ras/Akt-activation and poor prognosis and the other with no obvious Ras/Akt activity and a better outcome. This finding is in contrast to adult GBM in which Ras pathway is activated in most tumors (Faury

et al., 2007). This will be discussed later, together with the results of our first article in this chapter.

MGMT

In adult GBM the association between O6-methylguanine-DNA methyltransferase (*MGMT*) methylation status and response to treatment with the DNA alkylating agent temozolomide (TMZ) is well established. The DNA repair enzyme MGMT removes methyl groups from the O6-position of guanine and the expression of MGMT is therefore thought to inhibit the cytotoxic effect of TMZ. *MGMT* gene cytosine hypermethylation, which is observed in approximately 40% of adult GBM patients, results in a marked down-regulation in the synthesis of MGMT transcript and protein; consequently, the MGMT protein whose activity is critical to the repair of alkylated DNA is either absent or greatly impaired in these tumors (Hegi et al., 2005).

The observation that *MGMT* inactivation through promoter methylation especially appears to be associated with a favorable prognosis in adult patients with GBM who receive TMZ and other alkylating agents has led to the evaluation of the significance of *MGMT* promoter methylation in pediatric GBM. A strong correlation with OS was observed irrespectively of treatment, which suggests that *MGMT* methylation may be a prognostic factor for survival in pediatric GBM, as well as a possible marker for TMZ sensitivity (Donson et al., 2007). Similarly, the association between MGMT expression status and outcome in pediatric HGG, with use of tumor samples from the CCG-945 study, has been investigated. Over-expression of MGMT was associated with an unfavorable prognosis in children with HGG who had received alkylator-based chemotherapy as a component of a multimodality treatment (Pollack et al., 2006b).

However in two studies on pediatric DIPG the use of temozolomide has not resulted in any survival benefit (Chiang et al.; Jalali et al., 2010) and could actually be deleterious. This could be explained by the fact that MGMT does not appear to be overexpressed in tumors from patients with DIPG and therefore cannot account for the temozolomide resistance (Zarghooni et al., 2010).

BRAF

Recent studies have reported significant *BRAF* gene alterations in brain tumors, most notably tandem duplications among LGG and the V600E mutation in tumors grades 1 to 4 in both pediatric and adult populations, the latter showing a high incidence in pleomorphic xanthoastrocytomas (around 60%, in a cohort of mixed adult and pediatric patients with mutations found in 2/5 PXA patients under 18 years), (Dias-Santagata et al., 2011) and in 9/18 gangliogliomas in an exclusively pediatric series (Dougherty et al., 2010). Schiffmann et

al have also studied a pediatric cohort and described the V600 mutation in 2/12 grade II astrocytomas, 3/9 grade III astrocytomas and 2/11 GBMs. Mutations were coincident with CDKN2A homozygous deletions in 5/7 cases (Nicolaides et al., 2011; Schiffman et al., 2010). Albeit a potential predictive/prognostic role of these alterations, as anti-BRAF targeted therapies directed to this specific V600E mutation in melanoma patients have recently been reported with encouraging results (Bollag et al., 2010), their role in clinical management for brain tumors has yet to be established.

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Introduction to third article

One other area were biomarkers are actively studied is the prediction of the efficacy of a given targeted therapy. In adult oncology, numerous examples of their usefulness exist and in some cases these biomarkers have been taken into account to guide the prescription of these targeted therapies: *HER2* amplification/overexpression for trastuzumab, HERCEPTIN® in breast cancer, *EGFR* amplification/overexpression without *K-Ras* mutation for cetuximab, ERBITUX® in colon cancer, *EML4-ALK* translocation for crizotinib, XALKORI® in non-smal cell lung carcinoma... In pediatric oncology, however, phase I and II studies have only seldom been done together with a companion diagnostic test. The European consortium ITCC has pioneered such approaches with targeted agents. The target-driven exploratory study of imatinib mesylate, GLIVEC®, enrolled only patients with tumors overexpressing either c-kit, PDGFRA or PDGFRB on immunohistochemistry (Geoerger et al., 2009). The phase I study of erlotinib, TARCEVA®, in newly diagnosed brainstem gliomas and relapsing/refractory brain tumors explored in parallel the EGFR pathway status of the patients (Geoerger et al., 2011).

We show here the feasibility of biomarker studies in pediatric malignant glial tumors, including diffuse intrinsic pontine gliomas (DIPG), from patients participating in this erlotinib phase I trial. Immunohistochemistry and FISH could be applied to define the status of the EGFR pathway and PTEN in tumor tissue before treatment. EGFR was overexpressed in 8/20 DIPG samples (40%); slightly less than in supratentorial high-grade gliomas in which 6/8 tumors (75%) were positive for EGFR. Novel data showing here the frequent PTEN loss in DIPG by immunohistochemistry (15/19 = 80%) should alter the design of trials using agents targeting the tyrosine-kinase activity of growth receptors in this disease. In contrast with a recent report in patients with DIPG (Li et al., 2012) where *EGFRvIII* mutants were detected in 6 out of 11 cases, we did not find any of these mutants in our series. In addition, we show preliminary evidence that patients with DIPG most likely to benefit from the addition of erlotinib to radiation therapy are those with EGFR-positive tumors or oligodendroglial differentiation. Future trials could therefore be designed with treatment allocation on the basis of the molecular profiling of the tumor, even for DIPG at diagnosis.

One could make the hypothesis that the enrichment of phase I/II studies for targeted agents with patients whose tumors harbor the corresponding biological abnormalities could improve the global results of these treatments in the future. In this respect, the BATTLE trial for personalizing therapy in lung cancer paved the way by using an adaptative trial design to test four targeted therapies for the treatment of non-small cell lung cancer to determine which patients should receive each therapy based on the molecular makeup of a patient's tumor based on a biopsy. One of the main findings of this trial was that patients prescribed

treatment with existing drugs based on their tumor biomarkers benefited more than patients whose treatment was not based on their tumor biomarkers (Kim et al, 2011).

Similar trials are under development in children with DIPG, both in Europe (BIOlogical MEdicine for DIPG Eradication, BIOMEDE) or in the USA (Molecularly Determined Treatment of Diffuse Intrinsic Pontine Glioma, NCT01182350). The BIOMEDE trial will allocate the patient to a treatment with erlotinib (TARCEVA®), dasatinib (SPRYCEL®) or everolimus (AFINITOR®) in addition to radiation therapy based on the status of the EGFR, PDGFRA or mTOR pathways, respectively while the North-American trial will explore the addition of erlotinib (TARCEVA®) or temozolomide (TEMODAL®) to a combination of radiation therapy and bevacizumab based on EGFR and MGMT status.

Biomarkers for the efficacy of targeted therapies are therefore required in order to tailor the treatment to the patients most likely to benefit from it. These developments have important economical implications for the Health System but also for the effective implementation of personalized cancer medicine.

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EGFR pathway status and response to erlotinib in children with refractory or recurrent brain tumors and newly diagnosed diffuse intrinsic pontine gliomas

Felipe Andreiuolo[1,2,3,4], Alexander Valent[4£], Pascale Varlet[5£], Stephanie Puget[1,2,3,6], Marie-Cecile Le Deley[7], Chris Jones[8], Alexandru Szathmari[9], Virginie Marty[4], Philippe Vielh[4,10], Catherine Miquel[5], Christian Sainte-Rose[6], Matthieu Vinchon[11], Nicolas André[12], Guirish Solanki[13], Concezio Di Rocco[14], Gilles Vassal[1,2,3,15], Birgit Geoerger[1,2,3,15*], Jacques Grill[1,2,3,15*].

1 University Paris-Sud IX, Vectorology and Anticancer Therapeutics, UMR 8203, Orsay, France.

2 CNRS, Orsay, Vectorology and Anticancer Therapeutics, UMR 8203, France

3 Institut Gustave Roussy, Vectorology and Anticancer Therapeutics, UMR 8203, Villejuif, France

4 Translational research laboratory, Institut Gustave Roussy, Villejuif, France

5 Department of Neuropathology, Hôpital Sainte–Anne, Paris, France

6 Department of Neurosurgery, Hôpital Necker Enfants-Malades, Université Paris V Descartes, Paris, France.

7 Biostatistics and Epidemiology, Institut Gustave Roussy, Villejuif, France

8 Section of Paediatric Oncology, The Institute of Cancer Research, Sutton, Surrey, UK

9 Department of Pediatric Neurosurgery, Lyon, France

10 Department of Pathology, Institut Gustave Roussy, Villejuif, France

11 Department of Neurosurgery, Hôpital Roger Salengro, Lille, France

12 Department of Pediatric Oncology, Hôpital de La Timone, Marseille, France

13 Department of Neurosurgery, Birmingham Childrens Hospital, UK

14 Department of Pediatric Neurosurgery, Universita Cattolica di Roma, Italy

15 Department of Pediatric and Adolescent Oncology, Institut Gustave Roussy, Villejuif, France

[£,*] These authors made equivalent contributions to the study.

Correspondence to: Birgit Geoerger, MD, PhD, Institut Gustave Roussy, Department of Pediatric and Adolescent Oncology, CNRS UMR 8203 Vectorology and Anticancer Therapeutics, 114 Rue Edouard Vaillant, 94805 Villejuif, France; Tel: +33 1 42 11 46 61, Fax: +33 1 42 11 52 45, Email: geoerger@igr.fr

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Running head: EGFR pathway and inhibition in pediatric malignant glial tumors

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Abstract

Purpose: EGFR pathway abnormalities in tumors may predict response to EGFR pharmacological blockade. Our study aimed to define molecular determinants of the EGFR pathway in pediatric recurrent brain tumors and newly diagnosed diffuse intrinsic pontine glioma (DIPG) treated with erlotinib alone or with radiation therapy within a multicentric phase I trial.

Experimental design: After central histological review, EGFR, EGFRvIII, PTEN, p-AKT and p-MAPK expression by immunohistochemistry, *EGFR* amplification and *PTEN* loss by FISH were available in 38/50 tumors of patients treated with erlotinib. An independent series of 21 DIPG treated with chemoradiation was used to estimate the prognostic impact of relevant EGFR pathway alterations.

Results: EGFR expression was most common in supratentorial glioma (6/8) and in DIPG (8/20) although *EGFR* gain was found only in one supratentorial glioma. Loss of PTEN expression was frequent in DIPG (15/19 *vs* 3/8 in supratentorial glioma) and 11 out of 15 DIPG displayed *PTEN* loss on FISH.

Children with DIPG treated with erlotinib and irradiation had a superior overall survival compared to our control group (median 14.9 *vs* 9.5 months, p=0.033). In these patients, EGFR overexpression was associated with a lower risk of progression (OR=0.35, p=0.058), and oligodendroglial *vs* predominant astrocytic differentiation was associated with a trend for improved overall survival (median 14.0 *vs* 8.5 months; p=0.10). This was not seen in DIPG patients not treated with erlotinib.

Conclusion: PTEN loss is frequent in DIPG. Children with EGFR-positive DIPG and oligodendroglial differentiation had an improved survival when treated with erlotinib and irradiation.

Translational Relevance:

Predictive biomarkers for efficacy of targeted therapies are required in order to tailor the treatment to patients most likely to benefit. This is the first study that showed the feasibility of biomarker assessment in pediatric malignant brain tumors including newly diagnosed diffuse intrinsic pontine gliomas (DIPG) in patients participating in an erlotinib phase I trial. IHC and FISH were applied to define the status of EGFR pathway and PTEN. The absence of *EGFR* amplification and our novel data showing the frequent PTEN loss in DIPG needs to be considered in the design of trials using targeted agents in these diseases. We show preliminary evidence that patients with DIPG most likely to benefit from the addition of erlotinib to radiation therapy are those with EGFR-positive tumors or oligodendroglial differentiation. Future trials could therefore be designed with treatment allocation based on molecular profiling of the tumor including DIPG.

Introduction

Central nervous system (CNS) tumors constitute 20% of all pediatric cancers and represent the first cause of cancer-related death and morbidity in this age group. Although an overall 55% cure rate is observed with multimodal intervention such as surgical resection, irradiation and chemotherapy, some subtypes such as diffuse intrinsic pontine gliomas (DIPG) or high-grade glioma are still associated with a dismal prognosis and new therapeutics are highly desirable (1).

Epidermal growth factor receptor (EGFR) contributes to cell growth and proliferation in adult malignant gliomas. Most frequently EGFR protein overexpression is associated with *EGFR* gene amplification or constitutively activating *EGFR* mutations (i.e. *EGFRvIII*); activation may result through alterations in its co-receptors (2-6). EGFR is overexpressed in about 40 to 60% adult high grade gliomas of which approximately 50 to 70% display the variant III mutation (7-10). When activated, the receptor both autophosphorylates and initiates downstream signaling through the RAS/RAF/MAPK and the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signal transduction cascades (11). Expression of EGFRvIII in adult glioblastoma multiforme (GBM) has been associated with poor prognosis, shorter interval to relapse (12-14) and increased resistance to chemotherapy and radiotherapy (15). Furthermore, EGFR tyrosine kinase inhibition has shown radiosensitizing effects in CNS tumor cell lines and xenograft models (16, 17). Anti-EGFR therapies have therefore been largely studied in adult high grade glioma (18-23).Various molecular mechanisms involved in both sensitivity and resistance of gliomas to anti-EGFR therapies have been suggested (24). Coexpression of EGFRvIII and PTEN was found significantly

associated with clinical response to EGFR inhibition in some studies (18, 25). According to other authors low levels of p-AKT together with EGFR overexpression were best predictors of response (19).

In pediatric high grade gliomas, EGFR was found variably overexpressed in 10% to 80% (26-30). A more recent series of pediatric supratentorial high grade glioma reported *EGFR* amplification in 8 of 74 cases (11%) and *EGFRvIII* deletions in 6 out of 35 cases (17%) (31). Other authors reported even lower frequency of *EGFR* amplification in 3% (2 of 78 samples) (32). In a series of 28 surgical biopsy and autopsy brainstem glioma specimens, EGFR overexpression had a positive correlation with higher histological grade. Two cases showed high level and one low level *EGFR* amplifications which did not correlate with EGFR immunohistochemical status (28). In another autopsy series, three out of 11 DIPG patients had strong EGFR positivity in immunohistochemistry (IHC), four had weak positivity and four were negative for EGFR. Interestingly one of the patients without EGFR expression had low level *EGFR* copy number gains (33). Thus, although EGFR pathway alterations are less common at the genomic level in pediatric gliomas than in their adult counterpart, its frequent overexpression could still qualify for a relevant therapeutic target. Based on this rationale, we had explored the EGFR tyrosine kinase inhibitor erlotinib (Tarceva®) within a phase I trial in recurrent brain tumors and newly diagnosed DIPG (34).

Here we present the first study to correlate the analysis of tumor samples and clinical data from children with DIPG at diagnosis. To our knowledge, this is also the first

study to explore EGFR pathway and response to EGFR targeting therapy in children with DIPG treated within a prospective study.

Patients and Methods

Patients and clinical response to erlotinib. Fifty patients were treated with erlotinib within a multicenter phase I study which aimed to establish the recommended dose of erlotinib administered orally as single agent or with radiotherapy in children with malignant brain tumor aged 1 to ≤ 21 years (34). The protocol (NCT00418327) was approved by independent ethics committees/institutional review boards and complied with the Declaration of Helsinki. Written informed consent was received for the clinical and the biological studies. All patients had histologically confirmed diagnosis as inclusion criteria including children with DIPG who underwent stereotactical biopsy procedure at study entry.

Twenty-nine patients with brain tumors refractory to or relapsing after conventional therapy were treated with erlotinib alone in Group 1. In Group 2, 21 newly diagnosed patients with DIPG received radiotherapy (54 Gy) and erlotinib. Overall, 28% of patients in Group 1 had stable disease (SD) (median PFS was 1.5 months); two patients with malignant glioma experienced clinical improvement and tumor regression of 45%. In Group 2, 17% of patients experienced partial response (PR) and 50% SD; the median overall survival (OS) was 12 months (95% CI: 9.3–14.0 months).

In order to assess the role of EGFR expression in patients with DIPG independent of erlotinib treatment, an independent control group of 21 patients who underwent

stereotactical biopsy and were treated with radiation and temozolomide at our institution were explored retrospectively (partly included in (35)).

Centralized histological review. Central histological review was performed by two neuropathologists (PV, FA) and final diagnosis was rendered as a consensus according to WHO 2007 guidelines (36) and Sainte-Anne Hospital classification (37, 38). In addition to hematoxylin-eosin staining, IHC was performed for diagnosis when needed. For DIPG, we morphologically and immunophenotypically assigned a predominant astrocytic or oligodendroglial differentiation as described previously described (39). Astrocytic differentiation was depicted by the presence of atypical cells, often elongated and strongly positive for GFAP and vimentin. Tumors with prominent oligodendroglial differentiation displayed monomorphous round nuclei and were strongly positive for OLIG2 and weak for GFAP and vimentin.

Immunohistochemistry. IHC analysis for EGFR, EGFRvIII, HER2/neu, PTEN, p-AKT and p-MAPK expression was performed on fixed and paraffin-embedded (FPE) tissue. Material was available for 38 patients, paraffin blocks for 26 patients and unstained slides for 12 patients. Antigen retrieval was performed by heating slides in a water bath at pH 6.0 (p-AKT, p-MAPK, EGFRvIII), pH 7.3 (HER2/neu) or pH 9.0 (PTEN) for 30-40 minutes. Protein blocking solution (DAKO, reference X0909) was applied to prevent non-specific binding, and sections were incubated with the following primary antibodies: the monoclonal mouse anti-human PTEN (clone 6H2.1, 1/400, 1 hour), EGFR variant III (clone L8A4, 1/100, overnight; kindly provided by Dr Darrell Bigner), the monoclonal rabbit anti-human p-AKT (SER 473, 1/50, overnight), p-MAPK (TYR 202/TYR 204, 1/100, overnight). HER2/neu stainings were performed

without prior protein blocking solution using the polyclonal rabbit anti-human anti-HER2 antibody (DAKO, reference A0485) incubated for 1 hour at 1/1000 for formalin and formalin zinc fixed material and 1/1500 for alcohol, formalin, acetic acid fixed material. For signal revelation, the Envision anti-mouse and anti-rabbit revelation kits (DAKO, references K4001 and K4003), and the R.T.U Vectastain ABC Elite universal kit (Vector, reference PK-7200) for EGFRvIII were used respectively. Sections were counterstained with hematoxylin. Staining for EGFR (clone 3C6, Ventana) was done using the Benchmark automated system following the manufacturers' instructions. This mouse monoclonal IgG1 antibody is directed against the extracellular domain of human EGFR and recognizes both wild type and variant III EGFR (40).

Immunostaining was scored semi-quantitatively, as adapted from (41), based on both cell number and staining intensity, as follows: 0 = no staining; 1 = less than 10% of cells showing mild to moderate intensity; 2 = 10% to 50% of cells showing mild to moderate staining or strong staining intensity in less than 10% of cells; and 3 = more than 50% of cells showing mild to moderate staining intensity or strong staining intensity in more than 10% of cells. Scores of 2 or 3 were considered positive, ie indicative of overexpression. For PTEN and p-AKT, nuclear and cytoplasmic staining was considered separately. Staining for PTEN on blood vessels was used as internal control and as a parameter for the intensity of staining as described elsewhere (42). For p-MAPK, scoring was done as previously described: a negative score designating light or patchy staining whereas a positive score was assigned to cases with heavy staining in most tumor cells (43). Scoring was performed in consensus between the two neuropathologists blinded to clinical data.

Fluorescence in situ hybridization (FISH). FISH for the genes *EGFR* (c-ErbB1) and *PTEN* was performed on FPE tumor sections of 4 µm, using LSI *EGFR* and *PTEN* probes (Vysis/Abbott, Abbott Molecular Diagnostics, Downers Grove, Illinois, USA) according to the manufacturer's recommendations. FISH for *PTEN* was performed for all DIPG with available material and for supratentorial gliomas showing PTEN loss by IHC. Briefly, the probe was co-denatured 5 min at 84°C on the slide with the tissue and than incubated overnight at 44°C. Slides were washed with post-hybridization wash buffer (0.5x SSC/0.1% SDS) 5 min at 72°C, air-dried and counterstained with DAPI dissolved in an anti-fade mounting solution. At least 100 tumor cells were analyzed and the number of fluorescent signals within the nuclear boundary of each evaluable interphase tumor cell was counted using an Axiophot-ZEISS fluorescent microscope at 1000x magnification. Only nuclei with unambiguous centromeric (7p11.1-q11.1 D7Z1 locus) or (10p11.1-11.1) hybridization green signals (as control) and *EGFR* (7p12) or *PTEN* (10q23) probe signals were scored. For a diagnosis of monosomy, a minimum of 20% of cells showing the loss of one chromosome was considered as a criterion. Normal status corresponded to 2 chromosomes and 2 genes per cell (disomy).

RT-PCR and sequencing for *EGFR variant III.* Reverse-transcriptase (RT)-PCR for detection of the *EGFRvIII* mutation from FPE sections was performed as previously described (31). The presence of the *EGFRvIII* fragment was confirmed in duplicates by direct sequencing.

Statistical analysis. Kaplan-Meier estimates were used for survival curves, Chi square test or log rank test to estimate statistical difference.

Results

Histological diagnosis and central review

Histological review was performed in 41 out of 50 patients treated within the clinical trial; material was not available for 4 supratentorial gliomas, 4 DIPG and 1 PNET. Additional immunostainings were performed for diagnostic purposes in 28 patients. Comparative diagnoses among institutional and central review are summarized in Table 1.

Local pathologist diagnosis was confirmed in 28 of 41 patients for whom both were available (68%). For 8 patients, minor modifications were applied in regard to grading or histological subtype. In five patients, 4 of them with DIPG, histological grade was modified from Grade III to IV or vice versa (#13, #38, #40, #35, #20); three grade III gliomas were considered as mixed oligo-astrocytoma rather than pure oligodendroglioma (#1, #23, #51). In four cases, the modification of the diagnosis was considered as major: one choroid plexus carcinoma (#4) and one DIPG (#17) were reclassified as respectively choroid plexus papilloma and exophytic pontine tumor. An atypical teratoid rhabdoid tumor or malignant glioma not otherwise specified (NOS) (#5) was classified as grade III ependymoma and one anaplastic astroblastoma as choroid plexus carcinoma (#49). One biopsy initially considered to be non-tumoral was reviewed as DIPG based on presence of atypical Ki67 positive infiltrating cells (#21).

Considering central diagnosis when available (n=41) and local diagnosis in the remaining 9 cases, Group 1 was mainly composed of supratentorial malignant glioma (n=12), DIPG (n=6) and anaplastic ependymoma (n=7).

Among the newly diagnosed DIPG in Group 2, according to 2007 WHO classification 2 were astrocytoma grade II, 1 oligodendroglioma grade II, 1 oligoastrocytoma grade II, 3 oligoastrocytoma grade III, 7 grade IV, 6 infiltrative gliomas NOS and 1 exophytic pontine glioma. Of note, all 7 lesions classified as GBM among DIPG showed morphological evidence of astrocytic differentiation and lacked OLIG2 positivity by IHC.

EGFR analysis

EGFR IHC and FISH analysis were performed in 38 patients (Table 2). EGFR overexpression was seen in 17/38 tumors (45%). It was more frequent in supratentorial HGG (6/8 = 75%) than in DIPG (8/20 = 40%) or ependymomas (3/7 = 43%). Most cases displayed cytoplasmic staining. Consistent with prior findings (41), EGFR staining was heterogeneously distributed in 15/17 cases (Figure 1A in a supratentorial GBM and 1B in a DIPG). Tumor cells in mitosis were frequently marked for EGFR as shown in Figure 1B. In our DIPG control group not treated with erlotinib, EGFR overexpression was found in 66% (14 of 21 samples).

EGFR (7p11.2) gene analysis by FISH revealed alterations in chromosome 7 copy numbers in 23 of the 31 interpretable cases. Thirteen cases had polysomy harboring 3 to 5 copies of chromosome 7. Monosomy of chromosome 7 was seen in 10 cases. The alterations of copy number (*EGFR* gene and chromosome 7 centromere) were balanced in all cases but one. A GBM or malignant glioneuronal tumor according to Sainte Anne classification (#15) exhibited a gain of *EGFR* in 30% of tumor cells (Figure 1C); most cells had polysomy and only 20% were disomic for chromosome 7. No cases of *EGFR* amplification were observed.

Three cases showed weak positivity for the EGFRvIII in IHC. RT-PCR confirmed the presence of the *EGFRvIII* mutation in one grade III oligoastrocytoma; presence of the mutation could not be confirmed in two ependymomas due to the poor quality of extracted RNA.

Distribution of *EGFR* gene alterations did not differ significantly between the different tumor types.

Comparing *EGFR* gene alterations with EGFR overexpression, 7/13 (54%) patients with polysomy of chromosome 7 and 6/10 (60%) patients with monosomy were positive for EGFR versus 2/8 (25%) patients with a normal profile (p=NS, Chi square test).

HER2/neu expression

HER2/neu expression was absent in all 36 samples tested.

PTEN analysis

PTEN expression was found in 19 out of 38 tumors evaluated by IHC. Expression had mostly a cytoplasmic pattern, except for one DIPG which showed nuclear staining, and one ependymoma and the medulloblastoma with strong cytoplasmic and nuclear staining. Eighteen cases had PTEN loss (Figure 1D). Interestingly, 15 out of 19 DIPG studied had loss of PTEN expression as compared to 3 out of 18 patients with other tumor types (79% *versus* 17%, p<0.001).

FISH for 10p23 was assessed in all 15 patients with DIPG for whom material was available (11 with PTEN loss, 3 expressing PTEN in IHC, and one with not

interpretable IHC result) and in 3 supratentorial gliomas with PTEN loss. *PTEN* loss (10p23) was observed in 7 cases (Figure 1 E) and chromosome 10 monosomy in two (Figure 1F); one sample showed both abnormalities. Nine cases had no abnormality despite lack of PTEN expression in IHC. All three DIPG with PTEN expression in IHC had 10p23 loss.

p-AKT and p-MAPK expression

Sixteen out of 38 cases studied (42%) were positive for p-AKT expression (3/8 gliomas, 9/20 DIPG, 2/7 ependymomas, 2/3 miscellaneous diagnoses). Variable staining patterns (i.e., nuclear, cytoplasmic, or both) were observed.

Positive staining for p-MAPK was observed in 24 out of 37 patients tested (65%): 5/8 gliomas, 13/19 DIPG, 4/7 ependymomas, 2/3 miscellaneous diagnoses. Staining was always of strong intensity and each case displaying both nuclear and cytoplasmic distribution. Distribution of p-AKT and p-MAPK expression did not differ significantly between the main groups of diagnoses.

There was no correlation between the biomarkers tested. Among the 12 glioma samples with p-AKT expression detected, seven were also negative for PTEN expression, four were positive and one could not be assessed.

EGFR pathway in supratentorial high grade glioma and response to erlotinib

Two patients with supratentorial high-grade glioma experienced tumor regression close to 50% when treated with erlotinib. The oligoastrocytoma grade III (#23) for which material was available exhibited strong EGFR expression and chromosome 7

monosomy in 35% of cells without PTEN loss and low levels of p-AKT and p-MAPK (Figure 2).

The patient with an oligoastrocytoma grade III (#51) exhibiting *EGFRvIII* mutation with 7p polysomy in 80% of cells, activated p-AKT and p-MAPK without PTEN loss, progressed to erlotinib treatment.

One patient with recurrent, rapidly progressing GBM died from intratumoral hemorrhage on day 4 of erlotinib treatment (#15). The tumor had a gain of *EGFR* in 30% of cells (Figure 1C) and strong overexpression of EGFR (Figure 1A), monosomy of chromosome 10 in 30% of cells together with a loss of PTEN expression and *PTEN* locus in 23%. AKT and MAPK pathway were both activated.

EGFR pathway in DIPG and response to erlotinib

Among the 20 tumors available, 18 samples at diagnosis and 2 at relapse, 8 overexpressed EGFR which was often observed in elongated cells with scant cytoplasm as well as in cells in mitosis. Ten out of the 14 DIPG samples with interpretable FISH analysis exhibited an abnormal profile: 6 had polysomy and 4 monosomy of chromosome 7. Thirteen of 19 tumors were positive for p-MAPK, 9 of 20 for p-AKT. A high incidence of loss of PTEN expression was found (15/19) with only 4 cases examined being positive; one had non-interpretable results (#14). By FISH, 4 among these 15 samples had *PTEN* gene loss (#1, 11, 36, 46), and 3 showed chromosome 10 monosomy alone (#14, 26, 41).

We further evaluated prognostic factors in children with newly diagnosed DIPG treated with erlotinib combined with irradiation. Children receiving erlotinib together with irradiation had a superior OS when compared to a control group of 21 children

treated with irradiation and temozolomide (median, 12.0 mo *vs.* 10.7 mo; 95% CI=10.59-13.33 and 4.49-16.9, respectively; p=0.033, log rank test; Figure 3A and B).

EGFR overexpression was associated with a decreased risk of progression (mean PFS = 10.7 *vs* 6.6 months; p=0.058, log-rank test) in patients treated with erlotinib. In the control group of DIPG treated with chemoradiation, EGFR expression was not correlated with the risk of progression (mean, 7.1 *vs.* 6.9 months; p=NS, log-rank test; Figure 3D and F). There was also a better OS for patients with EGFR overexpression treated with erlotinib (mean, 16.9 *vs.* 11.9; p = 0.20, log rank test) while OS in the control group was independent of EGFR expression (mean, 10.2 *vs.* 8.6; p=NS, log rank test; Figure 3 C and E).

Tumors with oligodendroglial differentiation showed a trend for better OS than astrocytic tumors when treated with erlotinib (median, 14.0 *vs.* 8.5; p=0.10, log-rank test) while this was not the case in the control group (median, 7.2 *vs.* 14.0; p=0.NS, log-rank test; Table 3).

Discussion

This is the first study to correlate biomarkers with treatment efficacy in pediatric patients with brain tumors including DIPG. The study showed that biomarker analysis using IHC and FISH is feasible on stereotactic biopsy samples in DIPG which has only recently been re-introduced in medical practice (44).

We confirmed overexpression of EGFR in pediatric supratentorial malignant gliomas and DIPG, the former being more frequently positive for EGFR and PTEN. Despite the presence of EGFR protein overexpression, *EGFR* gene amplification is rare in pediatric gliomas, as suggested already in previous studies (28-33). The incidence of EGFR overexpression in our study was similar to those reported in the series of Gilbertson et al (13 out of 28 cases) when using an equivalent scoring scale (28) and of Zarghooni et al (7 out of 11 cases) (33). In the series of Gilbertson, 2 cases of DIPG were found with high-level *EGFR* amplification and one with low-level (28). In addition to the findings in these prior reports, we observed a high number of chromosome copy abnormalities, such as chromosome 7 monosomy and polysomy. The functional significance of this is so far not known.

The absence of correlation between chromosome 7 monosomy, polysomy and EGFR immunohistochemical status may be an evidence of important post-translational regulation of this gene. Our findings of heterogeneous, sometimes focal EGFR expression by different cells in a given tumor as well as the heterogeneity of chromosomal alterations in cell subpopulations may suggest also for pediatric gliomas a co-existence of different clones within the same tumor (45) and highlight

the importance in performing IHC and FISH techniques for this assessment as compared to expression arrays, and as already suggested for adult glioma (41).

Similarly to EGFR, PTEN status should be determined by both IHC and FISH. On one hand, *PTEN* gene loss was not always associated with absent PTEN protein and on the other hand the absence of PTEN expression can not solely explained by the loss of the *PTEN* locus. *PTEN* loss determined by FISH has been reported as a marker of bad prognosis for supratentorial childhood glioblastomas (46). Our evaluation in newly diagnosed DIPG revealed a high percentage of tumors with PTEN loss. The loss of PTEN function activates the PI3K/AKT/mTOR pathway thus enhancing cell proliferation and reduced apoptotic death. AKT phosphorylation was seen in conjunction with PTEN loss in the majority of cases. Four p-AKT positive cases expressed PTEN confirming alternative mechanisms to activate this pathway. PI3KCA mutations recently reported in DIPG (47) could be one of them. One could therefore postulate that mTOR inhibitors could be valuable drug candidates especially in patients with DIPG. In this respect, the phase II trial of the rapamycin analogue temsirolimus in pediatric patients with relapsed high-grade glioma showed interesting prolonged tumor stabilization in 5 out of 10 DIPG treated (48). In addition, radiosensitization with mTOR inhibitors has been obtained in *PTEN* null cells (49) and may suggest the use of these agents upfront in combination with radiotherapy for children with PTEN negative DIPG.

Following reports in adult glioma patients correlating EGFR pathway status with outcome treated in clinical trials with EGFR tyrosine kinase inhibitors (18, 19), the same biomarkers were explored in our pediatric trial cohort. Two of 8 patients with

supratentorial glioma experienced clinical benefit and close to 50% tumor regression with erlotinib single agent treatment at relapse. For the one whose tumor material was available for biomarker studies (anaplastic oligoastrocytoma), we found strong expression of EGFR and persistence of PTEN expression together with the absence of AKT and MAPK activation. This finding is in concordance with the profiles of responders previously described in the literature (18, 19, 25). One patient with a supratentorial glioblastoma displaying *EGFR* gain and strong EGFR expression in IHC experienced intratumoral hemorrhage on day 3 of erlotinib treatment. Although this subtype of gliomas is known for rapid growth and tendency to hemorrhages, a possible intratumoral necrosis due to tumor response to erlotinib could also be speculated. EGFR may be therefore a therapeutic target in a subset of recurrent pediatric gliomas displaying a similar profile.

In children with newly diagnosed DIPG treated with erlotinib and irradiation, these with EGFR expressing tumors appeared to have a better PFS compared to those that were negative for EGFR expression (PFS 12 months *vs* 6 months). In contrast, there was no difference in PFS for EGFR positive *versus* negative tumors in a comparable population of children treated with chemoradiation suggesting EGFR overexpression may be a predictive marker of tumor response to the combination of erlotinib and radiation and not a prognostic marker in DIPG. Moreover, the patients who seemed to benefit the most with erlotinib were those with oligodendroglial type of DIPG, which do usually represent the most aggressive form of DIPG (39). As a whole, the survival of the patients treated with erlotinib in combination with radiotherapy was better that the contemporary controls. These findings led us to design a phase II study of this combination for patients with EGFR positive tumors. Whether the frequent loss of

PTEN expression in DIPG could limit the response to EGFR inhibition needs to be further explored.

Conclusion

EGFR overexpression is present in pediatric high-grade glioma, often associated with chromosome 7 monosomy or polysomy, but rarely *EGFR* gene amplification or *EGFRvIII* mutation. The study highlights the importance of biological correlates and biomarker studies in the development of targeted agents for the treatment of pediatric brain tumors, although the complexity of biological findings and limited numbers of samples may preclude definitive conclusions with respect to response to treatment. Our results suggest an advantage of EGFR targeting with tyrosine-kinase inhibitors for supratentorial and pontine gliomas when EGFR is overexpressed and needs to be confirmed in a larger setting. The combination with inhibitors of the PI3K/AKT/mTOR pathway warrants future development in these tumors in which PTEN loss is frequent.

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Pat No	Local Diagnosis	Revised WHO Diagnosis	Revised St Anne Diagnosis
Group1			
Malignant Glioma (n=12)			
#15	GBM/MGNT	GBM	MGNT
#45	GBM/MGNT	GBM	MGNT
#44	GBM	GBM	MGNT
#50	Glial tumoral infiltration	Gliomatosis	Gliomatosis
#48	Oligodendroglioma gr II/ gr B	Oligodendroglioma gr II	Oligodendroglioma gr B
#35	GBM	Oligodendroglioma gr III	Oligodendroglioma gr B
#23	Oligodendroglioma gr III	Oligoastrocytoma gr III	Oligoastrocytoma gr B
#51	Oligodendroglioma gr III	Oligoastrocytoma gr III	Oligoastrocytoma gr B
#28	Malignant Glioma	NR	
#30	Malignant Glioma	NR	
#32	Malignant Glioma	NR	
#43	Malignant Glioma	NR	
Pontine glioma (n=6)			
#25	DIPG	DIPG NOS	DIPG NOS

Pat No	Local Diagnosis	Revised WHO Diagnosis	Revised St Anne Diagnosis
#18	Difficult diagnosis	DIPG NOS	DIPG NOS
#29	Astrocytoma gr III	Astrocytoma gr III	Oligoastrocytoma gr B
#13	Astrocytoma gr III	GBM	GBM
#24	DIPG possibly high grade	No viable tumor in block	
#33	DIPG	NR	
Ependymoma (n=7)			
#22	Ependymoma gr III	Ependymoma gr III	Ependymoma gr III
#27	Ependymoma gr III	Ependymoma gr III	Ependymoma gr III
#42	Ependymoma gr III	Ependymoma gr III	Ependymoma gr III
#2	Ependymoma, partially papillary	Ependymoma gr III	Ependymoma gr III
#6	Poorly differentiated ependymoma/neurocytoma	Clear cell ependymoma gr III	Clear cell ependymoma gr III
#12	Myxopapillary ependymoma	Myxopapillary ependymoma	Myxopapillary ependymoma
#5	ATRT or well differentiated glial tumor	Ependymoma gr III	Ependymoma gr III
Others (n=4)			
#3	Desmoplastic medulloblastoma	Desmoplastic medulloblastoma	Desmoplastic medulloblastoma
#4	Choroid plexus carcinoma	Choroid plexus papilloma	Choroid plexus papilloma

Pat No	Local Diagnosis	Revised WHO Diagnosis	Revised St Anne Diagnosis
#49	Anaplastic astroblastoma	Choroid plexus carcinoma	Choroid plexus carcinoma
#37	Cerebral PNET/Pineoblastoma	NL	
Group 2			
#11	GBM or oligoastrocytoma gr III	GBM	GBM
#31	Astrocytic high gr glioma	GBM	GBM
#41	GBM/ MGNT	GBM	MGNT
#36	Giant cell glioblastoma/MGNT	Giant cell glioblastoma	MGNT
#9	DIPG	Oligodendroglioma gr II	DIPG, grade ND
#14	DIPG	Oligoastrocytoma gr II	DIPG, grade ND
#10	DIPG	DIPG NOS	DIPG NOS
#19	Diffuse astrocytoma	DIPG NOS	DIPG NOS
#39	Astrocytoma gr II	DIPG NOS	DIPG NOS
#47	Low gr glioma	DIPG NOS	DIPG NOS
#34	Astrocytoma gr II	Astrocytoma gr II	Oligodendroglioma gr A
#26	Astrocytoma gr II/gr B	Astrocytoma gr II	Oligoastrocytoma gr B
#46	Oligoastrocytoma gr III/oligoastrocytoma gr B	Oligoastrocytoma gr III	Oligoastrocytoma gr B

Pat No	Local Diagnosis	Revised WHO Diagnosis	Revised St Anne Diagnosis
#38	Astrocytoma/oligoastrocytoma gr III	GBM	GBM
#1	Oligodendroglioma gr III	Oligoastrocytoma gr III	Oligoastrocytoma gr B
#40	Astrocytoma gr III	GBM	Oligoastrocytoma gr B
#20	Astrocytoma gr IV	Oligoastrocytoma gr III	Infiltrative brain stem glioma; grade ND
#17	Malignant infiltrative brain stem glioma	Exophytic brain stem glioma	Exophytic brain stem glioma
#21	Non representative biopsy	DIPG NOS	DIPG NOS
#7	Oligoastrocytic tumor, difficult to grade (NOS)	NL	
#8	GBM	NL	

Table 1: Local and reviewed histological diagnosis

GBM, glioblastoma; MGNT, malignant glioneuronal tumor; gr, grade; DIPG , Diffuse intrinsic pontine glioma; NOS, not otherwise specified; NR, no material received for review; NL, no tumor left or non representative slides, ND not determined due to lack of imaging

Table 2: EGFR pathway expression by FISH and IHC in patients treated with erlotinib

Pat No	Diagnosis WHO Classification	EGFR IHC	*EGFR* FISH	PTEN IHC	*PTEN* FISH	p-AKT IHC	p-MAPK IHC
Supratentorial glioma		**6+/8**		**5+/8**		**3+/7**	**5+/8**
#48	Oligodendroglioma gr II	2	Normal	3	ND	1	1
#35	Oligodendroglioma gr III	0	Monosomy 7 35%	0	*PTEN* loss 60%	0	0
#23	Oligoastrocytoma gr III	3	Monosomy 7 35%	2	ND	1	0
#51	Oligoastrocytoma gr III	3	Polysomy 7 80% (3 to 4 copies)	3	ND	2	1
#15	GBM	2	Polysomy 7 80% (3 to 5 copies) Low level gains 30%	1	Monosomy 10 30% *PTEN* loss 23%	3	1
#44	GBM	1	Normal	1	Normal	0	1
#45	GBM	2	Monosomy 7 61%	2	ND	3	1
#50	Gliomatosis	3	Polysomy 7 (3 copies) 25%	3	ND	0	0
Pontine glioma		**8+/20**		**4+/19**		**8+/20**	**12+/19**
#9	Oligodendroglioma gr II	2	Monosomy 7 60%	1	Normal	1	1
#26	Astrocytoma gr II	1	Normal	1	Monosomy 10 25% Balanced trisomy 27 %	3	1
#34	Astrocytoma gr II	3	Monosomy 7 30%	0	Normal	3	1
#14	Oligoastrocytoma gr II	1	Monosomy 7 30%	NE	Monosomy 10 33%	2	2
#1	Oligoastrocytoma gr III	0	Normal	3	*PTEN* loss 25%	1	1
#20	Oligoastrocytoma gr III	0	2/2 100%	0	Normal	0	1
#46	Oligoastrocytoma gr III	2	Polysomy 7 (3 to 4 copies) 47%	0	Polysomy 10 and *PTEN* loss 30%	1	0
#11	GBM	0	Polysomy 7 (3 to 5 copies) 65%	0	Polysomy 10 and *PTEN* loss 51% Polysomy 10 without loss 32%	1	1
#31	GBM	0	No signal	1	Normal	3	0
#38	GBM	1	No signal	0	No signal	2	1
#40	GBM	1	Monosomy 7 52%	3	ND	1	0
#41	GBM	0	Trisomy 7 11%	3	Monosomy 10 50%	3	1
#13	GBM[§]	3	Trisomy 7 54%	0	Normal	0	0

#36	Giant cell GBM	0	Polysomy 7 (3 to 5 copies) 90%	3	Polysomy 10 and *PTEN* loss 85%	3	1
#17	Exophytic brain stem glioma	0	No signal	0	Normal	0	0
#19	DIPG NOS	0	No signal	0	No signal	0	0
#21	DIPG NOS	2	Trisomy 7 17%	0	Normal	0	1
#39	DIPG NOS	2	No signal	0	No signal	0	ND
#47	DIPG NOS	2	No signal	0	ND	3	1
#25	DIPG NOS§	3	Normal	0	Normal	2	1
Ependymoma		**3+/7**		**7+/7**		**2+/7**	**4+/7**
#2		3	Monosomy 7 49%	3	ND	1	1
#5		0/3*	Polysomy7 (3 to 4 copies) 51%	3/3*	ND	1/0*	0/0*
#6		0/0*	Monosomy 7 25%	3/3*	ND	3/3*	1/1*
#12		1	Trisomy 7 70%	2	ND	1	1
#22		3	Monosomy 7 23%	3	ND	2	1
#27		0	Normal	2	ND	1	0
#42		2	Trisomy 7 60%	2	ND	0	0
Others		**0+/3**		**3+/3**		**2+/3**	**2+/3**
#3	Desmoplastic medulloblastoma	0	Trisomy 7 55%	3	ND	3	0
#4	Choroid plexus papilloma	0	No signal	3	ND	2	1
#49	Choroid plexus carcinoma	1	Normal	3	ND	1	1

ND, not done; NE, not evaluable; * shows results for samples at diagnosis and at relapse and § for samples at relapse only in two patients; FISH was preformed on the sample at diagnosis; results at diagnosis are used for the analysis

For EGFR, PTEN and p-AKT expression by IHC, 2 and 3+ were considered as positive, 1+ and 0 as negative; for p-MAPK expression 1 was considered as positive, 0 as negative.

Tumor heterogeneity is expressed in % of all different clones found by FISH. Four genetic categories of *EGFR* gene alterations in tumors were found: monosomy (blue), polysomy (green), gain (red), or clones with disomy (2 copies of chromosome 7 disomy considered as normal). Three genetic categories of *PTEN* gene were found: disomy (2 copies of chromosome 10, considered as normal) loss of the gene (red) and loss of one whole chromosome 10 (blue).

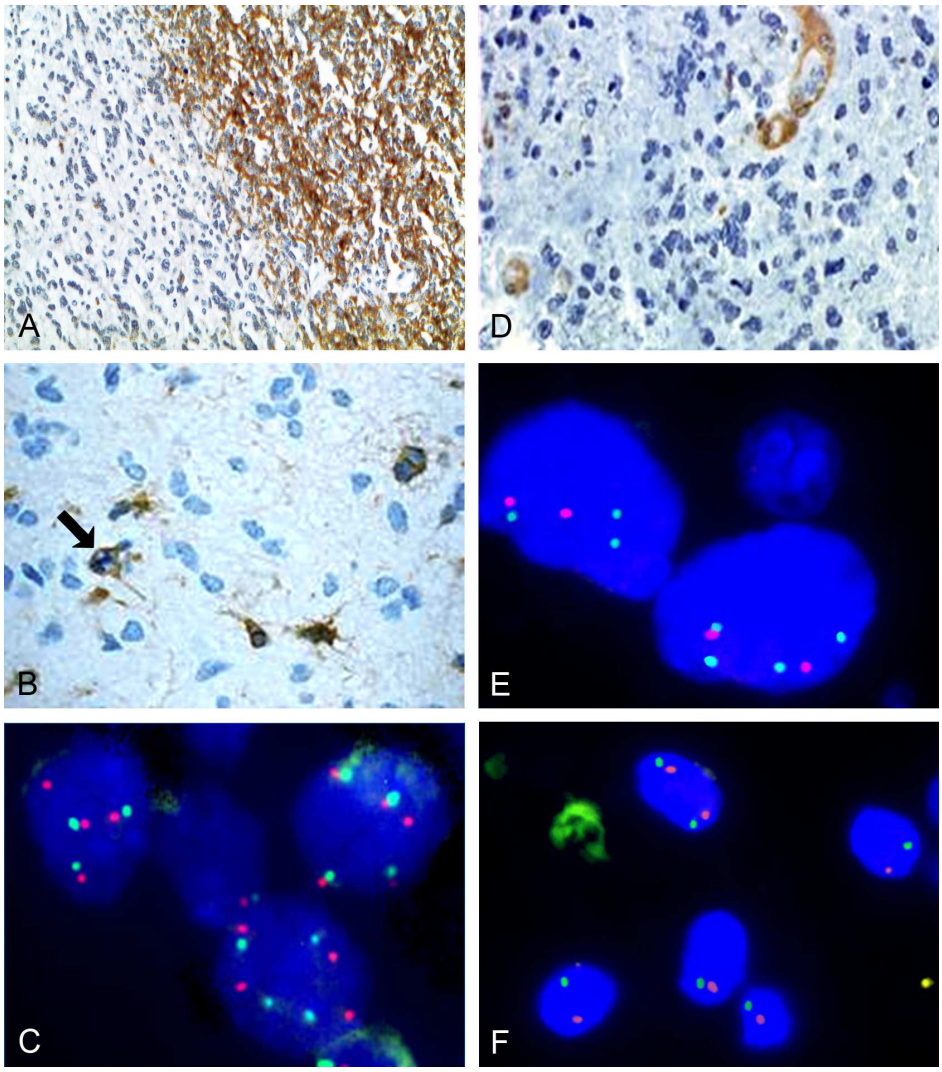

Figure 1. EGFR and PTEN expression by IHC and FISH analysis

(A) Strong but heterogeneous EGFR expression in a supratentorial glioblastoma and (B) a DIPG including in cells in mitosis (arrow). (C) *EGFR* low level gain in a glioblastoma with chromosome 7 polysomy. The tumor cell in on the upper left corner (arrow) presents with four red signals (*EGFR* gene) and three green signals (chromosome 7 centromer) whereas the other 2 cells have balanced polysomy. (D) Loss of PTEN expression in a DIPG: tumor cells show no staining as compared to positive blood vessels. (E) *PTEN* loss in a DIPG with chromosome 10 polysomy; PTEN signals (red) are outnumbered by chromosome 10 centromer signals (green). (F) Chromosome 10 monosomy in DIPG: 4 of 5 cells show only one copy of both *PTEN* and chromosome 10 centromer.

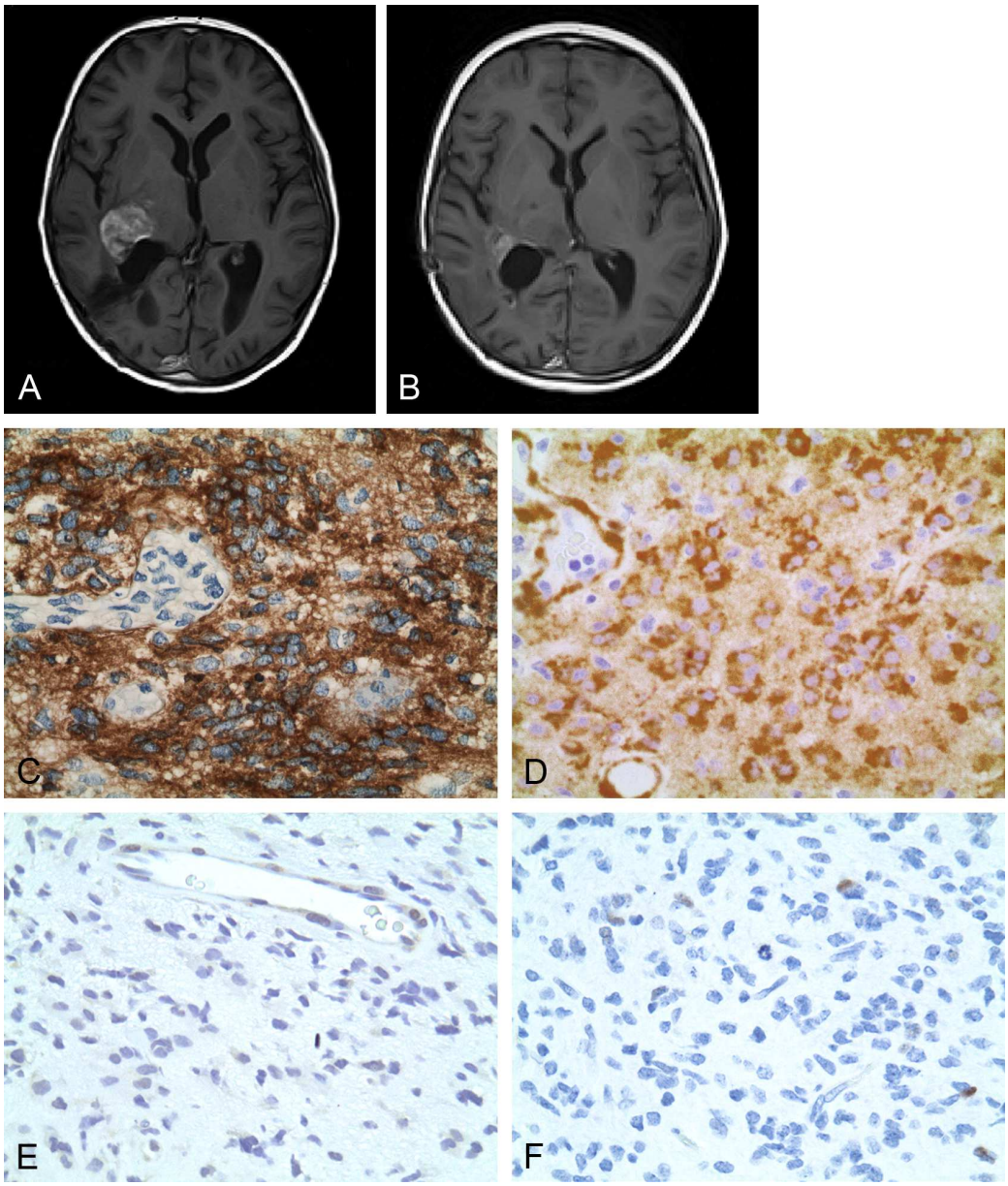

Figure 2. Patient with a grade III oligoastrocytoma experiencing tumor regression to erlotinib

Contrast-enhanced MRI scans at baseline (A) and after 2 cycles of erlotinib treatment (B). Immunohistochemistry shows strong EGFR expression (C), no PTEN loss (D), and no activation/posphorylation of AKT (E) and MAPK (F).

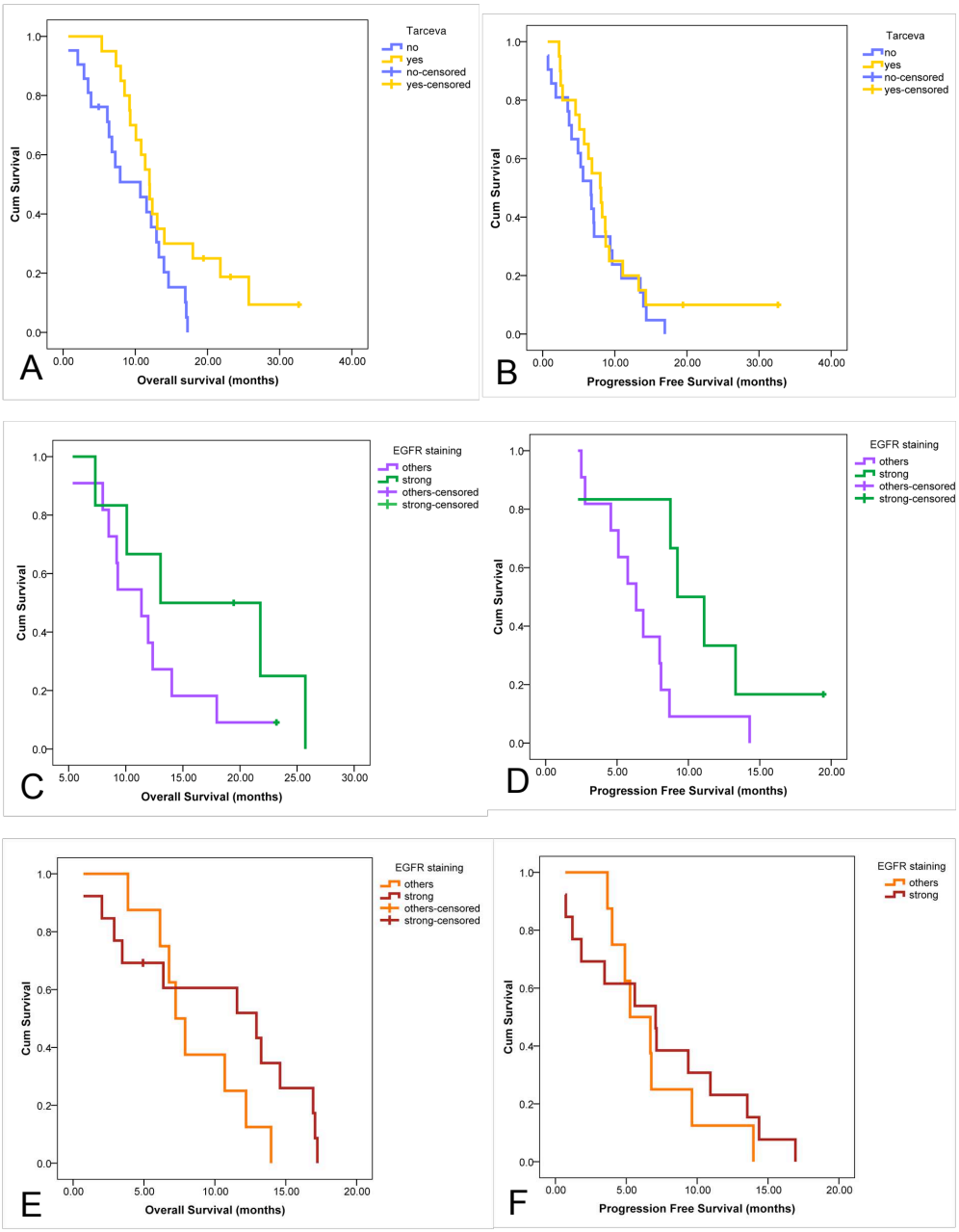

Figure 3. Outcome in children with DIPG and EGFR expression

(A) Children with DIPG treated with erlotinib combined with irradiation (yellow) had a superior OS as compared to a control group treated with radiation and temozolomide (blue; p= 0.033). (B) PFS was similar for both groups. When treated with erlotinib and irradiation, children with DIPG overexpressing EGFR (green) had a trend for better OS (p= 0.2; C) and PFS (p=0.057; D) than those without EGFR overexpression (violet). EGFR overexpression had no impact on OS (E) and PFS (F) in the control group of children treated with temozolomide and radiation.

Introduction to fourth article

The prognosis for children with DIPG is dismal, with a median survival of 9 months and most of the children dying before two years from diagnosis. Current therapies are mostly based on focal radiotherapy, which provides only temporary improvement. To the present, no significant benefit from chemotherapy has been reported. Effective therapies for this disease are dramatically lacking.

Most of the data on biology of DIPG has been drawn from post-treatment autopsy specimens. With the recent demonstration of the feasibility of stereotaxic biopsies for newly diagnosed DIPG with minimal morbidity, we studied a series of 20 cases by comprehensive mutation analysis.

The Oncomap® system we chose here interrogates 983 different mutations in 115 known oncogenes and tumor-suppressor genes (the complete list of genes follows the article), many of which are "drugable" targets of small inhibitor molecules. The other advantage of this system is the use from a relatively small amount of DNA (200ng) from each sample.

In this study we report mutations identified in 12/20 samples, of which 8 harbored mutations of *TP53*, 3 showed mutations of *PI3KCA* and 1 patient had mutations of both *ATM* and *MPL*, the latter 2 genes also involved in the Pi3K pathway. The mutations in *PI3KCA* constitute the first identification of oncogenes mutations in DIPG reported so far. *PTEN* loss and *PDGFRA* amplification were detected in 2 and 3 cases mutated for *TP53* respectively.
The lack of mutation in other known oncogenes and tumor suppressor genes such as *Ras* genes suggests particular mechanisms of oncogenesis in this disease that should be evaluated by exome or whole genome sequencing and through epigenetic profiling.
Although our cohort here is relatively small, the fact that *TP53* and *PIKCA* mutations were mutually exclusive is another evidence of subgroups of patients with distinct driving biological mechanisms; and is in keeping with data from the literature, which show different molecular subgroups in this disease. We have reported a "mesenchymal" subgroup with pro-angiogenic characteristics and an "oligodendroglial" subgroup, driven by PDGFRA and with a worse outcome (Puget et al) (Appendix 2.2).

The following table shows there was not a clear correlation between the mutations and the two gene expression groups previously defined. For further details on gene expression subgroups see Appendix 2.2.

Mutations and gene expression subgroups in DIPG samples

Gene	Mutation	Patient Number	GE group
ATM	P604S	13	TBD
MPL	W515L	13	TBD
PI3KCA	H1047R	1	1
PI3KCA	H1047L	4	1
PI3KCA	E542K	7	2
TP53	R248W	6	2
TP53	R273H	9	2
TP53	V157F	12	2
TP53	R213*	16	TBD
TP53	R273L	17	1
TP53	R282W	18	1
TP53	R273H	19	1
TP53	R273L	21	2

GE= gene expression; TBD= to be defined

This indicates that the two gene expression profiles of DIPG that we have evidenced (and that have been also found by Paugh et al, JCO 2011) are not driven by mutation in common oncogenes or tumor suppressor genes.

Recently it has been shown that a high percentage of DIPG present mutations in genes coding for histones H3.3 (60%) and H3.1 (18%) (Wu et al). The data on other mutations is not available to date. We have shown that gene expression of the mesenchymal group of DIPG is not driven by copy-number changes and it could be hypothesized that epigenetic changes consecutive to specific histone mutations may influence gene expression more dramatically.

Finding therapeutic targets in DIPG is of paramount importance; the approach that we have developed in order to find drugable mutations is rather pragmatic. By virtue, PI3KCA seems to be a possible new target along with EGFR, PDGFRA, or MET, the last two being shown amplified or gained in DIPG by two independent teams (Paugh et al., 2011; Puget et al., 2012). In addition, the loss of PTEN expression, frequently found in DIPG (as reported in the first article in chapter 2), is also pointing towards the deregulation of the mTOR pathway. Whether true PI3KCA inhibitors or dual PI3KCA/mTOR inhibitors should be used has still to be tested in vitro in relevant cell lines. In this respect, encouraging results have been shown in some patients with recurrent DIPG treated with temsirolimus (Geoerger et al., 2012).

Further developments will consist in incorporating new recurrent mutations in the drug screen for DIPG found by exome or whole genome sequencing and to correlate these mutations with functional aspects of the biology of these neoplasms (oligodendroglial differentiation, mesenchymal transition, angiogenesis...). Finally, good candidate mutations

will have to be tested in relevant preclinical models, recapitulating the complex and original biology of DIPG.

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PRIORITY REPORTS
Critical Oncogenic Mutations in Newly Diagnosed Pediatric Diffuse Intrinsic Pontine Glioma

Jacques Grill, MD, PhD, [1*y] Stephanie Puget, MD, PhD,[2] Felipe Andreiuolo, MD,[3,4] Cathy Philippe,[4] Laura MacConaill, PhD,[5z] and Mark W. Kieran, MD, PhD6§

Diffuse intrinsic pontine gliomas (DIPG) can not be cured with current treatment modalities. Targeted therapy in this disease would benefit from advanced technologies detecting relevant drugable mutations. Twenty patients with classic newly diagnosed DIPG underwent stereotactic biopsies and were analyzed for the presence of 983 different mutations in 115 oncogenes and tumor-suppressor genes using OncoMap, a mass spectrometric method of allele detection. Our results identified oncogenic mutations in TP53 (40%), PI3KCA (15%), and ATM/MPL (5%) while none were identified in a large number of other genes commonly mutated in malignant gliomas. The identification of oncogenic mutations in the PI3K pathway offers the potential of a therapeutic target at initial diagnosis in this devastating disease. Pediatr Blood Cancer 2012;58:489–491. ß 2011 Wiley Periodicals, Inc.

Key words: ATM; brainstem; PI3KCA; stereotactic biopsy; targeted therapy; TP53

INTRODUCTION

Diffuse intrinsic pontine gliomas (DIPG) are highly aggressive brain neoplasms of childhood that are inoperable and universally fatal. Diagnosis is based on the combination of characteristic symptoms at presentation and classic MRI appearance and historically have not required biopsy. Standard treatment remains focal radiation therapy; no improvement in outcome has been achieved with the addition of chemotherapy, radiation sensitizers, or other experimental approaches [1,2]. While DIPGs were thought to be similar to adult high-grade gliomas, increasing data suggests that there may be significant molecular differences between these tumors [3–5]. For DIPGs, most of the molecular information has been derived from autopsy cases after therapy [4,6,7]. PDGFRA and MET amplification were the most common genomic imbalance detected in most of these studies. With the demonstrated safety and feasibility of stereotactic biopsies in newly diagnosed DIPG [8], we now present a comprehensive mutational analysis and target identification in 20 consecutive cases.

Recent advances in molecular biology have resulted in adapted high-throughput genotyping for known oncogene mutations [9]. This method was optimized for both fresh frozen and formalin-fixed paraffin-embedded material and is called OncoMap. It has been applied to a number of adult and pediatric tumors and resulted in the discovery of the BRAF mutation in pediatric gangliogliomas [10]. Ideally suited for oncogenes, where a limited number of mutations at specific residues can result in constitutively activated genes products, common mutations in tumor suppressor genes can also be identified, although certain inactivating mutations can be missed.

METHODS

DNA was extracted from biopsy samples obtained at diagnosis from 20 patients with DIPG and were directly snap frozen in the operating room; all samples were obtained from patients with classical clinical and radiological criteria for DIPG on an IRB approved protocol [11,12]. All 20 samples were histologically proven high grade glial neoplasms. We used 200 ng of DNA from each sample in an optimized profiling platform called ''OncoMap'' to interrogate 983 unique mutations in 115 known oncogenes and tumor suppressor genes [13,14], many of which are targets of small molecule inhibitors. DNA was quantified using picogreen analysis, then subjected to whole genome amplification and used for further analysis only if DNA fingerprinting demonstrated non-biased amplification. Whole genome amplified DNA was used as input for multiplex PCR using primers from OncoMap 3 and OncoMap 3 Extended which together comprise 1,047 independent assays interrogating 983 unique mutations across 115 genes. Single base primer extension was performed using iPlex Gold single base extension enzyme (Sequenom, San Diego, CA) and products were transferred to SpectroCHIPs for analysis by MALDI-TOF mass spectrometry. Allele peaks were flagged using a modified Sequenom algorithm followed by manual review by two independent reviewers of candidate calls which were classified as ''aggressive'' or ''conservative'' depending on their apparent robustness. Sample quality was considered adequate for analysis if more than 80% of the attempted genotypes resulted in identifiable products. Candidate mutations were validated using multi-base hME extension chemistry and a

[1] Brain Tumor Program, Department of Pediatric and Adolescent Oncology, Gustave Roussy Cancer Institute, Universite Paris Sud, Villejuif, France; [2]Department of Neurosurgery, Hopital Necker-Enfants Malades, Universite Paris Descartes, Paris, France; [3]Histo-Cytopathology Module, Translational Medicine Program, Gustave Roussy Cancer Institute, Universite Paris Sud, Villejuif, France; [4] CNRS 8203 ''Vectorology and Anticancer Treatments'', Research Pavillon 2, Gustave Roussy Cancer Institute, Villejuif, France; [5] Center for Cancer Genome Discovery, Personalized Cancer Medicine Partnership, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Boston, Massachusettes; [6]Pediatric Medical Neuro-Oncology, Dana-Farber Cancer Institute and Children's Hospital Boston, Pediatric Hematology/Oncology, Boston, Massachusettes

[y] Director.

[z] Scientific Director.

[§] Director.

*Correspondence to: Jacques Grill, MD, PhD, Brain Tumor Program, Department of Pediatric and Adolescent Oncology, Gustave Roussy Cancer Institute, Universite Paris Sud, Villejuif, France.
E-mail: grill@igr.fr

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bidirectional assay design interrogating both DNA strands independently from unamplified genomic DNA. Proportion of mutant alleles in each sample was determined by dividing the area of the mutant allele peak identified in hME by the sum of the areas of the mutant and wild-type allele peaks. Results of the mutation screen were analyzed in parallel with chromosomal imbalances detected with the Agilent 44K Whole Human Genome Array G4410B. The microarray data related to this article have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-TABM-1107.

RESULTS

Mutations were detected in 11/20 samples (Table I). In spite of the highly malignant nature of DIPG, only a limited set of cancer-driving mutations were detected in these 11 patients. In particular, we did not find mutations previously reported in adult or pediatric gliomas including PTEN, RB1, EGFR, MET, CTNNB1, N-, H-, or K-RAS, MLH1, EPHA genes or the tyrosine-kinase domain of KIT or PDGFRA.

Specific and recurrent mutations were identified for the tumor suppressor TP53 (40%) and the tumor oncogene PI3KCA (15%). TP53 mutations in eight patients, including five at the 273 arginine position were not associated with increased genome instability as measured by the ratio of chromosomal breaks in the CGHarray analysis run in parallel. Loss of the p53 gene was identified in four out of eight samples with mutations. IHC for p53 was positive in all samples with p53 mutations.

One sample had missense mutations in ATM and in MPL identified.

DISCUSSION

Our results demonstrate the clinical feasibility of high-throughput mutational profiling to query a large panel of ''actionable'' cancer gene mutations with limited amounts of DNA obtained through stereotactic biopsies from children with newly diagnosed DIPG. In the future, this type of approach may be incorporated into clinical decision making to guide the choice of targeted anticancer agents in DIPG or other diseases. The risks associated with stereotactic biopsy in the brainstem [8] in centers with appropriate expertise needs to be balanced by the potential of finding relevant therapeutic targets. Using the findings in this study, modifications of the OncoMap panel used for further target identification in this disease can be performed.

The frequency of p53 mutations found in this study is in line with a previous report that detected mutations in five out of seven cases by sequencing [15]; five of these samples were autopsy cases and our study shows that p53 alterations are present at the time of diagnosis. Interestingly, one of the hotspot tested, R273H, was more frequently mutated in DIPG. This mutation, which is localized to the DNA-contact domain, does not completely alter the transcriptional activity of the protein [16,17] but has been associated with the induction of drug resistance [18], a feature characteristic of DIPG. Our screen likely underestimate the frequency of p53 abnormalities since it covers only the most frequent mutations (V157F, G245S, R248W, R248Q, R273C, R273H, R306).

Our study reveals PI3KCA as the first mutated oncogene described in DIPG. PI3KCA mutations were not detected in the samples with amplified PDGFRA or PTEN loss, the most frequent alterations described so far at the genomic level in DIPG [4]; this finding is consistent with the fact that these oncogenic events would likely be redundant.

PI3KCA mutations were also not associated with BRAF or RAS mutations as reported in other cancers [19,20]. The apparently mutually exclusive occurrence of mutations involving the PDGFR and PI3K pathways may indicate that DIPG could segregate into different subgroups. Most importantly, these mutations are drugable and PI3K/AKT/mTOR inhibitors may be useful drug candidates for a subset of these patients.

The two other mutations described were identified from the same patient and are also linked to the PI3K pathway. ATM is a member of the PI3K family that can also regulate p53 activity. Although missense mutations in ATM have been associated with breast cancer, their functional role is still debated [21]. MPL (CD110) is a cytokine receptor which also signals through PI3K and the W515L mutation results in the constitutional activation of JAK2 [22].

TABLE I. Validated Mutations Detected in the 20 Diagnostic Samples Analyzed

Patient	Gene	Mutation	p53 CNA	p53 IHC	PDGFRA CNA	PTEN CNA
1	ATM	P604S	À0.5292	À	À0.0375	À0.0224
1	MPL	W515L	À0.5292	À	À0.0375	À0.0224
2	PI3KCA	H1047R	0.0155	À	À0.0142	0.0002
3	PI3KCA	H1047L	0.0033	þþ	À0.0088	0.0010
4	PI3KCA	E542K	0.0421	þþþ	À0.0165	À0.0033
5	TP53	R248W	À0.4546	þþ	2.5200	À0.0153
6	TP53	R273H	À0.5166	þþ	À0.0386	À0.2063
7	TP53	V157F	À0.8058	þ	À0.0076	À0.3552
8	TP53	R273Ã	À0.1597	NA	À0.0191	À0.2676
9	TP53	R273L	0.0011	þþþ	À0.0257	0.0006
10	TP53	R282W	0.0174	þþþ	2.2870	À0.3614
11	TP53	R273H	À0.3997	þþþ	1.7514	0.0019
12	TP53	R273L	0.0101	NA	À0.0079	À0.2159

Results of the CGHarray with Agilent 44K Whole Human Genome Array G4410B are indicated by the ratio of chromosomal imbalance; values above 1.5 were considered as amplifications and those below À0.3 were considered as losses. IHC for p53 (DO-7 antibody) was graded in a semi-quantitative way (cut-off 10%).

The presence of frequent p53 abnormalities in the context of PDGFR amplification or PI3k mutations, both of which can lead to the activation of the PI3k pathway in newly diagnosed DIPG patients raises the possibility that this signaling pathway constitutes a major component of the pathogenesis of this disease.

Finally, the absence of mutations in the other common oncogenes and tumor suppressor genes in newly diagnosed DIPG samples suggests that additional novel mechanisms are driving their oncogenesis; they will need to be evaluated using additional molecular methodologies including next-generation sequencing or epigenetic profiling. Gene expression profiling may also be used to describe molecular pathways participating in their oncogenesis. The limited number of mutations identified in these highly malignant and rapidly fatal tumors was unexpected and suggests that this type of approach can impact our understanding of rare diseases while redirecting efforts to particular pathways and novel tumor cellular mechanisms.

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ONCOMAP MUTATION SET

ASSAY_ID	GENE	MUTATION (AA)	MUTATION (BP)	FORWARD PCR PRIMER	REVERSE PCR PRIMER	EXTENSION PROBE
OM_00018	ABL1	G250E	g.132728170G>A	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ACGTTGGATGACTTACCATGCCACTTTCCC	GGGACAAAGAATTGGATCTG
OM_00019	ABL1	Q252H	g.132728177G>C	ACGTTGGATGACTTGGGAGGTATCCACATC	ACGTTGGATGTTCTGGTGCCACTACCACAG	TGCCTTTACCACTCAGA
OM_00020	ABL1	Y253H	g.132728178T>C	ACGTTGGATGCAACATCTCCGAAAGCCAAC	ACGTTGGATGAGGTTCAGAGCCATGGACCC	cgCCGAAAGCCAACAAGGAAATCCTC
OM_00021	ABL1	Y253F	g.132728179A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	ATATTCTCGACACAGCAGGTCA
OM_00022	ABL1	E255K	g.132728184G>A	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ACGTTGGATGACTTACCATGCCACTTTCCC	aaAGATTACAGTGGGACAAAGAATTG
OM_00023	ABL1	E255V	g.132728185A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	TTCTCGACACAGCAGGT
OM_00024	ABL1	D276G	g.132737341A>G	ACGTTGGATGCTAAATTTTCTCTTGGAAAC	ACGTTGGATGCTTCTACGTTGATTTCAGAG	ATTTTCTCTTGGAAACTCCCATT
OM_00026	ABL1	F317L	g.132738111C>G	ACGTTGGATGTGGGCAAAGAGGTGCTCC	ACGTTGGATGTCGGGCTACGCAAGGATTTG	tAAGAGGTGCTCCAGGACTG
OM_00027	ABL1	M351T	g.132738212T>C	ACGTTGGATGCAACATCTCCGAAAGCCAAC	ACGTTGGATGAGGTTCAGAGCCATGGACCC	ggggtAAAGCCAACAAGGAAATCCTC
OM_00028	ABL1	E355G	g.132738224A>G	ACGTTGGATGGGGAAAGTGGTGAAGATATG	ACGTTGGATGCATTGCCCCTGACAACATAG	aagagATGTGACTTTGGATTGGCT
OM_00029	ABL1	F359V	g.132738235T>G	ACGTTGGATGAGGCCTGCTGAAAATGACTG	ACGTTGGATGCTGTATCGTCAAGGCACTCT	caTTGTGGTAGTTGGAGCTG
OM_00030	ABL1	H396R	g.132740177A>G	ACGTTGGATGTCTGCAGAACTGCCTATTCC	ACGTTGGATGACCTGTACCATCTGTAGCTG	aATCTGTAGCTGGCTTTCA
OM_00263B	ABL1	M244V	g.132728151A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	ACTCTTGCCCACACCGC
OM_00264	ABL1	L248V	g.132728163C>G	ACGTTGGATGAAGCTGACAAGTGGGGCTTC	ACGTTGGATGAGGATCCAGGGCACTGATG	GGGAAGTGTTTAGTGGC
OM_00970	AKT1	E17K	g.104317596C>T	ACGTTGGATGTCACCATCTCCAGCTGGTC	ACGTTGGATGTCACACCCTTCTCCCTTCG	cctGAGAAGCTCCCGCCACCGCCGTCG
OM_00032	AKT2	S302G	g.45434060T>C	ACGTTGGATGTCCAGGAAGCCTACGTGATG	ACGTTGGATGACGGTGGAGGTGAGGCAGAT	gaGTGATGGCCAGCGTGGAC
OM_00033	AKT2	R371H	g.45433700C>T	ACGTTGGATGAGCCCCTGGTGCTCCATGA	ACGTTGGATGGCTTCGCTTACCAGAGTCG	TGGTGCTCCATGAGGAGACA
OM_00317	APC	R1114*	g.112202530C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	ctccGTGCGCTTTTCCCAACACCA
OM_00318	APC	Q1338*	g.112203202C>T	ACGTTGGATGTTTCTTCCAGATATCCTCGC	ACGTTGGATGACGAGGAGGAGAACTTCTAC	CTCGCTGGGCGCCGGGG
OM_00319	APC	Q1367*	g.112203289C>T	ACGTTGGATGTCTGGAAGAAATTCGAGCTG	ACGTTGGATGAACGTAGGAGGGCGAGCAGA	ggTGCCCACCCCGCCCCTGT
OM_00320A	APC	R1450*	g.112203538C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ggACTGGTGGTGGTTGGAGCA
OM_00320B	APC	R1450*	g.112203538C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	GCGCTTTTCCCAACACCAC
OM_01087	APC	R876*	g.112201816C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	attgcCAGTGCGCTTTTCCCAACACCA
OM_01088	APC	E1306*	g.112203106G>T	ACGTTGGATGTACAAGTCAACAACCCCCAC	ACGTTGGATGCTTGACAAAGCAAATAAAGAC	AAGCAAATAAAGACAAAGCCAAC
OM_01091	APC	E1309fs*4	g.112203117_112203121_delAAAGA	ACGTTGGATGACGGTGGAGGTGAGGCAGAT	ACGTTGGATGTCCAGGAAGCCTACGTGATG	ccGGCGGCACACGTGGGGGTTG
OM_01092	APC	Q1378*	g.112203322C>T	ACGTTGGATGTGTGACCGCAACGTAGGAG	ACGTTGGATGCAGCGAGGATATCTGGAAG	gGGAAGAAATTCGAGCTGCTGCCCA
OM_01093	APC	E1379*	g.112203325G>T	ACGTTGGATGGTTGGCTTTGTCTTTATTTGC	ACGTTGGATGGCGTGCAGATAATGACAAGG	ttTCAAGATCATTTTTTGTTAAAGTA
OM_01094	APC	Q1429*	g.112203475C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	cacaaGTGGTGTTGGGAAAAGC
OM_01095	APC	S1465fs*3	g.112203582_112203583delGA	ACGTTGGATGTAGCGCCTGGAAGAGAAAAG	ACGTTGGATGGACCCTATGCAGTAAATGG	GAAGAGAAAAGGAGATTACAGCTT
OM_01115	APC	T1667fs*9	g.112203851delA	ACGTTGGATGCGCTATCAAGGAATTAAGAG	ACGTTGGATGACACAGCAAAGCAGAAACTC	ctataAAGGAATTAAGAGAAGCAACA
OM_00034	BRAF	G464R	g.140127887C>G	ACGTTGGATGAGTCCATTTGACAAAGCCCG	ACGTTGGATGAATCAGACTTAATAGTCCGC	ctTGCATAACAGCCTAATCTCGT
OM_00035	BRAF	G464V	g.140127886C>A	ACGTTGGATGCCTGAAATAATTTCACCTTCG	ACGTTGGATGTCAAGAACTCTTCCACCTCC	CGTTTTTTTCCTTCTGCAGGAGG
OM_00036	BRAF	G466A	g.140127880C>G	ACGTTGGATGCTCACGTTTCCTTTAACCAC	ACGTTGGATGCAAAGATTTGTGATTTTGGTC	ccCATAATTAGAATCATTCTTGATGT
OM_00037	BRAF	G466R	g.140127881C>G	ACGTTGGATGGATTTGTGATTTTGGTCTAG	ACGTTGGATGGAATGGGTACTCACGTTTCC	GATTTTGGTCTAGCCAGAG
OM_00039	BRAF	G469R	g.140127872C>G	ACGTTGGATGCTCACGTTTCCTTTAACCAC	ACGTTGGATGCAAAGATTTGTGATTTTGGTC	CATAATTAGAATCATTCTTGATGT
OM_00040	BRAF	G469R	g.140127872C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	CGCTTTTCCCAACACCA
OM_00041	BRAF	G469S	g.140127870_140127872TCC>GCT	ACGTTGGATGGTTGGATCATATTCGTCCAC	ACGTTGGATGTGGTGGCGTAGGCAAGAGT	ggTTCTGAATTAGCTGTATCGT
OM_00042	BRAF	G464E	g.140127886C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	gtgaGCTTTTCCCAACACCAC
OM_00043A	BRAF	G466V	g.140127880C>A	ACGTTGGATGGGCTGTATTTCTTCCACACG	ACGTTGGATGCATCACCATGAAGCACAAGC	CACGCCCTCGTACACCT
OM_00043B	BRAF	G466V	g.140127880C>A	ACGTTGGATGGGCTGTATTTCTTCCACACG	ACGTTGGATGCATCACCATGAAGCACAAGC	tcaACACGCCCTCGTACACC
OM_00044	BRAF	G466E	g.140127880C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	caGCTTTTCCCAACACCAC
OM_00045	BRAF	G469A	g.140127871C>G	ACGTTGGATGGAATGGGTACTCACGTTTCC	ACGTTGGATGGATTTGTGATTTTGGTCTAGC	gaACATAATTAGAATCATTCTTGATG
OM_00046	BRAF	G469E	g.140127871C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	gggaaAAACTGGTGGTGGTTGGAGCA
OM_00047	BRAF	G469V	g.140127871C>A	ACGTTGGATGTACATGGCCACTCAGATCTC	ACGTTGGATGCCCTACCTGTGGATGAAGTT	AGCCATGGAGTACCTGG
OM_00048	BRAF	F595L	g.140099619A>C	ACGTTGGATGGTGGTAGTGGCACCAGAATG	ACGTTGGATGGAAAAGCGGCTGTTAGTCAC	TTCCAGAGTCCAGGTAA
OM_00049	BRAF	G596R	g.140099618C>G	ACGTTGGATGCAAAGATTTGTGATTTTGGTC	ACGTTGGATGCTCACGTTTCCTTTAACCAC	GATTTTGGTCTAGCCAGA
OM_00050	BRAF	L597V	g.140099615G>C	ACGTTGGATGTTCTGGTGCCACTACCACAG	ACGTTGGATGACTTGGGAGGTATCCACATC	ccccaGTGCCACTACCACAGCTCCTT
OM_00051	BRAF	L597S	g.140099614_140099615AG>GA	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ACGTTGGATGAGGCCTGCTGAAAATGACTG	ggaCGTCAAGGCACTCTTGCCTACGC
OM_00052A	BRAF	V600E	g.140099605A>T	ACGTTGGATGCATGTACTGGTCCCTCATTG	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ggggCTCATTGCACTGTACTCCTC
OM_00054	BRAF	V600R	g.140099605_140099606AC>CT	ACGTTGGATGTCCAGGAAGCCTACGTGATG	ACGTTGGATGACGGTGGAGGTGAGGCAGAT	agcCGTGATGGCCAGCGTGGACAACC
OM_00055	BRAF	K601N	g.140099601T>A	ACGTTGGATGACCCCGCCACTCTCACCCGA	ACGTTGGATGACCACCAGCGTGTCCAGGAA	aCCACTCTCACCCGACCCGTG
OM_00056	BRAF	K601E	g.140099603T>C	ACGTTGGATGTCACATCGAGGATTTCCTTG	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	gTGTTGGCTTTCGGAGAT
OM_00057	BRAF	D594G	g.140099623T>C	ACGTTGGATGTCCAGGAAGCCTACGTGATG	ACGTTGGATGACGGTGGAGGTGAGGCAGAT	TGGCCAGCGTGGACAAC
OM_00058	BRAF	D594V	g.140099623T>A	ACGTTGGATGACCACCAGCGTGTCCAGGAA	ACGTTGGATGACCCCGCCACTCTCACCCGA	tcAGCCCTCCCGGGCAGCGTCGT
OM_00059	BRAF	L597R	g.140099614A>C	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ACGTTGGATGACTGGCAGCAACAGTCTTAC	tCACCAGAATGGATTCCA
OM_00060	BRAF	L597Q	g.140099614A>T	ACGTTGGATGCATGTACTGGTCCCTCATTG	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	tGTCCCTCATTGCACTGTACTCCTC
OM_00061	BRAF	T599I	g.140099608G>A	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ACGTTGGATGACTTACCATGCCACTTTCCC	gaacGTGGGACAAAGAATTGGATCT
OM_00062	BRAF	V600L	g.140099606C>G	ACGTTGGATGAGGCTCGACTACCTGTGAAG	ACGTTGGATGACCAGACGTCACTTTCAAAC	ggCTACCTGTGAAGTGGATGGCACCT
OM_00329	BRAF	R444W	g.140127947G>A	ACGTTGGATGACTTACCATGCCACTTTCCC	ACGTTGGATGTGGGCAGATTACAGTGGGAC	TTGTAGACTGTTCCAAATGAT
OM_00335	BRAF	N581S	g.140099662T>C	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	ACGTTGGATGTCACATCGAGGATTTCCTTG	gggagAAATTCCCGTCGCTATCAA
OM_00337	BRAF	E586K	g.140099648C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	GGTGGTGGTTGGAGCAG
OM_00338	BRAF	D587A	g.140099644T>G	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ACGTTGGATGAGGCCTGCTGAAAATGACTG	AGGCACTCTTGCCTACGCCAC
OM_00339	BRAF	D587E	g.140099643G>C	ACGTTGGATGACTTGGGAGGTATCCACATC	ACGTTGGATGTTCTGGTGCCACTACCACAG	CAGGATTGCCTTTACCACTCAGA
OM_00340	BRAF	I592V	g.140099630T>C	ACGTTGGATGCCTTCTCTCTCTGTCATAGG	ACGTTGGATGAATTCCTTGATAGCGACGGG	ccgtaATAGGGACTCTGGATCCCA
OM_00341	BRAF	I592M	g.140099628T>C	ACGTTGGATGTCCAGGAAGCCTACGTGATG	ACGTTGGATGTGAGGCAGATGCCCAGCAG	TACGTGATGGCCAGCGTG
OM_00342	BRAF	D594E	g.140099622A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	tcTATTCTCGACACAGCAGGTCA
OM_00343	BRAF	F595S	g.140099620A>G	ACGTTGGATGGACGGAATATAAGCTGGTGG	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ctCACTCTTGCCCACACCG
OM_00344	BRAF	V600M	g.140099606C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	gCAGTGCGCTTTTCCCAACAC
OM_00347	BRAF	S605N	g.140099590C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	CAGTGCGCTTTTCCCAACA
OM_00354	BRAF	T599_V600insTT	g.140099607T>CGTAGTA	ACGTTGGATGAGCAGAAACTCACATCGAGG	ACGTTGGATGAATTCCCGTCGCTATCAAGG	gagTTCCTTGTTGGCTTTCG
OM_00355	BRAF	V600D	g.140099604_140099605CA>AT	ACGTTGGATGACTGGCAGCAACAGTCTTAC	ACGTTGGATGGCCTTTACCACTCAGAGAAG	GTCTTACCTGGACTCTG
OM_00359	BRAF	S605F	g.140099590_140099591CT>AA	ACGTTGGATGGAAAAGCGGCTGTTAGTCAC	ACGTTGGATGGTGGTAGTGGCACCAGAATG	gggaAGCAACAGTCTTACCTG
OM_01022	BRAF	V600A	g.140099605A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	ACTCTTGCCCACACCGC
OM_01023	BRAF	V471F	g.140127866C>A	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	ACGTTGGATGAGCCCCCGTTCTATATCATC	tcGTCCAGGAGGTTCCCGTAGGTCAT
OM_01105	BRAF	K601del	g.140099601_140099603delTTT	ACGTTGGATGTGTGACTTTGGATTGGCTCG	ACGTTGGATGGTAGGAAATAGCAGCCTCAC	gggTTTGGATTGGCTCGAGATAT
OM_00064	CDK4	R24H	g.56431697C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	TGGTGGTGGTTGGAGCAGGT
OM_00372	CDKN2A	R58*	g.21961186G>A	ACGTTGGATGACTTACCATGCCACTTTCCC	ACGTTGGATGTGGGCAGATTACAGTGGGAC	CTTTCCCTTGTAGACTGTTC
OM_00373A	CDKN2A	E61*	g.21961177C>A	ACGTTGGATGTTTGGCCTGAGCAGGTTGAT	ACGTTGGATGGGTGCAGTCCATTTGATGGG	cccGGGACACCTACACAGCCC
OM_00373B	CDKN2A	E61*	g.21961177C>A	ACGTTGGATGACAAGTGGGAGATGGAACGC	ACGTTGGATGTCGTACACCTCCCCGTACTG	cgaccTCACCATGAAGCACAAG
OM_00374A	CDKN2A	E69*	g.21961153C>A	ACGTTGGATGTCGTACACCTCCCCGTACTG	ACGTTGGATGACAAGTGGGAGATGGAACGC	gggcgCCCCGCCCAGCTTGTGCTTCA
OM_00374B	CDKN2A	E69*	g.21961153C>A	ACGTTGGATGCCCTACCTGTGGATGAAGTT	ACGTTGGATGTACATGGCCACTCAGATCTC	cttcTTCTTCTCCAGGTACTCC
OM_00375A	CDKN2A	H83Y	g.21961111G>A	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ACGTTGGATGACTTACCATGCCACTTTCCC	TGGGACAAAGAATTGGATCTG
OM_00375B	CDKN2A	H83Y	g.21961111G>A	ACGTTGGATGACTTACCATGCCACTTTCCC	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ccaaTTTCCCTTGTAGACTGTT
OM_00376	CDKN2A	D84Y	g.21961108C>A	ACGTTGGATGCATCACCATGAAGCACAAGC	ACGTTGGATGGGCTGTATTTCTTCCACACG	GAAGCACAAGCTGGGCG
OM_00377	CDKN2A	R80*	g.21961120G>A	ACGTTGGATGACTTACCATGCCACTTTCCC	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ccgGCCACTTTCCCTTGTAGACTGTTC
OM_00378	CDKN2A	D108Y	g.21961036C>A	ACGTTGGATGTACATGGCCACTCAGATCTC	ACGTTGGATGTGGCCAGGCCCCTACCTGT	cTGGAGTACCTGGAGAAGAAAAAC
OM_00379	CDKN2A	W110*	g.21961028C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	cccaaTGGTGGTGGTTGGAGCAGGTG
OM_00380	CDKN2A	P114L	g.21961017G>A	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ACGTTGGATGACTTACCATGCCACTTTCCC	tgacGAATTGGATCTGGATCATTTG
OM_00381	CDKN2A	R80*	g.21961120_21961121GG>AA	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ACGTTGGATGACTGGCAGCAACAGTCTTAC	gggAGTGGCACCAGAATGGATT
OM_01030A	CDKN2A	E88*	g.21961096C>A	ACGTTGGATGGGCTGTATTTCTTCCACACG	ACGTTGGATGCATCACCATGAAGCACAAGC	TCGTACACCTCCCCGTA
OM_01030B	CDKN2A	E88*	g.21961096C>A	ACGTTGGATGGGCTGTATTTCTTCCACACG	ACGTTGGATGCATCACCATGAAGCACAAGC	ccgaCCTCGTACACCTCCCCG
OM_00636	CSF1R	L301*	g.149433237A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	ccTGGATATTCTCGACACAGCAGGT
OM_00679	CSF1R	L301S	g.149433237A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	ACTCTTGCCCACACCGC
OM_01098	CSF1R	Y969*	g.149413837A>T	ACGTTGGATGCATGTACTGGTCCCTCATTG	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	aTCCCTCATTGCACTGTACTCCTCT
OM_01099	CSF1R	Y969*	g.149413837A>C	ACGTTGGATGACTGGCAGCAACAGTCTTAC	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ctcctCAACAGTCTTACCTGGACT
OM_01100	CSF1R	Y969C	g.149413838T>C	ACGTTGGATGTCACATCGAGGATTTCCTTG	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	CTTGTTGGCTTTCGGAG
OM_01101	CSF1R	Y969F	g.149413838T>A	ACGTTGGATGGCAGGTACCGTGCGACATC	ACGTTGGATGTGGACGTGCGCGATGCCTG	cccCTCCTCAGCCAGGTCCACG
OM_01102	CSF1R	Y969H	g.149413839A>G	ACGTTGGATGGACGGAATATAAGCTGGTGG	ACGTTGGATGAATGGTTCTGGATCAGCTGG	gATAAGCTGGTGGTGGTGGGCGCCG
OM_00411	CTNNB1	A21T	g.41241068G>A	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ACGTTGGATGACTTACCATGCCACTTTCCC	cAAGAATTGGATCTGGATCATTTG
OM_00412	CTNNB1	V22A	g.41241072T>C	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	ACGTTGGATGTCACATCGAGGATTTCCTTG	ccTCCCGTCGCTATCAAGG
OM_00415A	CTNNB1	D32Y	g.41241101G>T	ACGTTGGATGCATTATCAACTTTGGTACTGG	ACGTTGGATGTTCTTTCCTTTGTAGTGTCC	ggggACTTTGGTACTGGTATCAATTT
OM_00415B	CTNNB1	D32Y	g.41241101G>T	ACGTTGGATGTTCTTCACTTTGCATATGCC	ACGTTGGATGTTCTGACTACTTTTACATC	TTTGCATATGCCATACATGGAA
OM_00416	CTNNB1	D32N	g.41241101G>A	ACGTTGGATGCCACTCCATCGAGATTTCAC	ACGTTGGATGTCTTCATGAAGACCTCACAG	AGATTTCACTGTAGCTAGAC
OM_00417	CTNNB1	D32H	g.41241101G>C	ACGTTGGATGCTCAGGATTGCCTTTACCAC	ACGTTGGATGACAGTCTTACCTGGACTCTG	ccatgGCCTTTACCACTCAGAG
OM_00418	CTNNB1	D32G	g.41241102A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	tctCACTCTTGCCCACACCG
OM_00419	CTNNB1	D32A	g.41241102A>C	ACGTTGGATGACTGGCAGCAACAGTCTTAC	ACGTTGGATGGCCTTTACCACTCAGAGAAG	cccccGCAACAGTCTTACCTGGACT
OM_00420	CTNNB1	D32V	g.41241102A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	cATATTCTCGACACAGCAGGTC
OM_00423B	CTNNB1	S33C	g.41241105C>G	ACGTTGGATGACCAGACGTCACTTTCAAAC	ACGTTGGATGAGGCTCGACTACCTGTGAAG	gggGTATACACAGTTGAAAATGCTTT
OM_00424A	CTNNB1	S33F	g.41241105C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	cGTGCGCTTTTCCCAACAC
OM_00424B	CTNNB1	S33F	g.41241105C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	TGGTGGTTGGAGCAGGT
OM_00425A	CTNNB1	S33Y	g.41241105C>A	ACGTTGGATGAGGTGCCATCATTCTTGAGG	ACGTTGGATGTCTCACCACCCGCACGTCT	CGCCAGGTCTTGATGTACT
OM_00425B	CTNNB1	S33Y	g.41241105C>A	ACGTTGGATGGGCTGTATTTCTTCCACACG	ACGTTGGATGCATCACCATGAAGCACAAGC	CTCGTACACCTCCCCGT
OM_00427	CTNNB1	G34R	g.41241107G>C	ACGTTGGATGTTCTGGTGCCACTACCACAG	ACGTTGGATGACTTGGGAGGTATCCACATC	CACTACCACAGCTCCTT
OM_00428A	CTNNB1	G34V	g.41241108G>T	ACGTTGGATGTCCTCAGACATTCAAACGTG	ACGTTGGATGGCATGAAGACCGAGTTATAG	cCGTGTTTTGATCAAAGAAGAG
OM_00428B	CTNNB1	G34V	g.41241108G>T	ACGTTGGATGTTCTGCATTGGTGCTAAAAG	ACGTTGGATGATAAATATAATGAACTTACC	AAAAGTTTCTTGGATCACATTTT
OM_00429	CTNNB1	G34E	g.41241108G>A	ACGTTGGATGTCTTCATGAAGACCTCACAG	ACGTTGGATGCCACTCCATCGAGATTTCAC	ccccGAAGACCTCACAGTAAAAT
OM_00433	CTNNB1	S37A	g.41241116T>G	ACGTTGGATGAGGCCTGCTGAAAATGACTG	ACGTTGGATGCTGTATCGTCAAGGCACTCT	TTGTGGTAGTTGGAGCT
OM_00434	CTNNB1	S37P	g.41241116T>C	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	ACGTTGGATGTCACATCGAGGATTTCCTTG	aaacgTCCCGTCGCTATCAAGGA
OM_00435	CTNNB1	S37F	g.41241117C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	TGCGCTTTTCCCAACAC
OM_00436A	CTNNB1	S37C	g.41241117C>G	ACGTTGGATGCCCACAGAAACCCATGTATG	ACGTTGGATGTGTGTTGGGTCTATGTAAAC	gaacaCATGTATGAAGTACAGTGGA
OM_00437	CTNNB1	S37Y	g.41241117C>A	ACGTTGGATGGTCCTTCTTAAGCAGCCCAG	ACGTTGGATGATCCTCATGGAAGAGATCCG	ggggaGCCTCGGGGCTGAGCGTG
OM_00440A	CTNNB1	T41A	g.41241128A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	cTGGTCAGCGCACTCTTGCCCACAC
OM_00441	CTNNB1	T41S	g.41241128A>T	ACGTTGGATGCATGTACTGGTCCCTCATTG	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	TTGCACTGTACTCCTCT
OM_00442	CTNNB1	T41P	g.41241128A>C	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ACGTTGGATGACTGGCAGCAACAGTCTTAC	gggtCACCAGAATGGATTCCA
OM_00443A	CTNNB1	T41I	g.41241129C>T	ACGTTGGATGTGTCCTCATGTATTGGTCTC	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	taTCTCATGGCACTGTACTCTTCTT
OM_00451B	CTNNB1	S45P	g.41241140T>C	ACGTTGGATGTCACATCGAGGATTTCCTTG	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	GAGGATTTCCTTGTTGGCTTTCGG
OM_00452	CTNNB1	S45A	g.41241140T>G	ACGTTGGATGAGGCCTGCTGAAAATGACTG	ACGTTGGATGCTGTATCGTCAAGGCACTCT	AAACTTGTGGTAGTTGGAGCTG
OM_00453A	CTNNB1	S45F	g.41241141C>T	ACGTTGGATGGACTGAGTACAAACTGGTGG	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	cggGGTGGTTGGAGCAGGTG
OM_00453B	CTNNB1	S45F	g.41241141C>T	ACGTTGGATGAGTGGTTCTGGATTAGCTGG	ACGTTGGATGGACTGAGTACAAACTGGTGG	agtATTGTCAGTGCGCTTTTCCCAACA
OM_00454	CTNNB1	S45Y	g.41241141C>A	ACGTTGGATGGATGGCCACATCAAGATCAC	ACGTTGGATGCAGAAGGTTTTCATGGTGGC	CCTCTGCAAAGAGGGCATC
OM_00455	CTNNB1	S45C	g.41241141C>G	ACGTTGGATGCCCACAGAAACCCATGTATG	ACGTTGGATGTGTGTTGGGTCTATGTAAAC	gCATGTATGAAGTACAGTGG
OM_00469	CTNNB1	V22_G38del	g.41241071_41241121delGTTAGTCACTGGCAGCAACAAATCCATTCTG	ACGTTGGATGAGCCCCGCCTGCAGGATG	ACGTTGGATGTGGCCCCTGAGCGTCATCT	ccGGGCCGGTGCGGGGAGC
OM_00472	CTNNB1	W25_D32del	g.41241081_41241104delGGCAGCAACAGTCTTACCTGGACT	ACGTTGGATGACTCCGTGTTTGCCCACGA	ACGTTGGATGAGGGACCCCTCACATTGTTG	cttTTTGCCCACGACCTGCT
OM_01081	CTNNB1	A13T	g.41241044G>A	ACGTTGGATGACTTACCATGCCACTTTCCC	ACGTTGGATGTGGGCAGATTACAGTGGGAC	ccaATGCCACTTTCCCTTGTAGACTGT
OM_00069	EGFR	E746_A750del	g.55209959_55209973delGGAATTAAGAGAAGC	ACGTTGGATGACGACTCCGTGTTTGCCCA	ACGTTGGATGACCAGTGGCCCTTCACGTC	aCGTGTTTGCCCACGACCTGC
OM_00071A	EGFR	S752_I759del	g.55209978_55210001delTCTCCGAAAGCCAACAAGGAAATC	ACGTTGGATGCATTGCCCCTGACAACATAG	ACGTTGGATGGGGAAAGTGGTGAAGATATG	TAGTTGGAATCACTCATGATAT
OM_00071B	EGFR	S752_I759del	g.55209978_55210001delTCTCCGAAAGCCAACAAGGAAATC	ACGTTGGATGGGGAAAGTGGTGAAGATATG	ACGTTGGATGCATTGCCCCTGACAACATAG	TTTGGATTGGCTCGAGA
OM_00073	EGFR	L747_P753>S	g.55209964_55209981delTAAGAGAAGCAACATCTC	ACGTTGGATGCACCCCGTAGCTGAGGATG	ACGTTGGATGATGTCTTTGCAGCCGAGGAG	gggaCCCGTAGCTGAGGATGCCTGCA
OM_00074	EGFR	L747_T751del	g.55209964_55209978delTAAGAGAAGCAACAT	ACGTTGGATGATGTCTTTGCAGCCGAGGAG	ACGTTGGATGCACCCCGTAGCTGAGGATG	cccCTGACGAGGCGGGCAGTGTGT
OM_00075	EGFR	L747_T751>P	g.55209963_55209974TTAAGAGAAGCAA>C	ACGTTGGATGTCACATCGAGGATTTCCTTG	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	gattgCCTTGTTGGCTTTCGGAGATG
OM_00077	EGFR	L747_S752del	g.55209963_55209980delTTAAGAGAAGCAACATCT	ACGTTGGATGCATTGCCCCTGACAACATAG	ACGTTGGATGGGGAAAGTGGTGAAGATATG	TAGTTGGAATCACTCATGATAT
OM_00079	EGFR	E746_A750>V	g.55209961_55209975AATTAAGAGAAGCAAC>T	ACGTTGGATGACCTCGTCTTCTACTTCTGG	ACGTTGGATGAGCTTCTGTTCCCGGGAATC	GGTCTATGCCCACCAGATGG
OM_00082	EGFR	E746_T751>A	g.55209961_55209975delAATTAAGAGAAGCAA	ACGTTGGATGCCTTCTCTCTCTGTCATAGG	ACGTTGGATGAATTCCTTGATAGCGACGGG	tctccCTGTCATAGGGACTCTG
OM_00083	EGFR	E746_S752>A	g.55209961_55209978delAATTAAGAGAAGCAACAT	ACGTTGGATGGAAAACACATCCCCCAAAGC	ACGTTGGATGTCCTTCCTGTCCTCCTAGCA	CCAAAGCCAACAAAGAAATCTTA
OM_00084	EGFR	E746_S752>D	g.55209962_55209979delATTAAGAGAAGCAACATC	ACGTTGGATGATACCCTCTCAGCGTACCCT	ACGTTGGATGAGAAGGCGGGAGACATATGG	AGCATACGTGATGGCTGGT
OM_00085	EGFR	L747_E749del	g.55209963_55209971delTTAAGAGAA	ACGTTGGATGCATTGCCCCTGACAACATAG	ACGTTGGATGGGGAAAGTGGTGAAGATATG	CATAGTTGGAATCACTCATGATA
OM_00089	EGFR	D770_N771insG	g.55216507_55216508insGGT	ACGTTGGATGTGGTGGCGTAGGCAAGAGTG	ACGTTGGATGGTTGGATCATATTCGTCCAC	gggagGGCAAGAGTGCCTTGACGATA
OM_00095	EGFR	P772_H773insV	g.55216511_55216512insGGT	ACGTTGGATGGTTGGATCATATTCGTCCAC	ACGTTGGATGTGGTGGCGTAGGCAAGAGTG	ccctACAAAATGATTCTGAATTAGCT
OM_00100	EGFR	D770_N771insN	g.55216510_55216511insAAC	ACGTTGGATGACTGGCAGCAACAGTCTTAC	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ACAGTCTTACCTGGACTCT
OM_00103	EGFR	N771_P772>SVDNR	g.55216508_55216511ACCC>GCGTGGACAACCG	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ACGTTGGATGAGGCCTGCTGAAAATGACTG	cccTCAAGGCACTCTTGCCTACG
OM_00107A	EGFR	T790M	g.55216565C>T	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	ACGTTGGATGTGTCCTCATGTATTGGTCTC	TGGACATACTGGATACAGCTGGAC
OM_00107B	EGFR	T790M	g.55216565C>T	ACGTTGGATGTGTCCTCATGTATTGGTCTC	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	ggCTCTCATGGCACTGTACTCTTCT
OM_00108A	EGFR	L858R	g.55227009T>G	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ACGTTGGATGAGGCCTGCTGAAAATGACTG	aGTCAAGGCACTCTTGCCTACGCCA
OM_00108B	EGFR	L858R	g.55227009T>G	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ACGTTGGATGAGGCCTGCTGAAAATGACTG	TCAAGGCACTCTTGCCTACGCCAC
OM_00110	EGFR	G719A	g.55209202G>C	ACGTTGGATGCAACAGTCTTACCTGGACTC	ACGTTGGATGCAGGATTGCCTTTACCACTC	tctgtAATCCATTCTGGTGCCACTA
OM_00111	EGFR	G719C	g.55209201G>T	ACGTTGGATGTATTCCCACAGTGTATCGGC	ACGTTGGATGCTAATTCTGGGTGCTCAGAC	ATCGGCTAGCCTATCTC

OM_00112	EGFR	G719S	g.55209201G>A	ACGTTGGATGTCTTCATGAAGACCTCACAG	ACGTTGGATGGACCCACTCCATCGAGATTT	GTAAAAATAGGTGATTTTGGTC
OM_00486	EGFR	G719D	g.55209202G>A	ACGTTGGATGGGTGATTTTGGTCTAGCTAC	ACGTTGGATGATGGATCCAGACAACTGTTC	TGGTCTAGCTACAGTGAA
OM_00490A	EGFR	L730F	g.55209912C>T	ACGTTGGATGTGTCCTCATGTATTGGTCTC	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	TCTCATGGCACTGTACTCTTC
OM_00491	EGFR	W731*	g.55209917G>A	ACGTTGGATGATGAGATCTACTGTTTTCC	ACGTTGGATGCCTATTTTACTGTGAGGTC	TTCCTTTACTACTACACCTCAG
OM_00492	EGFR	P733L	g.55209922C>T	ACGTTGGATGTGTCCTCATGTATTGGTCTC	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	gCTCTCATGGCACTGTACTCTTCTT
OM_00493	EGFR	E734K	g.55209924G>A	ACGTTGGATGATAGGTGATTTTGGTCTAGC	ACGTTGGATGATGGATCCAGACAACTGTTC	TTTGGTCTAGCTACAGTG
OM_00494	EGFR	G735S	g.55209927G>A	ACGTTGGATGTCTTCATGAAGACCTCACAG	ACGTTGGATGGACCCACTCCATCGAGATTT	GTAAAAATAGGTGATTTTGGTC
OM_00495	EGFR	V742A	g.55209949T>C	ACGTTGGATGTCACATCGAGGATTTCCTTG	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	cccgCCTTGTTGGCTTTCGGA
OM_00496	EGFR	K745R	g.55209958A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	CGCACTCTTGCCCACAC
OM_00497	EGFR	E746K	g.55209960G>A	ACGTTGGATGTCTTCATGAAGACCTCACAG	ACGTTGGATGTTCAAACTGATGGGACCCAC	CCCACTCCATCGAGATT
OM_00499A	EGFR	A750P	g.55209972G>C	ACGTTGGATGCAACAGTCTTACCTGGACTC	ACGTTGGATGCAGGATTGCCTTTACCACTC	acGGAATCCATTCTGGTGCCACT
OM_00501	EGFR	S752Y	g.55209979C>A	ACGTTGGATGCTAATACCCTGCAAATAGC	ACGTTGGATGTGCTGGAACTTCGGTCACAG	aaCCTGCAAATAGCAGAAATAAAAGA
OM_00502	EGFR	P753S	g.55209981C>T	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	ACGTTGGATGTGTCCTCATGTATTGGTCTC	TGGACATACTGGATACAGCTGGA
OM_00503	EGFR	D761N	g.55210005G>A	ACGTTGGATGCCATCGAGATTTCACTGTAG	ACGTTGGATGTTTCTTCATGAAGACCTCAC	GCTAGACCAAAATCACCTA
OM_00507	EGFR	H773R	g.55216514A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	cctccGCGCACTCTTGCCCACA
OM_00516	EGFR	G810S	g.55216624G>A	ACGTTGGATGTCTTCATGAAGACCTCACAG	ACGTTGGATGCCACTCCATCGAGATTTCAC	AGTAAAAATAGGTGATTTTGGT
OM_00529A	EGFR	L858M	g.55227008C>A	ACGTTGGATGCAGGTGGTGTTATTGTAATAG	ACGTTGGATGCCTCAAGTAACATCTGAGAC	gATTGTAATAGTCTGAGCTGTAAA
OM_00529B	EGFR	L858M	g.55227008C>A	ACGTTGGATGGACCTAGTTCCAATCTTTTC	ACGTTGGATGAATCAGACGACACAGGAAGC	caTAGTTCCAATCTTTTCTTTTATTT
OM_00561	EGFR	L747_A750>P	g.55209962_55209972ATTAAGAGAAG>GC	ACGTTGGATGAGGCCTGCTGAAAATGACTG	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ggtTGGTAGTTGGAGCTGGT
OM_00571	EGFR	L747_P753>Q	g.55209963_55209982TTAAGAGAAGCAACATCTCC>CA	ACGTTGGATGTCACATCGAGGATTTCCTTG	ACGTTGGATGGAAAGTTAAATTCCCGTCGC	gtttaGTTGTTGGCTTTCGGAGAT
OM_00572	EGFR	L747_T751>S	g.55209964_55209975delTAAGAGAAGCAA	ACGTTGGATGCACCCCGTAGCTGAGGATG	ACGTTGGATGATGTCTTGCAGCCGAGGAG	GAGGATGCCTGCATACACAC
OM_00574	EGFR	L747_R748>FP	g.55209965_55209968AAGA>CCCG	ACGTTGGATGTCCAGGAAGCCTACGTGATG	ACGTTGGATGACGGTGGAGGTGAGGCAGAT	agGATGGCCAGCGTGGACA
OM_00587	EGFR	E746_T751del	g.55209960_55209977delGAATTAAGAGAAGCAACA	ACGTTGGATGGACCCCTATGCAGTAAATGG	ACGTTGGATGTAGCGCCTGGAAGAGAAAAG	cttAGATTACAGCTTCCCCAGACT
OM_01103	EGFR	D761Y	g.55210005G>T	ACGTTGGATGCTAATTCTGGGTGCTCAGAC	ACGTTGGATGTATTCCCACAGTGTATCGGC	tAAGGCGTTCACAAAGTGTATTTAG
OM_01104	EGFR	G810D	g.55216625G>A	ACGTTGGATGTCTTCATGAAGACCTCACAG	ACGTTGGATGCCACTCCATCGAGATTTCAC	gaaaAGTAAAAATAGGTGATTTTGGT
OM_01114	EGFR	p.E746_A750del	g.55209960_55209974delGAATTAAGAGAAGCA	ACGTTGGATGGTCTCCTGTAATTCTCAGGC	ACGTTGGATGTGCCCTTCCAGTGTATACTC	gtaagTCTCAGGCTTGACTAGGCTCC
OM_00117	ERBB2	L755P	g.35133745_35133746TT>CC	ACGTTGGATGACGTACTGGTGAAAACACCG	ACGTTGGATGTCTTTCTCTTCCGCACCCAG	ggCAAGATCACAGATTTTGGGC
OM_00118	ERBB2	G776S	g.35134523_35134524insA	ACGTTGGATGTGCAGCACCACAGGGTGT	ACGTTGGATGCCACTCTCACCGCACCCGT	acccGCGTGTCCAGGAAGCCCT
OM_00120	ERBB2	G776VC	g.35134523_35134524insTTT	ACGTTGGATGTCCCACCTTTCAGCAGGTCA	ACGTTGGATGGTTTGAGAACATCGGGAAGG	TCAGCAGGTCAGAGAGC
OM_00608	ERBB2	L755S	g.35133746T>C	ACGTTGGATGGAAAGTTAAAATTCCCGTCGC	ACGTTGGATGTCACATCGAGGATTTCCTTG	gggacAATTCCCGTCGCTATCAAG
OM_00609	ERBB2	D769H	g.35133787G>C	ACGTTGGATGCAACAGTCTTACCTGGACTC	ACGTTGGATGCAGGATTGCCTTTACCACTC	cccCTGGAATCCATTCTGGTGCCACT
OM_00612A	ERBB2	V777L	g.35134526G>T	ACGTTGGATGTCTTTCAAGGTTGAAAATC	ACGTTGGATGCTTGCATCTAGATCTTTATT	TTCAAGGTTGAAAATCTTTCTAAA
OM_00612B	ERBB2	V777L	g.35134526G>T	ACGTTGGATGCACCTCTTCATCAAGGTTAC	ACGTTGGATGGTGAATGACTTCACTTATTG	aTCTTCATCAAGGTTACTTTTTC
OM_00127	FGFR1	S125L	g.38405095G>A	ACGTTGGATGAATCTCCCAATCATCACTCG	ACGTTGGATGCTGTTTGGCTTGACTTGAC	agTCTCCCAATCATCACTCGAGTCCC
OM_00128	FGFR1	P252T	g.38401366G>T	ACGTTGGATGGTAGCGATACAAACTTGGAG	ACGTTGGATGCTTACTGATTTAAGCATGG	CTTGGAGTTCGCTTGTATTAC
OM_00634	FGFR2	S252W	g.123269667G>C	ACGTTGGATGCAGGATTGCCTTTACCACTC	ACGTTGGATGCAACAGTCTTACCTGGACTC	gCAGAGAAGGAGCTGTGG
OM_01106	FGFR2	N549K	g.123248025A>C	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ACGTTGGATGACTGGCAGCAACAGTCTTAC	CACCAGAATGGATTCCA
OM_01107	FGFR2	N549K	g.123248025A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	aaGATATTCTCGACACAGCAGGTC
OM_01108	FGFR2	K310R	g.123269493T>C	ACGTTGGATGTCTGGATCCCAGAAGGTGAG	ACGTTGGATGTTGGCTTTCGGAGATGTTGC	AAAATTCCCGTCGCTATCAAG
OM_01109	FGFR2	Y375C	g.123264784T>C	ACGTTGGATGTCTCTTGAGGATCTTGAAGG	ACGTTGGATGCCTTATACACCGTGCCGAAC	gaAATTCAAAAAGATCAAAGTGCTGG
OM_01110	FGFR2	S372C	g.123264793G>C	ACGTTGGATGTTACCACTCAGAGAAGGAGC	ACGTTGGATGCATGGAACCAGACAGAAAAG	GAGCTGTGGTAGTGGCA
OM_01111	FGFR2	C382R	g.123264764A>G	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACGTTGGATGGACGGAATATAAGCTGGTGG	AGCGCACTCTTGCCCACAC
OM_00129A	FGFR3	R248C	g.1773362C>T	ACGTTGGATGTGTCCTCATGTATTGGTCTC	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	ATGGCACTGTACTCTTCT
OM_00129B	FGFR3	R248C	g.1773362C>T	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	ACGTTGGATGTGTCCTCATGTATTGGTCTC	gaTGGACATACTGGATACAGCTGGAC
OM_00130B	FGFR3	S249C	g.1773366C>G	ACGTTGGATGCATGTGATTACCAAGGTAAC	ACGTTGGATGTTAGCGAGTGCCCATTTGAC	gggaCAAGGTAACTCAGGACTT
OM_00131A	FGFR3	G370C	g.1775887G>T	ACGTTGGATGAGAGCCATGCAAGGGATTC	ACGTTGGATGAGAAGGCAACTTGACAAGAG	cccTGCAAGGGATTCCATGATTC
OM_00131B	FGFR3	G370C	g.1775887G>T	ACGTTGGATGGCAAGGGATTCCATGATTCG	ACGTTGGATGAGAAGGCAACTTGACAAGAG	cttagTCCATGATTCGATGTTCACATC
OM_00132B	FGFR3	Y373C	g.1775897A>G	ACGTTGGATGATGTACTGGTCCCGCATGG	ACGTTGGATGTGTTGGACATCCTGGATACC	GGCGCTGTACTCCTCCT
OM_00136	FGFR3	K650Q	g.1777687A>C	ACGTTGGATGACTGGCAGCAACAGTCTTAC	ACGTTGGATGGCCTTTACCACTCAGAGAAG	ccACAGTCTTACCTGGACT
OM_00641	FGFR3	S371C	g.1775890A>T	ACGTTGGATGTGGAGAAACCTGTCTCTTGG	ACGTTGGATGCATGTACTGGTCCCTCATTG	gTGGATATTCTCGACACAGCAGGTC
OM_00645	FGFR3	L794fs*23	g.1778747T>GA	ACGTTGGATGCTGTATCGTCAAGGCACTCT	ACGTTGGATGAGGCCTGCTGAAAATGACTG	acGGCACTCTTGCCTACGC
OM_01021	FGFR3	G697C	g.1778129G>T	ACGTTGGATGTTCAAGGTGATCAGTTGGTC	ACGTTGGATGGTTTCATATAGGATTCACC	ATCAGTTGGTCCTTCTC
OM_00661	FLT3	Y572C	g.27506341T>C	ACGTTGGATGTCTCTTGAGGATCTTGAAGG	ACGTTGGATGCCTATACACCGTGCCGAAC	CAAAAAGATCAAAGTGCTG
OM_00665A	FLT3	D835Y	g.27490642C>A	ACGTTGGATGTGCTGGAACTTCGCTCACAG	ACGTTGGATGCTAATACCCTGCAAATAGC	AGCTGACCTAGTTCCAATCTTTT
OM_00665B	FLT3	D835Y	g.27490642C>A	ACGTTGGATGTGCTGGAACTTCGCTCACAG	ACGTTGGATGCTAATACCCTGCAAATAGC	cCCTAGTTCCAATCTTTTCTTT
OM_00666	FLT3	D835H	g.27490642C>G	ACGTTGGATGGTTGTTGAGGAGATAAATGG	ACGTTGGATGCTGGGAAACTCCCATTTGTG	ccctcACATAGACCCAACACAAC
OM_00667	FLT3	D835V	g.27490641T>A	ACGTTGGATGTGGAGCAGCAGCAGCTCCG	ACGTTGGATGGCTCTGACCATTCTGTTCTC	ccCAGCAGCTCCGCCACTC
OM_00668	FLT3	D835E	g.27490640A>C	ACGTTGGATGCAACAGTCTTACCTGGACTC	ACGTTGGATGCAGGATTGCCTTTACCACTC	CTTACCTGGACTCTGGAATCCATT
OM_00669	FLT3	D835E	g.27490640A>T	ACGTTGGATGTTTTGCACAGGGCATTTTGG	ACGTTGGATGGCACAGTGAATTTTCTTGCC	CAGGGCATTTTGGTTGTGTATATC
OM_00677	FLT3	D835del	g.27490640_27490642delATC	ACGTTGGATGTGAAAATTCCAGTGGCCATC	ACGTTGGATGGGGCTTACGTCTAAGATTTC	ATTCCAGTGGCCATCAAAGTGT
OM_00678	FLT3	I836del	g.27490637_27490639delGAT	ACGTTGGATGATGAAGATCGCAGACTTCGG	ACGTTGGATGAGGGCCGGGCTCACGTTGG	caaGTGCACAACCTCGACTACTACAAG
OM_01072	FLT3	I836M	g.27490637G>C	ACGTTGGATGGAGTCCAGGTAAGACTGTTG	ACGTTGGATGTTGATGGAGTTGGACATGGC	gtTAAGACTGTTGCTGCCAGTGACTA
OM_00138A	HRAS	G12V	g.524288C>A	ACGTTGGATGCTTCAGGAGCGAAATCTCCC	ACGTTGGATGGGGTCTCCTGAACATAGTG	CCTCCAAAAGTGGTGCT
OM_00138B	HRAS	G12V	g.524288C>A	ACGTTGGATGAAGTTCCAGCAGTGTCACAG	ACGTTGGATGGCCTGGCTGATTCTGAAGAT	ctAACCAAATCCAGCAGACTG
OM_00139	HRAS	G12D	g.524288C>T	ACGTTGGATGTGTCCTCATGTATTGGTCTC	ACGTTGGATGGTGAAACCTGTTTGTTGGAC	gccTGGCACTGTACTCTTCT
OM_00141	HRAS	G12R	g.524289C>G	ACGTTGGATGAGCAGAGAATGGGTACTCAC	ACGTTGGATGTGATTTTGGTCTAGCCAGAG	ggtcCTCACGTTTCCTTTAACCACATA
OM_00142	HRAS	G12C	g.524289C>A	ACGTTGGATGACTGACAGAAGTACATCTGC	ACGTTGGATGTCAGACACCCAAAAGTCCAC	GCTAAACATGAGTGGGGTCT
OM_00143A	HRAS	G13C	g.524286C>A	ACGTTGGATGATCTGCTAAACATGAGTGGG	ACGTTGGATGTCCCTCCAAAAGTGGTGCTC	CATGAGTGGGGTCTCCT
OM_00143B	HRAS	G13C	g.524286C>A	ACGTTGGATGTGGAGGTGTTTTACTTCTGC	ACGTTGGATGAGTGGCATTATAAGCCCCAG	CTGCTTGGTGGCATGGTTT
OM_00145	HRAS	G13R	g.524286C>G	ACGTTGGATGTGATTTGGTCTAGCCAGAG	ACGTTGGATGAGCAGAGAATGGGTACTCAC	cccGAGACATCAAGAATGATTCTAA
OM_00146	HRAS	G13S	g.524286C>T	ACGTTGGATGAAGTGAGGACGTACACTGCC	ACGTTGGATGAGATCTGTGACTTTGGCCTG	ttCCTTTCGACACATAGTTCGAAT
OM_00148	HRAS	Q61H	g.523873C>G	ACGTTGGATGCCCAACACAACTTCCTTATG	ACGTTGGATGAGCCCCTGTTTCATACTGAC	CACAAATGGGAGTTTCC
OM_00149	HRAS	Q61P	g.523874T>G	ACGTTGGATGAGGCCTGCTGAAAATGACTG	ACGTTGGATGCTGTATCGTCAAGGCACTCT	taaaaTTGTGGTAGTTGGAGCT
OM_00150	HRAS	Q61H	g.523873C>A	ACGTTGGATGTGACACAAAGACTGGCTTAC	ACGTTGGATGGGGAGCCAATGGTTCAGAA	cATTCCATGATTAGAACCCACTC
OM_00151A	HRAS	Q61L	g.523874T>A	ACGTTGGATGGAGCCCAACTGCGCCGACC	ACGTTGGATGACCAGCGTGTCCAGGAAGC	GACCCCGCCACTCTCAC
OM_00151B	HRAS	Q61L	g.523874T>A	ACGTTGGATGACCAGCGTGTCCAGGAAGC	ACGTTGGATGGAGCCCAACTGCGCCGACC	caCAGCGTCGTGCACGGGTC
OM_00152A	HRAS	Q61R	g.523874T>C	ACGTTGGATGTCTCTTGAGGATCTTGAAGG	ACGTTGGATGCCTATACACCGTGCCGAAC	gTCAAAAAGATCAAAGTGCTGG
OM_00152B	HRAS	Q61R	g.523874T>C	ACGTTGGATGTCTCTTGAGGATCTTGAAGG	ACGTTGGATGCCTATACACCGTGCCGAAC	ggtAATTCAAAAAGATCAAAGTGCTG

Appendix 2.1

Neuropathological and neuroradiological spectrum of pediatric malignant gliomas: correlation with outcome

Neuropathological and Neuroradiological Spectrum of Pediatric Malignant Gliomas: Correlation With Outcome

Ste´phanie Puget, MD, PhD*‡
Nathalie Boddaert, MD, PhD§
Anne-Sophie Veillard, MD{
Mathew Garnett, MDk
Catherine Miquel, MD, PhD#**
Felipe Andreiuolo, MD‡
Christian Sainte-Rose, MD, PhD*
Thomas Roujeau, MD*
Federico DiRocco, MD*
Marie Bourgeois, MD*
Michel Zerah, MD*
Francxois Doz, MD, PhD‡‡
Jacques Grill, MD, PhD‡§§
Pascale Varlet, MD, PhD#

*Department of Neurosurgery, Hoˆpital Necker Enfants Malades, Universite´ Paris Descartes, Paris, France; ‡UPRES EA3535, Gustave Roussy Institute, Villejuif, France; §Department of Neuroradiology, Hoˆpital Necker Enfants Malades, Universite´ Paris Descartes, Paris, France; {Biostatistics and Epidemiology Department, Gustave Roussy Institute, Villejuif, France; kDepartment of Neurosurgery, Addenbrookes Hospital, Cambridge, United Kingdom; #Department of Pathology, Sainte-Anne Hospital, Universite´ Paris Descartes, Paris, France; **UMR Inserm U894; Centre Paul Broca, Universite´ Paris Descartes, Paris, France; ‡‡Department of Pediatric and Oncology, Curie Institute, Paris, France; §§Department of Pediatric and Adolescent Oncology, Gustave Roussy Institute, Villejuif, France

Correspondence:
Ste´phanie Puget, MD, PhD,
Department of Neurosurgery,
Hopital Necker Enfants Malades,
Universite´ Paris Descartes,
149 Rue de Se`vres,
75015 Paris, France.
E-mail: stephanie.puget@nck.aphp.fr

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BACKGROUND: The diagnostic accuracy and reproducibility for glioma histological diagnosis are suboptimal.

OBJECTIVE: To characterize radiological and histological features in pediatric malignant gliomas and to determine whether they had an impact on survival.

METHODS: We retrospectively reviewed a series of 96 pediatric malignant gliomas. All histological samples were blindly and independently reviewed and classified according to World Health Organization 2007 and Sainte-Anne classifications. Radiological features were reviewed independently. Statistical analyses were performed to investigate the relationship between clinical, radiological, and histological features and survival.

RESULTS: Cohort median age was 7.8 years; median follow-up was 4.8 years. Tumors involved cerebral hemispheres or basal ganglia in 82% of cases and brainstem in the remaining 18%. After histopathological review, low-grade gliomas and nonglial tumors were excluded (n = 27). The World Health Organization classification was not able to demonstrate differences between groups and patients survival. The Sainte-Anne classification identified a 3-year survival rate difference between the histological subgroups (oligodendroglioma A, oligodendroglioma B, malignant glioneuronal tumors, and glioblastomas; P = .02). The malignant glioneuronal tumor was the only glioma subtype with specific radiological features. Tumor location was significantly associated with 3-year survival rate (P = .005). Meningeal attachment was the only radiological criteria associated with longer survival (P = .02).

CONCLUSION: The Sainte-Anne classification was better able to distinguish pediatric malignant gliomas in terms of survival compared with the World Health Organization classification. In this series, neither of these 2 histological classifications provided a prognostic stratification of the patients.

KEY WORDS: Children, High-grade astrocytomas, Histological classification, MRI

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Brain tumors are the most common solid tumors in children and have the highest mortality rate of all solid pediatric tumors. The glial cell tumors account for approximately 60% of all pediatric brain tumors, with 50% of them being high-grade tumors.[1] Despite neuro-oncological advances in multimodality therapy, pediatric malignant gliomas (pMGs) invariably have a poor survival, with 5-year survival rates of , 20% in most published series.[2-4]

ABBREVIATIONS: BGM, glioblastoma multiforme; MGNT, malignant glioneuronal tumor; pMG, pediatric malignant glioma; SA, Sainte-Anne; WHO, World Health Organization

At present, the treatment options and the prognosis for this group of tumors have been based on the clinical criteria and histopathological diagnosis.[4,5] The pMGs are heterogeneous in terms of histology; furthermore, histologically identical tumors can respond differently to the same treatment.[6]

The most widely used histological classification system of brain tumors is that of the World Health Organization (WHO).[6] This grading system was developed in an adult population and is based on subjective morphological criteria.

However, using it to classify high-grade gliomas by histological features can be challenging, thus leading to limited reproducibility and interobserver variability of 20% to 50% in some series.[7-12] In addition, despite similar histological terminology, adult and pediatric high-grade gliomas differ in terms of prognosis.[6,13]

In 1988, Daumas-Duport et al[14] proposed another grading scheme based on the summation of only 4 histological features for astrocytic tumors in adults. It has been reported as a simpler and more standardized grading system with thus more reproducibility than the WHO system.[15,16] The original Daumas-Duport or Sainte-Anne-Mayo grading system has been enhanced with the addition of further tumor grades or subtypes, the oligodendroglioma grades A and B[17,18] and the malignant glioneuronal tumor (MGNT),[19] and is now called the Sainte-Anne (SA) classification.

There is recent evidence that magnetic resonance imaging (MRI) appearances may be helpful in distinguishing histological tumor grades for glial lesions and may be helpful in assessing prognosis in adults with malignant gliomas.[5,20,21]

In this study, we retrospectively reassessed the histological classification of a series of pediatric patients who were originally diagnosed and treated for a malignant glioma. Both the WHO and SA classifications were used to investigate the concordance between these 2 grading systems. The effect that the histological grade had on the prognosis was also assessed. The initial MRI scans were retrospectively reviewed to investigate the possible correlation between both diagnosis and clinical outcome with MRI features. This study was a prerequisite to further genomic investigations, which may provide additional information to improve pMG classification, to guide treatment, and to predict outcome more accurately.

PATIENTS AND METHODS

Patients

All children who had undergone a surgical procedure at either the Necker Hospital in Paris or Wertheimer's Hospital in Lyon between 1991 and 2006 were considered. The inclusion criteria were initial histological diagnosis of malignant glioma (WHO grade 3 or 4) and no preoperative adjuvant treatment. Ninety-six children were eligible for the study. Clinical characteristics and survival information were collected from the medical notes. The location of the tumor and extent of surgical resection, defined as complete resection, subtotal resection, or biopsy, were assessed from the operative reports and postoperative imaging. We considered the resection to be complete if the MRI displayed no contrast enhancement and no hyper-T2 or hyper–fluid attenuated inversion recovery around the cavity.

Histological Review

All histopathological specimens, fixed in buffered formalin and embedded in paraffin, were reevaluated for histopathological diagnosis for the purposes of this study. All available hematoxylin and eosin–stained slides and immunohistochemical preparations were reviewed independently by 2 neuropathologists (C.M. and P.V.) who were masked to clinical condition and outcome. All tumors were classified according to 2007 WHO and SA classifications. Cases with discrepancies were reviewed again by both pathologists until a consensus was reached.

The 5 combined following histological features were used for the WHO 2007 grading system: increased cellular density, nuclear atypia, necrosis, vascular proliferation, and mitotic activity. The histological criteria used in the SA classification included architectural pattern (solid tumoral tissue and tumoral infiltration), microangiogenesis (endothelial hyperplasia, ie, all capillaries exhibiting hyperplastic endothelial cells in at least 1 low-power field and/or vascular proliferation), necrosis, and mitosis. Immunostaining such as neurofilament protein, synaptophysin, and neuronal nuclei was also used to help diagnose the MGNT.[19,22] In addition, the contrast enhancement on MRI was used to discriminate oligodendrogliomas grade A from grade B.[17,18]

Immunohistochemical Studies

Representative formalin-fixed specimens from each case were selected, and an array of immunohistochemical markers were systematically undertaken, including glial fibrillary acidic protein (1/200, clone 6F2, DAKO, Glostrup, Denmark), neurofilament (NF) protein (1/50, clone 2F11, DAKO), neuronal nuclei (1/500, clone VMA377, Abcys, Paris, France), synaptophysin (1/50, clone SY38, Progen, Heidelberg, Germany), Olig2 (1/100, R&D Systems, Abingdon, UK), Ini-1 (1/50, clone BAF47, BD Biosciences, San Jose, California), and Ki-67 (1/75, Mib-1, DAKO). Additional immunostaining was performed when indicated and included epithelial membrane antigen (1/1, clone E29, DAKO), chromogranin A (1/75, clone LK2H10, Immunotech, Marseille, France), and microtubule-associated protein 2 (1/100, clone HM2, Sigma, St. Louis, Missouri).

The paraffin sections were cut at 4 mm, deparaffinized, exposed to 30 minutes of microwave treatment at 98°C in citrate pH 6.0 buffer for antigen retrieval, and then treated with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity. Antibody binding was visualized with an Immunotech peroxidase kit; diaminobenzidine tetra hydrochloride (DAKO) was used as the chromogen. Automated immunohistochemical detection of glial fibrillary acidic protein was performed with an avidin-biotin-peroxidase complex on a Ventana (Benchmark, Ventana Systems, Strasbourg, France).

Imaging Analysis

The preoperative radiological features were assessed by a pediatric neuroradiologist (N.B.) and 2 pediatric neurosurgeons (S.P., C.S.R) blinded to clinical condition, histopathological data, and outcome. T1-weighted (before and after gadolinium) and T2-weighted MRIs were analyzed. The following image characteristics were evaluated: (1) tumor location; (2) cystic component, present vs absent; (3) meningeal attachment, present vs absent; (4) ependymal attachment, present vs absent; (5) ''grapelike'' appearance, ie, a tumor with coalescent nodular and cystic components, present vs absent; (6) contrast enhancement, present vs absent; (7) ratio of enhancement area to the hyper-T2 signal area; and (8) sharp vs indistinct border on T1. The images were scored by consensus.

Statistical Analysis

The primary end point of this study was overall survival, which was measured from the date of surgical resection to the last follow-up appointment or death. Median follow-up was computed with the reverse Kaplan-Meier method.[23] A multiple-correspondence analysis was used to

study the pattern of relationships between the different radiological features. Logistic regression analyses were performed to determine whether radiological features were related to histological subgroups, as defined by the WHO and SA classifications.

Survival curves were obtained with the Kaplan-Meier method and tested by the log-rank test. The Cox model was used to study prognostic factors on overall survival. Univariate and multivariate analyses were performed both for the whole series and after exclusion of the brainstem gliomas.

All reported P values were 2 sided. All analyses were performed with SAS software (version 8.2, SAS Institute, Cary, North Carolina).

RESULTS

The main characteristics of the population are summarized in Table 1.

Histological Classifications

In the 96 patients, it was not possible to classify 4 tumors because of a paucity of pathological material. In addition, 7 tumors were reclassified as nonglial on the basis of immunostaining such as Ini-1 and were therefore excluded. The 2 pathologists had similar findings in 92 cases (96%), and a consensus diagnosis was established in all cases.

The details of the histological results according to both classifications are summarized in Table 2. In this series, 12 and 16 of the tumors were classified as low-grade gliomas or benign mixed neuronal-glial tumors according to SA and WHO classifications, respectively. All brainstem tumors (13 patients) were diagnosed as infiltrative gliomas (brainstem infiltrative gliomas). Interestingly, half of the tumors were classified as glioblastoma multiforme (GBM) and anaplastic oligodendroglioma according to the WHO classification, whereas half were classified as MGNT and oligodendroglioma B according to the SA classification.

TABLE 1. Characteristics of the Pediatric Malignant Gliomas Cohort[a]

Characteristics		
Median age (range)	7.8 y (5 d-17 y)	
Sex, n (%)		
Male	39	56.5
Female	30	43.5
Symptoms at presentation, n (%)[b]		
Signs of raised intracranial pressure	53	76.8
Focal neurological impairment	27	39
Seizures	10	14.5
Tumor location		
Supratentorial hemispheric	28	41
Basal ganglia	28	41
Brainstem	13	18
Extent of surgery, n (%)		
Complete resection	13	18.8
Incomplete resection	41	59.4
Biopsy	15	21.7
Adjuvant treatment, n (%)		
Chemotherapy	60	87
Radiotherapy	46	66.7

[a] n = 69.

[b] Patients may present with . 1 symptom.

TABLE 2. Classification of Pediatric Gliomas According to the 2007 World Health Organization and Sainte-Anne Classifications[a]

WHO Classification (n = 85)	n	%	SA Classification (n = 85)	n	%
LGG	16	20	LGG	12	14
Anaplastic astrocytoma	4	5	Oligo A	4	5
Oligoastrocytoma III	8	9	Oligo B	18	21
Anaplastic oligodendroglioma	22	26	MGNT	28	33
Glioblastoma	20	24	Glioblastoma	3	4
BSIG	13	15	BSIG	13	15
Undefined	2	1	Undefined	7	8

[a] BSIG, brainstem infiltrative gliomas; LGG, low-grade gliomas; MGNT, malignant glioneuronal tumours; Oligo, oligodendrogliomas; SA, Sainte-Anne; WHO, World Health Organization.

Image Analysis

It was possible to review 69 preoperative MRI scans of the cohort of 85 patients. The imaging features for this series are summarized in Table 3. Some examples of the radiological features are shown Figure 1. The tumors could be divided into 3 anatomic locations, namely cerebral hemispheres, basal ganglia, and brainstem (41%, 41%, and 18%, respectively). All tumors except 2 displayed evidence of contrast enhancement. It was striking that the tumors with grapelike appearance were significantly associated with sharp limits, meningeal attachment, and the presence of cysts (P , .001, P = .002 and P = .010, respectively). Moreover, the tumors with sharp limits were associated with a well-defined ependymal attachment (P = .004). Peritumoral edema was associated with the ratio of enhancement area to the hyper-T2 signal area (P = .030).

Histopathology, Location, and MRI Characteristics

Logistic regression analysis was used to compare the radiological features with the WHO subtypes and grades. It failed to show any significant association between them. There were, however, imaging features that significantly correlated with the SA classification. In univariate analysis, the MGNT tumors were significantly associated with a grapelike appearance, presence of a cyst, and meningeal attachment (P = .001, P = .010, and P = .040, respectively). In multivariate analysis, however, only the grapelike appearance reached significance (P = .004; Table 4).

We applied a multiple correspondence analysis to study the pattern of relationships between the different radiological features (Figure 2). The variables location and histopathological subgroups according to WHO and SA were then added in this analysis as illustrative variables. According to WHO, grade 3 and

TABLE 3. Magnetic Resonance Imaging Characteristics of the Pediatric Malignant Gliomas

	Whole Series (n = 69)		Without Brainstem Infiltrative Gliomas (n = 56)	
	n	%	n	%
Grapelike appearance				
No	43	62	31	55
Yes	26	38	25	45
Sharp limits				
No	27	39	18	32
Yes	42	61	38	68
Enhancement T1 , T2				
No	37	54	35	63
Yes	32	46	21	37
Edema				
No	40	58	34	61
Yes	29	42	22	39
Cyst				
No	47	68	35	63
Yes	22	32	21	38
Meningeal attachment				
No	44	64	31	55
Yes	25	36	25	45
Ependymal attachment				
No	38	55	25	45
Yes	31	45	31	55

4 tumors were very closed, suggesting that similar radiological features characterize them. The MGNTs, from the SA classification, were associated with supratentorial location, grapelike appearance, cyst, sharp limits, and meningeal attachment.

Survival Analysis

The median follow-up in this cohort was 4.8 years (range, 0.15-10.3 years). The overall survival (Table 5) rates at 3 and 5 years were 37% (95% confidence interval, 26-49) and 23% (95% confidence interval, 14-36), respectively (Figure 3A). When the analysis was repeated after the exclusion of brainstem infiltrative gliomas, the survival rates at 3 and 5 years were 44% and 28%, respectively. There was no significant association in the cohort between the extent of surgical resection (complete vs incomplete) and survival even if the analysis was applied for each location group. When analyzed as a categorical variable, age at diagnosis had an effect on survival with a trend toward a better prognosis for the youngest patients (, 3 years; log-rank test, P = .09). Treatment with chemotherapy and/or radiotherapy failed to show prognostic value.

For both classifications, patients with a low-grade glioma had a significantly better 5-year survival compared with those patients with a malignant tumor (94% vs 18%, P , .001 for WHO; 92% vs 32%, P = .001 for SA).

In this cohort, using univariate analysis, the SA classification by subtypes was able to discriminate between survival (P = .02), whereas the WHO classification was not (P = .1; Figure 3B and 3C). The tumors described by the SA classification could be stratified into 3 groups, with a very poor prognosis for GBMs and brainstem infiltrative gliomas, a better prognosis for oligodendroglioma B tumors and MGNTs, and the best prognosis for the oligodendroglioma A tumors. When the WHO grades III and IV were compared, a different but nonsignificant 3-year survival (36% and 24%, respectively) was noted. Interestingly, it became a significant predictor of survival for patients . 3 years of age (P = .02).

The radiological characteristics of the preoperative imaging were considered in terms of the patient survival. Patients with a tumor located in the brainstem had a significantly worse outcome compared with the other patients (P = .001). The only

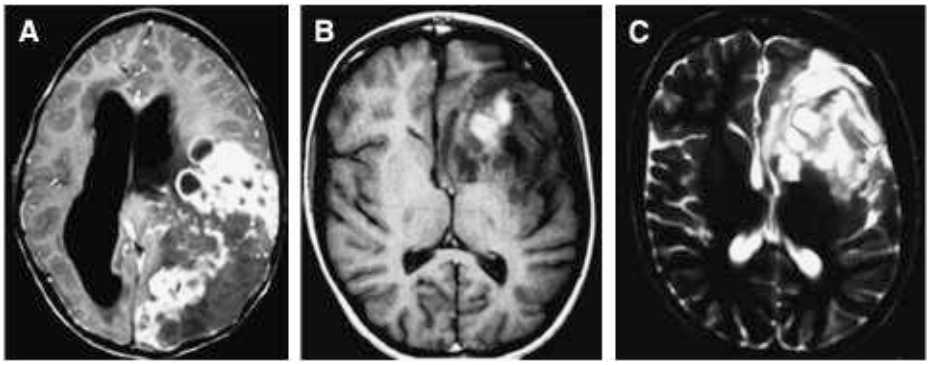

FIGURE 1. Examples of radiological findings for 2 different malignant gliomas. A, a tumor with a ''grapelike'' appearance with sharp limits and meningeal and ependymal attachments. B and C, a poorly circumscribed tumor with indistinct limits and an enhancement area less than the T2 area. There are also meningeal and ependymal attachments.

TABLE 4. Radiological Features of the Pediatric Malignant Gliomas Associated With the Sainte-Anne Classification: Comparison of the Malignant Glioneuronal Tumor Subgroups With the Others[a]

	Subgroup		Univariate Analysis MGNT vs Oligo-Astro			Multivariate Analysis MGNT vs Oligo-Astro		
	Oligo-Astro (n = 22)	MGNT (n = 28)	OR	95% CI	P (Global)	OR	95% CI	P (Global)
Grapelike appearance and limits					.001			.001
Sharp limits, no	12	3	1			1		
Sharp limits, yes	8	4	2.00	0.35-11		2.00	0.35-11	
Grapelike appearance, yes	2	21	42	6.1-288		42	6.1-288	
Enhancement , T2					.32			
Yes	13	12	1					
No	7	16	2.29	0.5-11				
Cyst					.01			.16
No	18	13	1			1		
Yes	4	15	5.2	1.40-19		3.4	0.62-19	
Meningeal attachment					.04			.96
No	16	12	1			1		
Yes	6	16	3.6	1.07-12		1.05	0.17-6.6	
Ependymal attachment					.06			.22
No	13	9	1			1		
Yes	9	19	3.0	0.95-9.8		3.1	0.51-19	

[a] CI, confidence interval; MGNT, malignant glioneuronal tumor; Oligo-Astro, oligoastrocytoma; OR, odds ratio.

other radiological finding that appeared to have an impact on survival was the presence of a meningeal attachment (P = .02, univariate analysis).

When all the variables were introduced in the Cox regression analysis, tumor location was the only factor that had a significant association with survival (P = .005).

DISCUSSION

In this study, we retrospectively analyzed a cohort of pediatric patients who had been treated for a malignant glioma over a 15-year period. The histological diagnoses of the surgical specimens were reevaluated with both the WHO 2007 and the SA classifications. The preoperative radiological characteristics of the tumor, together with its anatomical location, were also independently reviewed. The postoperative images were used to assess the extent of tumor resection. The histological classification, radiological characteristics, and extent of resection were then used to check for a correlation with survival.

In this study of 96 children treated for a malignant glioma, it was possible to reassess the histology in 92 patients. Of these patients, 7 (7%) were identified as having tumors that were nonglial in origin (eg, an atypical teratoid rhabdoid tumor). This is likely to reflect the greater array of immunohistochemical stains that are now available. Furthermore, 12 (14%) and 16 (20%) of the tumors, according to SA and WHO classifications, respectively, were identified as not having a high-grade tumor but a low-grade tumor. This yields a misclassification rate of 21% to 27%, which is in keeping with the previously reported literature of 20% to 50% disagreement for pediatric and adult gliomas.[7-12,24]

The patients who had a low-grade glioma, as assessed by either the WHO or the SA classifications, had a significantly better survival rate compared with the remaining patients. This is as expected and is in keeping with previous studies that have shown an improved outcome in children with a low-grade glioma as opposed to a high-grade glioma.[1,3,6,10,12]

In our series, the WHO 2007 grading had prognostic relevance when looking at patients . 3 years of age but not for the whole cohort. No impact of WHO grading on survival has been described in previous pediatric studies. One study used 1993 WHO histopathological classification for a large cohort of 340 children with supratentorial astrocytic tumors[10]; the second was a single-institution study of 39 purely nonbrainstem astrocytomas[25]; the third study of a multi-institutional cohort of 98 patients showed that histology had an independent association with outcome[26]; and the most recent study included about 43 pediatric high-grade gliomas.[27] In contrast, in others studies, there is a survival difference between grade 3 and 4 gliomas.[1,28-32] These series are large, but some are limited by the lack of central pathology review. In adult series, the distinction between glioma grades 3 and 4 affects prognosis.[6] This suggests that pediatric and adult malignant gliomas may be different tumors, in keeping with previous studies.[13,33,34] In the present series, there was a trend for a better prognosis for the youngest (, 3 years of age) patients, in accordance with a recent large series.[1] Previous studies have suggested that there may be biological differences that are stratified by age among the malignant pediatric gliomas.[35-38]

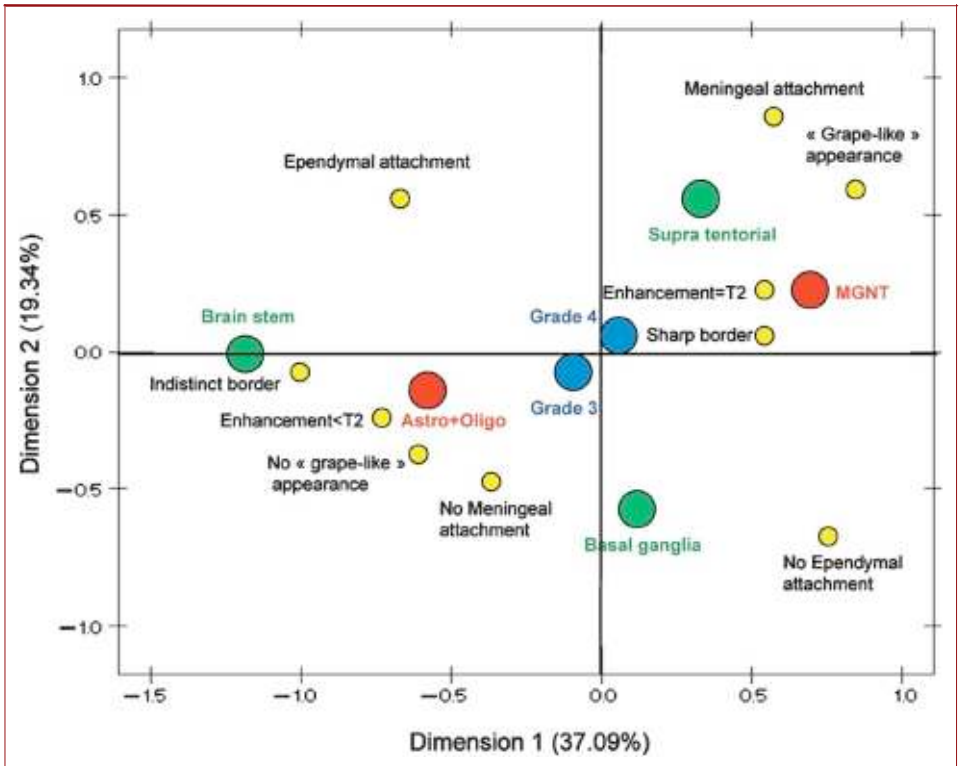

FIGURE 2. Multiple-correspondence analysis between radiological features (yellow), location (green), histopathological grades according to the World Health Organization (in blue), and the main subtypes according to the Sainte-Anne classifications (red). Astro, astrocytoma; MGNT, malignant glioneuronal tumors; Oligo, oligodendroglioma.

The prognostic significance of brain tumor classification and grading is particularly difficult to evaluate for several reasons. First, the classifications and histological subtypes have varied over time, with 4 editions of the WHO system since 1979.[6,39-42] Second, the grading criteria of gliomas have changed. Third, the interrater reproducibility of diagnoses and grades among pathologists, even for specialized pediatric pathologists, is low.[12,24,43] Finally, the impact on survival in terms of the anatomic location, presence of a mixed pathology (eg, oligoastrocytoma or neuroglial), and variety of therapeutic regimens that have been used in the last 20 to 30 years has not been fully evaluated. In addition, the evolution in immunophenotypical criteria (eg, the distinction of a malignant glioma from an atypical teratoid rhabdoid tumor with Ini-1 immunomarker) has identified tumors that now would not be considered malignant gliomas. The recent 2007 WHO classification is more rigorous in the definition of histological grading criteria, but it is still imprecise for some terms such as increased cellularity or moderate atypia, leading to inherent ambiguity even in well-trained pathologists.

The SA classification was proposed to standardize and improve diagnostic accuracy. The principle is based on a combination of limited and simple histological criteria with a particular focus on the infiltrative tissue surrounding the tumor. In the present study, the SA classification was able to discriminate between the different tumor subtypes and grades. This would be in keeping with a previous study on astrocytomas in adults.[15]

In the SA classification, the oligodendroglioma A tumors appeared to behave similarly to low-grade tumors, despite the latter being known to have a high potential of malignant transformation in adults. The oligodendroglioma A is a unique histological description to the SA classification. The histological feature is an infiltration by isolated tumoral oligodendrocytes exhibiting a clear swollen cytoplasm and a typical perineuronal satellitosis.[17,18] This is different from an oligodendroglioma B tumor in that oligodendroglioma grade A tumors are characterized by the absence of endothelial hyperplasia, which may be in accordance with its potential to behave in a fashion similar to the low-grade tumors. A large European review of oligodendroglioma in adults found that ''endothelial abnormalities'' were major histological features with prognostic significance.[44]

In both classifications, the histological definition for a glioblastoma is the same. In the SA classification, however, those tumors expressing neurofilament protein were reclassified as MGNTs. Interestingly, the SA classification could discriminate

TABLE 5. Variables With Predictive Value of Survival in the Univariate Analysis and With Independent Prognostic Value in the Multivariate Analysis[a]

Variable	Patients		Univariate Analysis			Multivariate Analysis		
	n	%	HR	95% CI	P	HR	95% CI	P
Grapelike appearance and limits						.39		
No sharp limits	27	39	1					
Sharp limits and no grapelike appearance	16	23	0.74	0.36-1.56				
Grapelike appearance	26	38	0.64	0.33-1.24				
Enhancement T1,T2						.12		
Yes	18	28	2.09	0.99-4.4				
No	47	72	1	0.87-3.8				
Cyst						.52		
No	47	68	1					
Yes	22	32	0.81	0.44-1.52				
Meningeal attachment						.02		
No	44	64	1					
Yes	25	36	0.47	0.25-0.90				
Ependymal attachment						.51		
No	38	55	1					
Yes	31	45	0.82	0.46-1.48				
Location						.005		.005
Supratentorial	28	41	1				1	
Basal ganglia	28	41	1.98	1.01-3.9			1.98	1.01-3.9
Brainstem	13	18	3.8	1.70-8.4			3.8	1.70-8.4
Age, y						.09		
, 3	14	17	1					
. 3	55	83	1.9	1.1-7.3				
WHO subtypes						.10		
Anaplastic astro	4	6	1					
Oligo-Astro	8	12	1.30	0.25-6.6				
Anaplasic oligo	22	33	1.03	0.23-4.5				
GBM	20	30	1.57	0.36-7.0				
BSIG	13	19	3.0	0.67-14				
Unclassified	2	...						
WHO grade						.21		
III	38	61	1					
IV	24	39	1.47	0.80-2.67				
SA subtypes						.02		
Oligo A	4	5	1					
Oligo B	18	25	3.4	0.44-26				
MGNT	28	38	4.6	0.62-35				
GBM	3	4	15	1.53-148				
BSIG	13	18	11	1.41-88				
Unclassified	7	10	4.3	0.50-37				
Complete resection						.31		
No	56	81	1					
Yes	13	19	0.67	0.30-1.47				

[a] BSIG, brainstem infiltrative gliomas; CI, confidence interval; GBM, glioblastoma multiforme; LGG, low-grade gliomas; MGNT, malignant glioneuronal tumours; Oligo, oligo dendrogliomas; Oligo-Astro, oligo astrocytoma; SA, Sainte-Anne; WHO, World Health Organization.

GBMs from MGNTs, the former having a uniformly limited survival. The description of a MGNT is unique to the SA classification. MGNTs represent a subgroup of malignant glial tumors that histologically resemble grade III or IV tumors according to the WHO 2007 classification but are characterized immunophenotypically by coexpression of glial and neuronal markers. The presence of NF-positive tumoral cells, including those in mitosis, is a hallmark diagnostic criterion of MGNTs because other neuronal markers are more inconstantly expressed. Recent evidence[19] suggests a distinct behavior between GBMs and MGNTs, the latter being characterized by no in situ tumor recurrence after gross total resection and metastases with frequent leptomeningeal extension. This would imply that in a series of patients with a GBM, in the WHO classification, there would

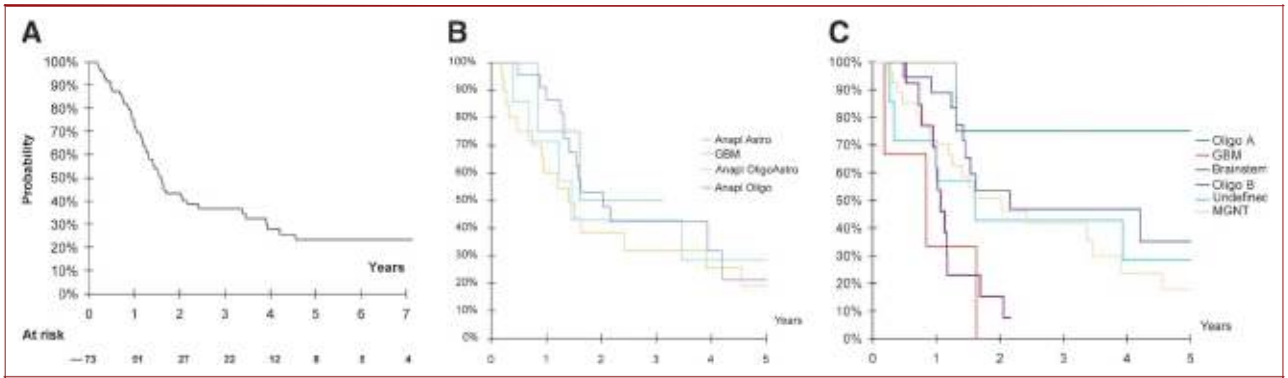

FIGURE 3. Overall survival for all pediatric malignant gliomas (A) according to 2007 World Health Organization subtypes (B) and Sainte-Anne classifications (C). Anapl Astro, anaplastic astrocytoma; Anapl Oligo, anaplastic oligodendroglioma; Anapl OligoAstro, anaplastic oligoastrocytoma; GBM, glioblastomas; MGNT, malignant glioneuronal tumor; Oligo A, oligodendroglioma A; Oligo B, oligodendroglioma B.

likely be tumors expressing neurofilament protein. If this subgroup of patients were to be removed, the remaining group of patients with a GBM would possibly have a poorer survival rate, as suggested by the small number of patients with a GBM in the present study.

The preoperative radiological evaluation indicated that the tumors in this cohort of patients could be classified into 3 groups: brainstem, hemispheric, and basal ganglia. When these 3 groups were compared, the patients with a brainstem tumor had a uniformly poor survival that was statistically significant. This would be in keeping with previous studies suggesting that malignant brainstem gliomas have a poor survival.[28,45] In addition, patients with a hemispheric tumor had a statistically significant better outcome than patients with a deep-seated basal ganglia tumor. The rate of the total resection in supratentorial tumors compared with the basal ganglia was higher in this cohort but insufficient to explain the survival difference.

In this cohort of pediatric patients, there was no significant association between the extent of resection (complete vs incomplete) and patient survival even when the analysis was repeated after the exclusion of brainstem infiltrative gliomas. The prognostic significance of the extent of resection in malignant gliomas in the literature is controversial.[46,47] Some evidence suggests that more extensive surgical resection is associated with longer life expectancy.[1,28-30,48] There is, however, no published prospective study on the extent of surgical resection and its impact on the survival of pMGs, and most of the published series did not benefit from a centralized radiological review. Furthermore, one of the reasons why MGNTs have an improved survival is believed to be that they are generally superficial hemispheric tumors and thus more surgically amenable to a radical resection.[19] In the present series, neither the location of the MGNTs nor the extent of resection was different from the others types of tumors. The benefit that this particular histological type of tumor may confer needs to be confirmed in a larger series.

There is some recent evidence in the literature that MRI appearances may be helpful in distinguishing histological tumor grades for gliomas and may also be helpful in assessing prognosis in adults with malignant gliomas.[5,20,21] In this pediatric study, the radiological features did not discriminate between the different types or grades of malignant glial cell tumors, according to the WHO classification. Conversely, the SA classification identified a tumor that was significantly associated with certain radiological features. The MGNT was significantly associated with a grapelike appearance, together with the presence of cysts and a meningeal attachment. Furthermore, the presence of a meningeal attachment was an independent variable in predicting outcome. This may reflect the improved survival of patients with an MGNT compared with other patients with a malignant glioma as previously described.[19]

In the present study, a multiple-correspondence analysis was used to study the relationships between radiological features, location, and histopathological groups. With this technique, it was possible to distinguish some of the tumors described in the SA classification. In particular, the MGNT could be identified as having a grapelike appearance, being supratentorial in location, and displaying a distinct radiological border with a meningeal attachment. This would be in keeping with the previously described features of the MGNT.[19] It was not possible to separate the tumors as defined by WHO 2007 with this method.

CONCLUSION

This study retrospectively analyzed a cohort of pediatric patients treated for a high-grade glioma. On reviewing the histology of the tumor samples, it was found that a significant number were neither glial cell in origin nor high grade.

The WHO classification is the most widely used classification for tumors. In this study, however, it was not found to be particularly helpful in terms of identifying radiological characteristics

of tumors or differentiating between patient survival, except for in patients . 3 years of age.

The SA classification, however, was able to identify certain radiological characteristics that were associated with some of the histological subtypes. Furthermore, this classification seems to better discriminate between the different histological groups and their survival, especially in distinguishing the MGNT from the GBM.

Further work is needed to confirm that the findings in this study are reproducible in a large, prospective cohort of patients. This study was a prerequisite to further genomic investigations, which may provide additional information to improve pMG classification, to guide treatment, and to predict outcome more accurately.

Disclosure

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Appendix 2.2

Mesenchymal transition and PDGFRA amplification/ mutation are key distinct oncogenic events in pediatric diffuse intrinsic pontine gliomas

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Mesenchymal Transition and PDGFRA Amplification/ Mutation Are Key Distinct Oncogenic Events in Pediatric Diffuse Intrinsic Pontine Gliomas

Stephanie Puget[1,2.], Cathy Philippe[2.], Dorine A. Bax[3], Bastien Job[4], Pascale Varlet[5], Marie-Pierre Junier[5], Felipe Andreiuolo[2], Dina Carvalho[3,6,7], Ricardo Reis[6], Lea Guerrini-Rousseau[2], Thomas Roujeau[1], Philippe Dessen[4], Catherine Richon[8], Vladimir Lazar[8], Gwenael Le Teuff[9], Christian Sainte-Rose[1], Birgit Geoerger[2,10], Gilles Vassal[2], Chris Jones[3], Jacques Grill[2,10]*

1 Department of Neurosurgery, Necker-Sick Children Hospital, University Paris V Descartes, Paris, France, 2 Unite Mixte de Recherche 8203 du Centre National de la Recherche Scientifique «Vectorology and Anticancer Therapeutics», Gustave Roussy Cancer Institute, University Paris XI, Villejuif, France, 3 Section of Pediatric Oncology, The Institute of Cancer Research/Royal Marsden Hospital, Sutton, Surrey, United Kingdom, 4 Formation de Recherche en Evolution 2939 du Centre National de la Recherche Scientifique, Integrated Research Cancer Institute in Villejuif, University Paris XI, Villejuif, France, 5 Team Glial Plasticity, Unite Mixte de Recherche 894 de l'Institut National de la Sante´ et de la Recherche Medicale and Department of Neuropathology, Sainte-Anne Hospital, University Paris V Descartes, Paris, France, 6 Life and Health Sciences Research Institute, University Do Minho, Braga, Portugal, 7 Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal, 8 Functional Genomics Unit, Gustave Roussy Cancer Institute, University Paris XI, Villejuif, France, 9 Department of Biostatistics and Epidemiology, Gustave Roussy Cancer Institute, University Paris XI, Villejuif, France, 10 Department of Pediatric and Adolescent Oncology, Gustave Roussy Cancer Institute, University Paris XI, Villejuif, France

Abstract

Diffuse intrinsic pontine glioma (DIPG) is one of the most frequent malignant pediatric brain tumor and its prognosis is universaly fatal. No significant improvement has been made in last thirty years over the standard treatment with radiotherapy. To address the paucity of understanding of DIPGs, we have carried out integrated molecular profiling of a large series of samples obtained with stereotactic biopsy at diagnosis. While chromosomal imbalances did not distinguish DIPG and supratentorial tumors on CGHarrays, gene expression profiling revealed clear differences between them, with brainstem gliomas resembling midline/thalamic tumours, indicating a closely-related origin. Two distinct subgroups of DIPG were identified. The first subgroup displayed mesenchymal and pro-angiogenic characteristics, with stem cell markers enrichment consistent with the possibility to grow tumor stem cells from these biopsies. The other subgroup displayed oligodendroglial features, and appeared largely driven by PDGFRA, in particular through amplification and/or novel missense mutations in the extracellular domain. Patients in this later group had a significantly worse outcome with an hazard ratio for early deaths, ie before 10 months, 8 fold greater that the ones in the other subgroup ($p = 0.041$, Cox regression model). The worse outcome of patients with the oligodendroglial type of tumors was confirmed on a series of 55 paraffin-embedded biopsy samples at diagnosis (median OS of 7.73 versus 12.37 months, $p = 0.045$, log-rank test). Two distinct transcriptional subclasses of DIPG with specific genomic alterations can be defined at diagnosis by oligodendroglial differentiation or mesenchymal transition, respectively. Classifying these tumors by signal transduction pathway activation and by mutation in pathway member genes may be particularily valuable for the development of targeted therapies.

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* E-mail: grill@igr.fr

. These authors contributed equally to this work.

Introduction

Brain tumors are the leading cause of cancer-related morbidity and mortality in children and adolescents, malignant gliomas carrying the worst prognosis among them [1]. Malignant gliomas that diffusely infiltrate the brainstem appear almost exclusively during childhood and adolescence and have a relatively homogenous presentation and dismal prognosis. DIPG represent the biggest therapeutic challenge in pediatric neuro-oncology with a median survival of 9 months despite collaborative efforts to improve treatment [2]. The vast majority of children succumb to their disease within 2 years of diagnosis. These tumors are unresectable and radiotherapy is the only treatment offering a significant but transient improvement. The addition of chemotherapy has not shown any benefit over the use of irradiation only [2,3]. The development of targeted therapies for DIPG has been hampered by the lack of knowledge of the biology of this devastating disease. Trials have been implemented so far based on the assumption that biologic properties of these brainstem gliomas of children are identical to cerebral high-grade gliomas of adults

[4,5]. Recent data suggest however that pediatric high-grade gliomas differ from their adult counterparts [6–9], and that there may be biological distinctions between childhood gliomas presenting in the brainstem compared with supratentorial ones [10].

Comprehensive genomic studies of a substantial number of DIPG at diagnosis have not yet been undertaken due to the lack of available tumor material. Indeed, diagnosis is usually based on the association of specific neurological signs, short clinical history with a typical radiological appearance on MRI [11]. A biopsy is not needed for diagnosis in most of the cases [12,13]. In addition, most of these lesions are infiltrating and grading according to the WHO classification does not correlate with outcome. Accordingly and despite the reported safety of the procedure [14], most of the neurosurgical teams limit the use of stereotactic biopsies to the lesions with unusual clinical or radiological characteristics. Therefore, only very limited data on true DIPG is available in the literature and confounded by the inclusion of autopsy – ie post-radiotherapy - cases [10,15–18].

Recently, our group started to use stereotactic biopsies of DIPG to obtain both pathological confirmation and immunohistochemical assessment of some specific biomarkers before the inclusion of patients in trials of targeted agents [19–21]. In this study, we sought to comprehensively define genetic alterations in DIPG at diagnosis by performing genome-wide array CGH and gene expression studies from frozen samples obtained by stereotactic biopsies. This study is the first to comprehensively define the biological alterations of DIPG at diagnosis, allowing the discovery of novel therapeutic targets directed specifically at these poor prognosis brain neoplasms.

Results

DIPG Biopsy Material

Over the 5 years of the study, 61 patients underwent stereotactic biopsies taking from one to eight tumor samples (median 3) in the Neurosurgery Department of Necker Sick Children's Hospital in Paris. In most instances, one or two biopsies were used for histological diagnosis and immunohistochemistry (Figure S1A). The remaining biopsies were snap-frozen with cytological control smears directly in the operating room, and nucleic acids extracted from representative samples. A median of 3.325 microg of DNA (range 0.805 to 21.5 microg) and 2.332 microg of RNA (range 0.048 to 15.84 microg) could be extracted from the biopsies, resulting in a total of 32 and 23 patients with sufficient quality and quantity of DNA and RNA, respectively, for microarray analyses without any amplification step.

A second set of surgical samples from pediatric non-brainstem high-grade gliomas of various histologies with arrayCGH (n = 34) and gene expression (n = 53) data acquired simultaneously on the same platform was used for comparative studies. Age distribution at diagnosis was similar in DIPG and in HGG.

DIPG Differ from Supratentorial High-grade Gliomas but Co-segregate with a Subgroup of Midline/thalamic Tumors

We first performed array CGH on the 32 frozen biopsies of newly diagnosed DIPG, and compared the high resolution DNA copy number profiles with a series of 34 pediatric supratentorial high grade gliomas. Unsupervised hierarchical clustering of the DIPG samples using the Euclidian distance defined two distinct subgroups, the first characterized by gain of chromosome 1q, and the second by numerous copy number losses and structural rearrangements (Figure S1B). There were no associations between array CGH subgroup and survival, age at onset, duration of symptoms before diagnosis, radiological characteristics or WHO grade according to the 2007 revision.

Amplifications at specific loci were detected by CGHarray for the oncogenes HRAS (5), PDGFRA (4), PDGFB (2), CAV1/2 (2), PTPRN2 (2), KDM5A (2), ETS1 (1), MYCN (1), WNT2 (1), RAB31 (1). Deletions were detected for PTEN (1), CDKN2A/B (1) and FAS (1). The oncogene H-RAS was gained or amplified in 7/32 (22%) and the TP53 tumor suppressor gene was lost in 7/32 (22%) of cases. Loss of TP53 locus was the only single chromosomal imbalance associated with a poorer outcome ($p = 0.01$, log-rank test) (Figure S1C). On immunohistochemistry, p53 overexpression was seen in 15/27 (55%) cases. A comprehensive list of minimal common regions of imbalances with a frequency superior to 15% is provided in Table S1.

It was not possible to clearly delineate DIPG and supratentorial tumors on the basis of the copy number profiles, as exemplified by an unsupervised principal component analysis (PCA) generated using all 42332 quality control passing probes (Figure S1D). By contrast, a similar PCA analysis of gene expression profiling using all 15231 quality control passing gene probes demonstrated the clustering of the DIPG samples distinct from the majority of supratentorial high-grade gliomas, with the exception of some midline (thalamic) tumors (Figure 1A).

Supervised analysis using the 76 samples (23 DIPG and 53 HGG) was used to identify the genes most closely associated with pediatric high-grade gliomas arising in the brainstem versus supratentorially, and resulted in an expression signature comprising 712 genes (p,0.005, Pearson correlation, Ward procedure) which could distinguish tumours based on location independent of WHO grade (Table S2). The corresponding heatmap showed that the GE profiles of midline tumors clustered in some cases with the ones of DIPG (Figure 1B). Figure 1C shows the distribution of the expression for transcription factors and neurogenesis regulators according to the three different locations. DIPG and supratentorial tumors could be distinguished by a different pattern of expression of specific homeobox and HLH genes. When analysing the expression levels of the major regulators of brainstem embryogenesis described in the literature, we observed a significant upregulation of GAL3ST1, MAFB, OLIG2 and HOXA2,3 and 4 in DIPG compared to supratentorial tumors (Figure S1E).

DIPG Comprise two Biological Subgroups with Distinct Survival and Pathological Characteristics

The unsupervised k-means algorithm was used to discover subgroups of DIPG based on their gene expression profiles. The most optimal Bayesian Information Criterion (BIC) value was obtained for the classification based on two clusters [22] (Figure S2A), as represented by the corresponding principal component analysis (Figure 2A). Supervised hierarchical clustering identified 643 genes differentially expressed between these two groups (False Discovery Rate (FDR) adjusted p-value,0.01) (Table S3 and Figure 2B). The first group had a significantly worse survival, with 70% (9/13) of children succumbing to the disease before the median overall survival time of 10.6 months (range 2 to 25 months) of the entire cohort, whilst only 10% (1/10) of the patients in the second group did so (Figure 2C). Since the risk of death was not proportional over time in the two groups, we use a Cox model with an interaction between group and time. The hazard ratio for early deaths, ie before 10 months, was 0.122 for group 2 vs group 1 ($p = 0.041$). Significant association of the 2 GE groups was observed neither with age nor with the array CGH classification described above.

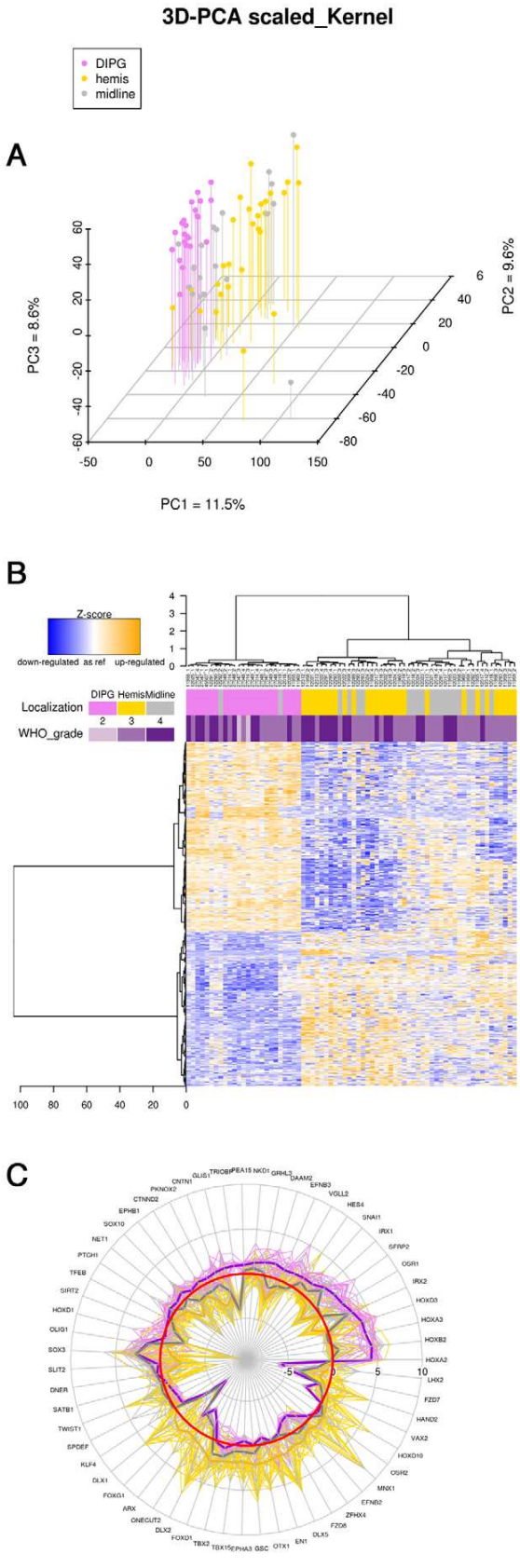

Figure 1. DIPG are different from supratentorial high-grade gliomas in children. Hemispheric, midline/thalamic tumors and DIPG are represented in gold, grey and violet respectively. Panel A: Gene expression of 23 DIPG and 49 supratentorial HGG were compared using a Principal Component Analysis on all 15231 quality control passing probes. Tumors are displayed according to their coordinates on the three first principal components, which describe 29.7% of the variance. Panel B: Heatmap of the 712 most differentially expressed genes between DIPG, midline and hemispheric tumors, selected using the moderated t-test of limma package of Bioconductor. Panel C: Radial plot of the expression of transcription factors and neurogenesis regulators according to the three tumor location, in log2 ratios related to normal brain stem. The zero red line represent the expression level of normal adult brainstem.
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Integrative analysis of the copy number and expression profiles using Spearman correlations demonstrated a significant influence of copy number on gene expression in group 1 tumours (306/15189 = 2% probes significantly correlated), however not for those in group 2 (3/15189 = 0.02% probes significantly correlated) (Figure 2D). These strong correlations were restricted to certain chromosomal abnormalities, in particular gain of 1q, loss of 19q, and amplification of 4q12. When considering both groups together the expression of 1460 genes (6% of the genome) was significantly correlated with their copy number; six of the twenty most correlated genes were located on chromosome 4q12 region: *CHIC2*, *SRP72*, *CLOCK*, *PPAT*, *SRD5A3* and *EXOC1* with Spearman correlation coefficient >0.9 and adjusted p<0.01 (Figure S2B).

Using gene set enrichment analysis [23], the expression profiles of the two groups were compared with the four subgroups of adult high grade gliomas recently described as proneural, neural, classical/proliferative and mesenchymal (http://tcga-data.nci.nih.goc/docs/publications/gbm_exp/) [24]. The proneural signature was highly enriched in the gene expression signature of group 1 (enrichment score = 0.66; nominal p = 0.004; FDR q = 0.089) (Figure 2E) while the mesenchymal signature was significantly associated with group 2 tumours (enrichment score = 0.8; nominal p = 0.004; FDR q = 0.007) (Figure 2F).

Mesenchymal Transition and a Pro-angiogenic Switch Define A Subset of DIPG

Since a mesenchymal gene expression signature was specifically represented in one of the two DIPG expression groups, we compared the expression of 53 transcription factors specific for this process as previously defined in adult high grade gliomas [25]. These genes were significantly upregulated in the group 2 DIPGs relative to the group 1 tumours (GSEA analysis: enrichment score 0.56, FDRq = 0.039, p nominal = 0.034), together with the master epithelial-mesenchymal transition regulators, *SNAI1* and *SNAI2/Slug* genes (Figure S3A). Expression of these genes alone was sufficient to distinguish group 1 and group 2 DIPG (Figure 3A). A subset of 7 transcription factors (*STAT3, BHLHE40, CEBPA and B, RUNX1, FOSL2 and ZNF238*) controlled most genes of the mesenchymal signature of gliomas; all but *ZNF238* were significantly upregulated in the group 2 tumours compared to the other DIPG (Figure 3B). This transcriptional module was associated with a mesenchymal phenotype with upregulation of *TNC*, *OSMR*, *VIM* and *YKL40/CHI3L1* and a more astrocytic histology (Table S3 & Figures 3A and 3C/D). Knowing that the *BRAF* V600E mutations could induce mesenchymal transition in some tumors [26] and that such mutations have been reported in a subset of pediatric glioma [27], we sequenced exon 15 of the *BRAF*

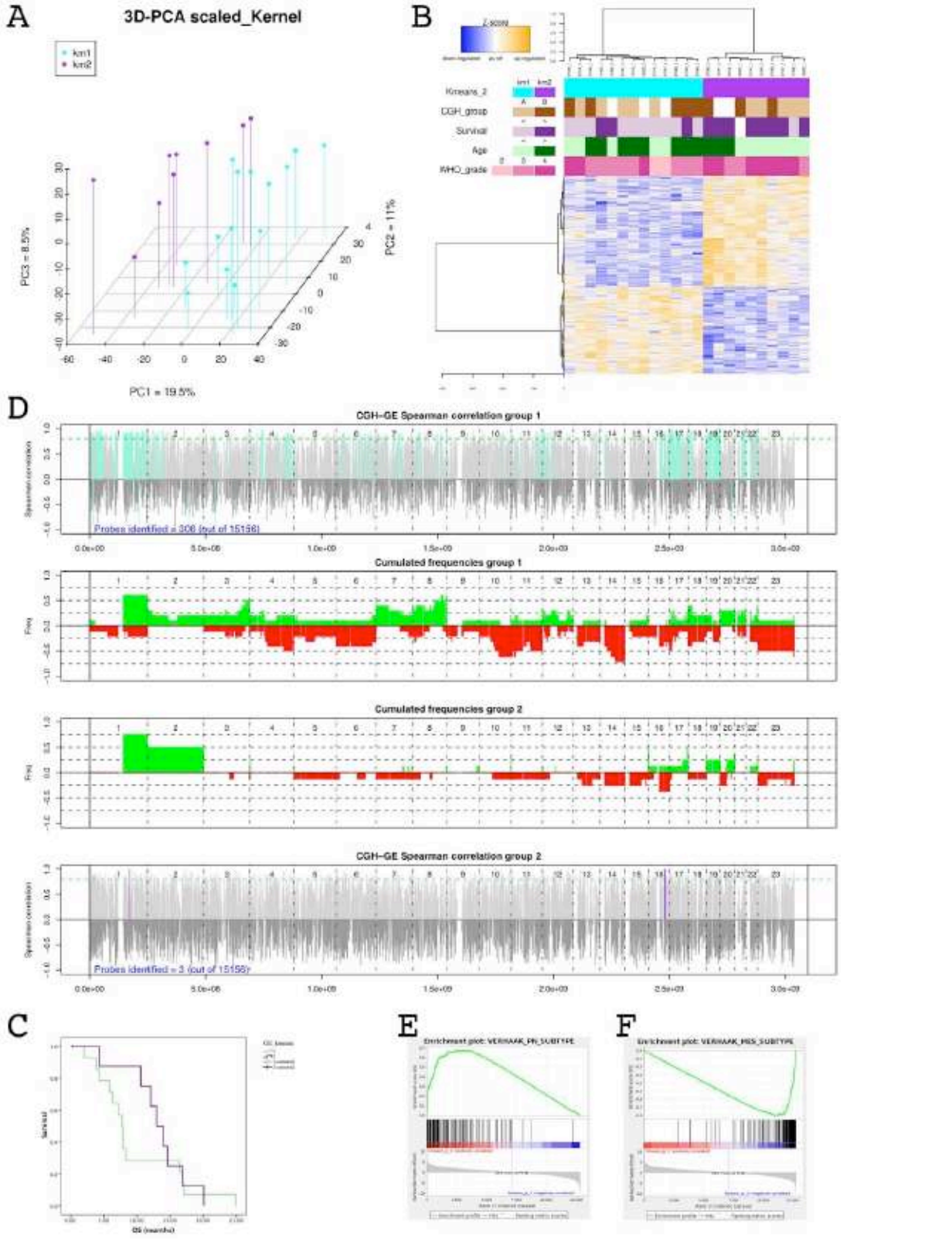
A
3D-PCA scaled_Kernel
km1
km2
PC3 = 8.5%
PC2 = 11%
PC1 = 19.5%
B
Z-score
Kmeans_2
CGH_group
Survival
Age
WHO_grade
D
CGH-GE Spearman correlation group 1
Probes identified = 306 (out of 15156)
Cumulated frequencies group 1
Cumulated frequencies group 2
CGH-GE Spearman correlation group 2
Probes identified = 3 (out of 15156)
C
Survival
OS (months)
E
Enrichment plot: VERHAAK_PN_SUBTYPE
F
Enrichment plot: VERHAAK_MES_SUBTYPE

Figure 2. DIPG are divided into two groups with different gene expression signatures. Gene expression levels of 23 DIPG were analysed using a unsupervised procedure. Panel A: K-means algorithm followed by a model selection procedure using BIC defined two separated groups of DIPG that can be also clearly seen with a PCA on all probes that passed the quality control. Panel B: Heatmap of the 643 most differentially expressed genes between the two groups of DIPG, selected using the moderated t-test of limma package of Bioconductor. Panel C: Overall survival curves of the two groups of DIPG defining a group of patients who died early (70% of cases before the median survival time of 10.6 months, light green curve) and a group of patients who died later (90% of cases after the median survival time of 10.6 months, purple curve), ($p = 0.004$, chi-square test). Panel D: Integrated genomic analysis using DR-Integrator (R package) showing the correlation between probes of copy number and gene expression mapped on the same genomic coordinates (Refseq HG19) of a gene. In the upper panel (resp. the lower panel), colored vertical lines (cyan for group 1, purple for group 2) show probes for which copy number and expression were significantly correlated. The two panels in the middle show the CNA frequencies for the group 1 (resp. for the group 2). Most of the correlations between GE and CGH were found in group 1. Gene set enrichment analysis (GSEA) plot comparing group 1 GE profile to the signatures described for adult type gliomas. Group 1 gene expression profiles were enriched for proneural genes (Panel E) while group A gene expression profiles were enriched for mesenchymal genes (Panel F).
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gene in 20 of the DIPG irrespective of their subgroup. No mutation was detected.

This mesenchymal phenotype was coupled with a hypoxia-induced angiogenic switch. Numerous proangiogenic genes were significantly overexpressed in this subgroup of DIPG compared to the other ones, including VEGFA, VWF, PECAM1, TREM1, OSMR and PLAU (Table S3 and Figure S3B). There was a strong correlation between VEGFA and SNAI2/Slug expression (Figure 3E), and between VEGFA and YKL40 (Figure 3F) across the entire dataset, with a clear separation of the tumors in the two groups defined by the gene expression profiling. Endothelial proliferation was present in 8/9 mesenchymal group 2 tumours vs 8/14 in group 1 (89% vs 57%, p = NS, chi square test). On the extended cohort of 54 FFPE samples where endothelial proliferation could be evaluated, there was no correlation with survival, however an inverse correlation with Olig2 immunopositivity, a core biomarker of the proneural signature was noted ($p = 0.01$, chi square test). This angiogenic switch was associated with the activation of the HIF1A pathway as shown by the higher expression of HIF1A in group 2 ($p = 0.058$, Student t-test) and by the significant overexpression compared to group 1 of 5/8 of the hypoxia-related genes whose promoter is known to be highly responsive to HIF1A: ENO2, HK1, HK2, LDHA, P4HA2 (Table S3).

This mesenchymal profile was further associated with a significant overexpression of numerous stem cell markers, including BMI1, CD34, CD44, CXCR4, LIF, DKK1, VIM and RUNX2, in group 2 versus group 1 tumours (Figure S3C). Association of mesenchymal and stem cell markers was conserved in tumor cells with stem-like properties derived from three independent DIPG biopsies. These tumor stem-like cells yielded phenocopies of the original tumors in intracerebral xenografts (for complete description see [28]) and had a molecular profile as seen by qPCR similar to fetal neural stem cells with respect to stem cell markers (ie SOX2, Musashi1, Nestin and FABP7/BLBP) while overexpressing the mesenchymal markers YKL40, SNAIL1 and SNAIL2 compared to normal neural stem cells (Figure 3G). Of note, none of these tumor stem cells cultures, showed PDGFRA overexpression or amplification. The gene expression profile obtained from one of these DIPG models resembled mesenchymal subtype of DIPG as shown by unsupervised clustering using PCA (Figure S3D).

Oligodendroglial Differentiation and PDGFRA Amplification/mutation Define the Remaining Subset of DIPG

The group 1 of DIPGs as identified by gene expression profiling was characterized by the overexpression of oligodendroglial markers compared to group 2 (Figure 4A). Blinded morphological assessment revealed a significantly greater degree of oligodendroglial differentiation in these tumours compared with the mesenchymal group (Figure 4B and C). Strong expression of Olig2 by immunohistochemistry was seen in 13/13 tumors in this group vs 3/8 in group 2 tumours (p value = 0.003, chi square test with McNemar correction) (Figure 4D and E). Of note, SOX10, a known transcription factor involved in oligodendrogliogenesis [29,30], was overexpressed in this subgroup compared to the other DIPG ($\log_2$ fold change 1.51 vs 0.21, adjusted p value = 0.0018). We used an extended cohort of 55 patients with histologically confirmed DIPG to study the impact of oligodendroglial differentiation on survival. Median overall survival of tumors with histological oligodendroglial features was 7.73 months versus 12.37 months for tumors that had predominantly astrocytic features ($p = 0.045$, log rank test) (Figure 4F).

The gene expression profile of group 1 DIPG was significantly enriched for the gene set describing the signature of PDGFRA amplified gliomas described in the TCGA [24] and in children [8] (GSEA analysis: enrichment score 0.59, FDRq = 0.038, p nominal = 0.052) (Figure 5A). Although PDGFRA was overexpressed in most of the tumors compared to normal brain, this overexpression was significantly stronger in the group 1 tumours ($p = 0.0055$) (Figure 4A). This overexpression was confirmed by immunohistochemistry on an independent cohort in 9/15 cases that were screened for the target-driven exploratory study of imatinib in children with solid malignancies [20] (Figure 5B & C). Eight of nine cases with gain/amplification of PDGFRA detected by arrayCGH were found in this subgroup; these imbalances were confirmed by FISH in six samples for which the analysis was possible (Figure 5D). Simultaneous amplification of PDGFRA and MET was observed in 4 samples (Figure 5E). A similar observation of co-amplification of two RTK was observed in one patient for EGFR and PDGFRA (Figure S4). The minimal common region of the PDGFRA amplicon also contained LNX1, RPL21P44, CHIC2, GSK2, KIT and KDR. Integration of copy number with gene expression data demonstrated a high degree of correlation only for CHIC2, KIT, KDR and PDGFRA only (Figure 5F).

Sequencing the PDGFRA gene in an extended series of DIPG samples revealed no mutations in the kinase domains, known hotspots in other tumors such as gastro-intestinal stroma tumors [31]. By contrast, novel missense mutations were observed in the extracellular domains in 3/34 (8.8%) cases, and in a further two high grade gliomas established as primary xenografts (Figure 5G). One of the mutations in the IGRG82 pediatric glioma xenograft has been previously described in an adult glioblastoma (C235Y) (http://tcga-data.nci.nih.goc/docs/publications/gbm_exp/). Both mutant-positive cases for which gene expression data was available were part of the group 1 DIPG, and harboured PDGFRA gene amplification, as did the additional case in the extended series.

DIPG subclasses signatures are enriched with genes of specific neural lineage

We conducted a GSEA to compare the GE profile of the two groups of DIPG to the gene list generated from 5 neural linages

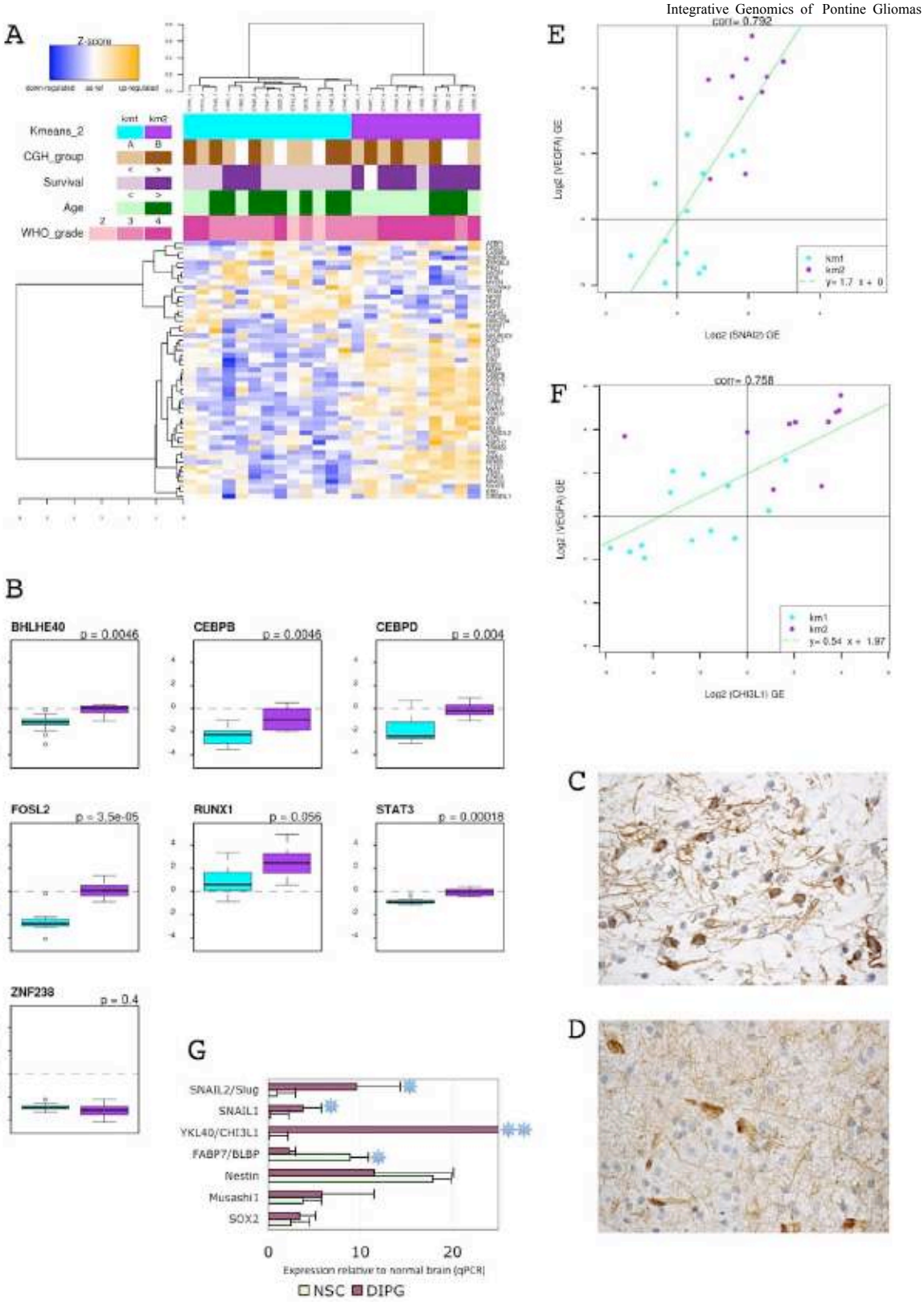
A
Z-score
down-regulated
up-regulated
km1
km2
Kmeans_2
A
B
CGH_group
Survival
Age
WHO_grade
2
3
4
B
BHLHE40
p = 0.0046
CEBPB
p = 0.0046
CEBPD
p = 0.004
FOSL2
p = 3.5e-05
RUNX1
p = 0.056
STAT3
p = 0.00018
ZNF238
p = 0.4
G
SNAIL2/Slug
SNAIL1
YKL40/CHI3L1
FABP7/BLBP
Nestin
Musashi1
SOX2
0
10
20
Expression relative to normal brain (qPCR)
NSC
DIPG
E
cor= 0.732
Log2 (VEGFA) GE
Log2 (SNAI2) GE
km1
km2
y= 1.7 x + 0
F
cor= 0.758
Log2 (VEGFA) GE
Log2 (CHI3L1) GE
km1
km2
y= 0.54 x + 1.97
C
D

Figure 3. Description of the mesenchymal type of DIPG. DIPG from group 2 gene expression profile was enriched with genes involved in mesenchymal transition, angiogenesis and stem cell maintenance. Panel A: Heatmap of the transcription factors linked with mesenchymal gene expression signature (MGES) in adult glioblastomas. Biomarkers of mesenchymal phenotype (VIM, CHI3L1 and TNC) and the two master regulators of epithelio-mesenchymal transition, SNAIL1 and SNAIL2/SLUG were added to the list provided by Carro et al (Carro et al., 2010). Panel B: Boxplots comparing the 7 transcription factors driving the MGES in adult glioblastomas (Carro et al., 2010) in the two groups of DIPG (group 1 in cyan, group 2 in purple). Relative expression in log2 ratio compared to normal brainstem control is indicated. Vimentine immunohistochemistry in tumors of group 2 shows the positivity of tumors cells (Panel C) while in group 1 only vessels and reactive astrocytes were positive (Panel D). Panel E: Spearman correlation of the expression of SNAI2 and VEGFA. Group 1 tumors (cyan dots) segregate clearly from tumors of group 2 (purple dots). Panel F: Spearman correlation of the expression of CHI3L1 and VEGFA. Group 1 tumors (cyan dots) segregate clearly from tumors of group 2 (purple dots). Panel G: Gene expression of stem cell and mesenchymal markers in DIPG tumorospheres derived from primary tumors of patients in stem cell medium as previously described (Thirant et al, 2011). Quantitative RT-PCR (qPCR) were performed using normal brain cortectomy as control. The spheroids cultured from three different DIPG were compared to normal neural stem cells (NSC) grown as neurospheres in the same medium.
doi:10.1371/journal.pone.0030313.g003

isolated from mouse brain developped by Lei et al [32] from the transcriptome database of Cahoy et al [33]. Tumors of group 1 DIPG were enriched with the gene signature of mature oligodendrocytes and to a lower extent with the one of oligodendrocyte precursor cells (OPC), ressembling in this respect to the proneural class of GBM glioblastoma (Figure 6). Conversely tumors of group 2 DIPG were enriched with the gene signatures of astrocytes and cultured astroglia (Figure 6). This later group of DIPG shared in this respect the GE signatures of the mesenchymal and classical classes of GBM that were enriched with the gene list of cultured astroglia and astrocytes, respectively.

Discussion

In this study, we report the first comprehensive genomic analysis of DIPG samples taken at diagnosis, and identify key biological features which distinguish them from other pediatric supratentorial HGG. The gene expression signatures associated with the location of a tumour was associated with differential reprogramming of embryonic signaling organizers, reflecting the discrete developmental origins of HGG presenting in different locations in the brain. Furthermore, our data indicate that DIPG arise from two distinct oncogenic pathways. The first group of DIPG exhibits an oligodendroglial phenotype associated with PDGFRA gain/amplification. Its gene expression profile is enriched for the proneural and PDGFRA-amplified glioma signatures. It comprises the most clinically aggressive tumours, independent of histological grade. The second group of DIPG exhibits a mesenchymal and pro-angiogenic phenotype orchestrated by a similar transcriptional module to that recently described in adult glioblastomas. These data greatly prolong our understanding of the molecular pathogenesis of pediatric DIPG and HGG, and have significant implications the future clinical management of children with these tumours.

DIPG Represent a Biologically Distinct Group of HGG in Children

Pediatric DIPG and supratentorial high-grade gliomas, although harboring overlapping patterns of chromosomal imbalances, could be clearly differentiated through their gene expression signatures. Among the most differentially expressed genes with respect to tumour location, we identified numerous homeobox and HLH genes that were associated with brainstem tumours, and likely represent embryonic signaling organizers that have undergone transcriptional reprogramming during oncogenesis. The concept of location driving tumorigenesis in the brain [34] has been applied to other tumor types like ependymoma [35–38] and pilocytic astrocytomas [39], where developmentally-restricted gene expression signatures could be related to the site of tumor growth. Interestingly, genes found to be overexpressed in DIPG compared to supratentorial HGG, such as LHX2 and IRX2, have been previously described to be overexpressed in posterior fossa pilocytic astrocytomas and ependymomas compared to their supratentorial counterparts [36,38,39]. The converse may also be true, with FOXG1 and ZFHX4 found to be upregulated in supratentorial HGG compared with DIPG, similar to data from ependymomas and pilocytic astrocytomas [36,38,39]. This suggests that there may be a common gene expression pattern related to the location and developmental origin of glial tumors irrespective of the histological diagnosis. Moreover, among the genes whose expression distinguished DIPG from the HGG in other location, we identified several genes involved in the SHh pathway such as PTCH1, GLIS1, GJA1, SLC1A6, KCND2, PENK, GAD1 (see Table S2) already shown to be upregulated in mouse models [40]. This is in line with data from Monje et al. who have recently shown the possible role of the Sonic Hedgehog pathway in the oncogenesis of DIPG [41].

Of particular significance was the similarity of gene expression profiles of HGG arising in the midline/thalamus with DIPG, and their distinction from hemispheric tumours, likely indicating expansion from closely-related precursor populations, in these tumours for which the cell(s) of origin are yet not known. Although the adoption of different treatment strategies for DIPG and supratentorial HGG is well-established in clinical practice, the biological resemblance of midline/thalamic tumors and DIPG raises questions regarding the management of these specific neoplasms, currently focused on strategies designed for supratentorial HGG [42].

Mesenchymal transition with a stem cell-like phenotype is the hallmark of a subset of DIPG

While a mesenchymal phenotype appears only infrequently represented in pediatric supratentorial HGG [8], almost half of the pediatric DIPG were characterized by the overexpression of biomarkers of mesenchymal transition, stemness and a hypoxia-induced angiogenic switch. The transcriptional module driving the mesenchymal gene expression signature in adult glioblastoma [25] was also specifically overexpressed in this group compared to the proneural group. The acquisition of a mesenchymal phenotype [43], stemness [44], as well as the expression of hypoxia-related genes [45,46] have been associated with resistance to treatment including radiotherapy. The enhanced self-renewing capability of this subtype of DIPG further points to a distinct development lineage from the more differentiated PDGFRA-driven DIPG. In this respect, the higher expression of STAT3 in the mesenchymal type of DIPG compared to the proneural one may play a key role in their opposite differentiation. Indeed, STAT3 elimination promote neurogenesis and inhibit astrogenesis in neural stem cell, ie the phenotype of group 1 DIPG [47]. Glioma stem cells are associated with a perivascular niche, and appear to modulate vascular proliferation via VEGF, itself regulated via the HIF pathway. These three phenomenons are closely interrelated in

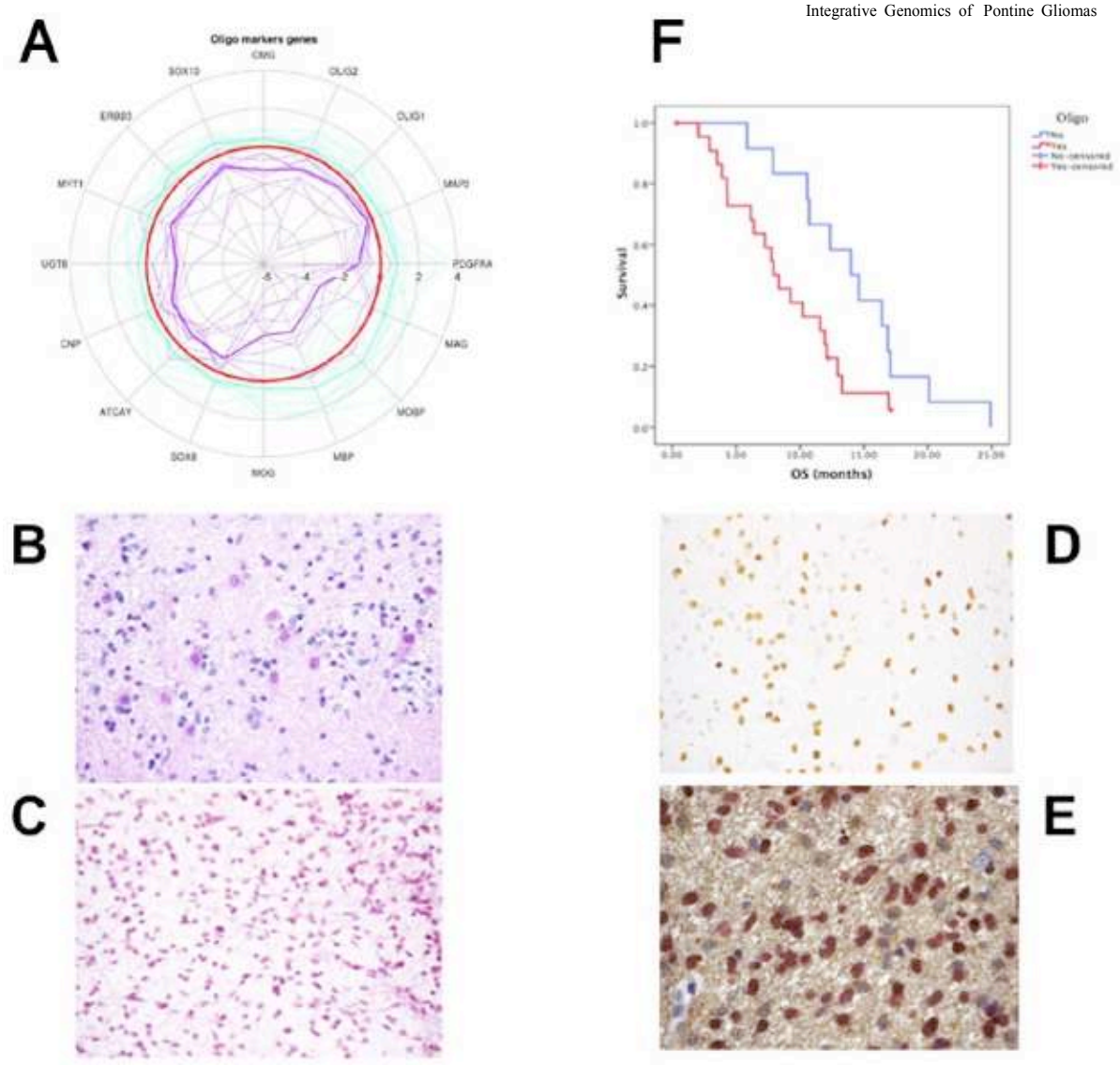

Figure 4. Description of the oligodendroglial/proneural type of DIPG. Panel A: Radial plot showing the expression of oligodendroglial markers in the two groups of DIPG, in log2 ratios related each other. The red circle represent the median expression level of the whole population of DIPG. Group 1 expresses higher levels of oligodendroglial markers than group 2 DIPG. Panel B: Morphological oligodendroglial differenciation in group 1 tumors (HES staining, 640). Panel C: Morphological astrocytic differenciation in group 2 tumors (HES staining 640). Panel D: Olig2 immunohistochemistry in a group 1 DIPG showing that probably not all cells in the biopsy are tumoral (640). Panel E: Dual immunohistochemistry for Olig2 and GFAP showing that tumor cells in mitosis are GFAP negative but Olig2 positive (6100). Panel F: Overall survival of 55 DIPG according to the presence (red) or absence (blue) of oligodendroglial differenciation. Median OS was shorter in patients with oligodendroglial type of tumors (7.73 vs 12.37, $p = 0.045$, log rank test).
doi:10.1371/journal.pone.0030313.g004

several cancers including glioblastoma [48–51], and open the possibility that agents which target angiogenesis and/or drive differentiation of tumour stem cells may find application in this subset of DIPG to increase the antitumor effects of ionizing radiation.

Despite the involvement of Ras pathway in epithelio-mesenchymal transition via SNAI2 [52] and its link with worse outcome of pediatric HGG [53], we did not find a correlation between H-RAS gain/amplification and its gene expression, nor activating mutations in the RAS genes including BRAF V600E already described in some pediatric supratentorial gliomas [27], again highlighting differential oncogenic mechanisms in DIPG compared to other pediatric HGG.

Proneural and oligodendroglial differentiation associated with PDGFRA amplification

We have identified through unsupervised gene expression clustering a group of DIPG characterized by a 'proneural'

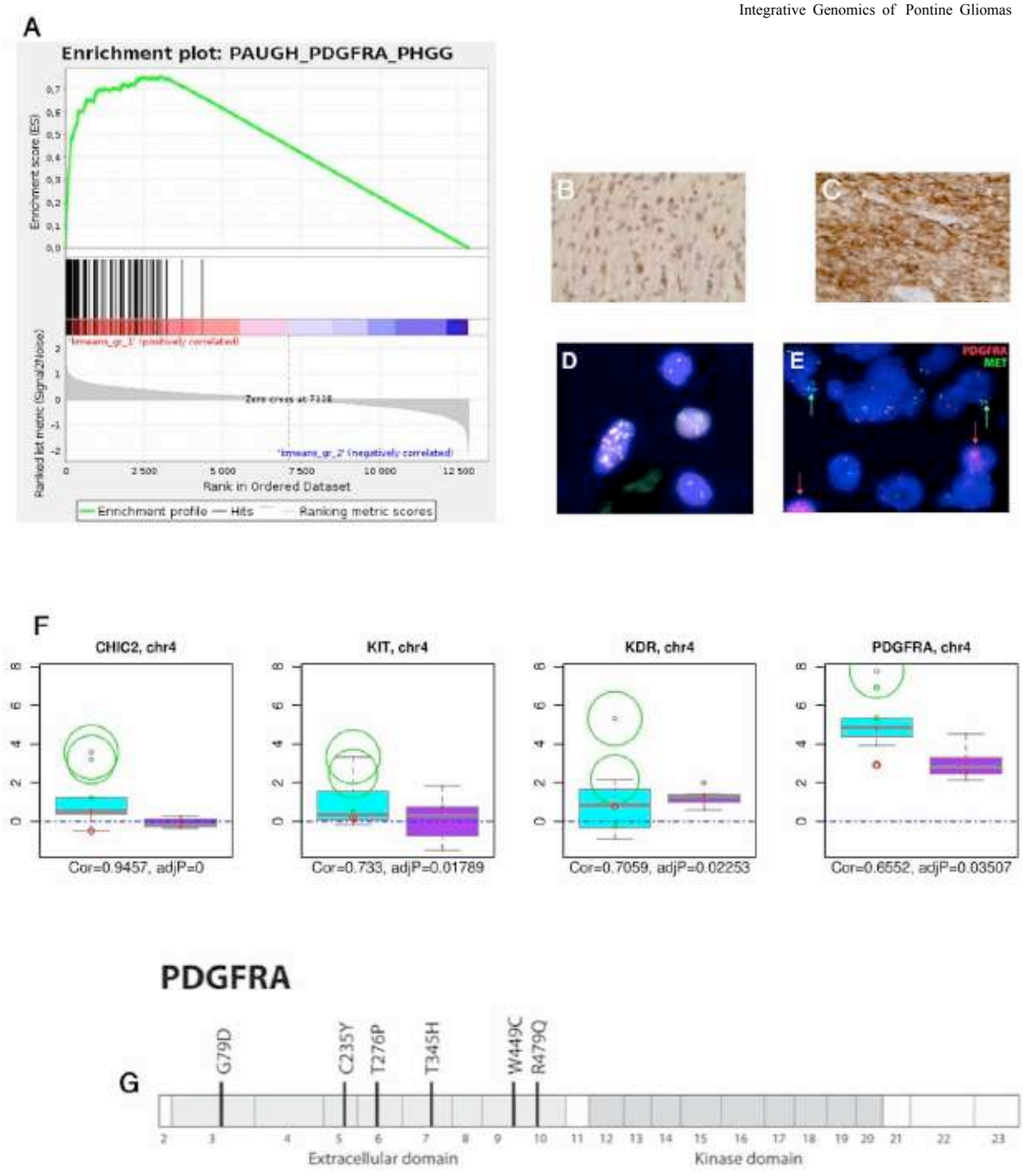

Figure 5. PDGFRA amplification/mutation is driving the oncogenesis of the oligodendroglial/proneural type of DIPG. Panel A: GSEA graph showing the enrichment of group 1 DIPG for the gene set describing the gene expression profile of PDGFRA amplified glioblastomas. Panel B: PDGFRA immunohistochemistry in the infiltrative part of a DIPG. Panel C: PDGFRA immunohistochemistry in the tumoral part of a DIPG. Panel D: FISH analysis of a DIPG using a FIP1L1/PDGFRA probe showing the amplification of the locus encompassing the two genes (most frequently seen). Panel E: Dual-FISH analysis of a DIPG with two probes one for PDGFRA and one for MET showing that the two oncogenes may be gained/amplified in different cells within the tumor. Panel F: Integrative genomic analysis using DR-Integrator (R package). Seven genes are present in the minimal common region (MCR) gained on chromosomal location 4q12 in DIPG. Boxplots represent the distribution of GE data and circles represent CNA data. The circles are centered on the corresponding GE measure on the distribution and their radii are proportional to the absolute value of CNA, red ones being losses and green ones gains. CNA and GE were highly correlated for four of these seven genes (CHIC2, KIT, KDR, PDGFRA). Panel G: Diagram of the PDGFRA gene showing the mutations discovered in DIPG samples and xenografts.

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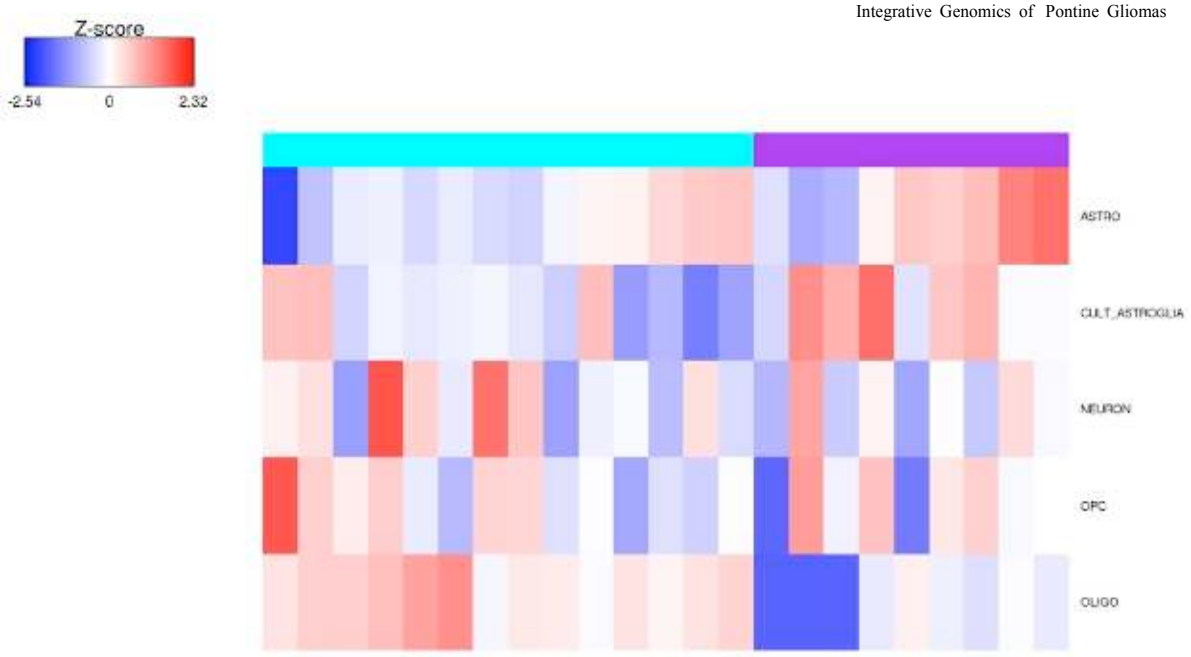

Figure 6. Comparison of gene expression signature of the two DIPG groups with specific neural lineages. A GSEA analysis was processed using the gene list previously described by Lei et al [32] and derived from the gene sets specifically enriched in astrocytes, oligodendrocytes, neurons, oligodendrocytes progenitors cells and cultured astroglial cells. Heatmap of the enrichment scores of each DIPG sample is represented with a red to blue color scale shows the range from the highest to lowest enrichment score.
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phenotype, an oligodendroglial differentiation, and PDGFRA amplifications/mutations. Moreover, the gene expression profile of group 1 DIPG was significantly enriched with genes describing the signature of PDGFRA amplified gliomas [8,24] supporting the hypothesis that PDGFRA amplification is associated with a robust gene expression profile across tumor location and patient's age. This association has been previously described in adult tumors [43,54–56], and include the expression of genes involved in neurogenesis and oligodendrocyte development, such as Olig transcription factors, Nkx2.2, PDGFRA and SOX10 [57]. DIPG with oligodendroglial phenotype and Olig2 overexpression exhibited an even worse evolution and resistance to radiation than the other DIPG in our series. This could be explained by the recent findings that the central nervous system-restricted transcription factor Olig2 opposes p53 response to genotoxic damage in neural progenitors and malignant glioma [58]. This is however in contrast with the adult gliomas where oligodendroglial differentiation and proneural phenotype are linked with a better prognosis [24]. Moreover, we did not observe IDH1/2 mutation in 10 DIPG [59] while in adult proneural gliomas IDH1 mutations are frequent [24]. In pediatric gliomas, IDH1/2 mutations are almost exclusively seen in adolescents [59,60] who indeed do not represent the target population of DIPG. The presence of IDH1 mutation in tumors from adolescents was not correlated with an oligodendroglial phenotype in our cohort of pHGG previously published [59]. Together with the fact that the group 1 DIPG is enriched preferentially with the signature of mature oligodendrocytes rather than oligodendrocyte progenitor cells, these data could suggest that this group of DIPG could be developed from a different oligodendroglial cell than their adult counterpart. This would be in line with the rarity of 1p19q co-deletion in pediatric gliomas with oligodendroglial features.

Integrative genomics showed that the gene expression of this group of DIPG was driven by copy number changes on the contrary to the other DIPG suggesting that chromosomal instability plays an important role in the phenotype of these tumors. Conversely, gene expression in the other group of DIPG may be more driven by epigenetic changes.

We found 28% (9/32) of PDGFRA gains or amplifications, all but one being included in the group 1 defined by unsupervised gene expression clustering. The PDGF autocrine/paracrine loop has been frequently implicated in oligodendrogliomas [61] and has been used to create preclinical models of glioma [62,63], including brainstem tumors [64,65]. PDGFRA amplification has been shown to be more frequent in pediatric HGG than in adult ones [8] and a recent report found PDGFRA gain or amplification in four out of eleven post-mortem samples of DIPG [10]. In one of our previous study, PDGFRA protein was also more frequently detected by IHC in DIPG than in other pediatric HGG [20].

We identified 10% of pediatric DIPG to harbor PDGFRA missense mutations, considerably more frequently than the 2/206 (1%) reported in adult GBM (http://tcga-data.nci.nih.goc/docs/publications/gbm_exp/). These mutations were located in exons coding for the extracellular domains of the protein, potentially disrupting ligand interaction, but not in the tyrosine-kinase domain. Their oncogenic role can be suspected, especially as they are found exclusively in concert with gene amplification. Similarly, mutations have been found in the ectodomain but not in the tyrosine-kinase domain of EGFR gene in adult GBM [66]; these mutations were shown to be oncogenic. Moreover, similar to EGFRvIII mutants, deletions in the extracellular domain of PDGFRA have been already reported in as many as 40% of glioblastomas with PDGFRA amplification and were associated with increased tyrosine-kinase activity [67]. Unfortunately, the

assay used for PDGFRA sequencing did not allow us to exclude the possibility of in frame deletions and this would need further analysis on new samples.

Translational implications of targeting genomic alterations in DIPG

Lack of insight into disease mechanisms impeded the development of effective therapies in DIPG for years, with the selection of therapeutic agents to be used in conjunction with irradiation determined empirically or based on their efficacy in adult high-grade gliomas. Changing the paradigm of the treatment of this disease requires a better understanding of the key biological events driving this type of neoplasm. Our clinical and biological program allowed us to discover new potential therapeutic targets previously overlooked or ignored. For the first time, rationale design of trials with targeted therapies could be implemented in the armentarium against these aggressive neoplasms. PDGFRA indeed seems to be the most exciting target given also the existence of several inhibitors with a known toxicity profile in children, including patients with DIPG at relapse [20] or at diagnosis after irradiation [68]. Despite significant drug concentrations reached inside the glioblastoma [69], imatinib has shown limited efficacy in recurrent or newly diagnosed glioblastoma in adults [70] and response to the drug was not increased in patients with PDGFRA immunopositivity [71]. No information on the histology of the brainstem tumors was available in the Pediatric Brain Tumor Consortium (PBTC) phase II trial of imatinib [68] where most of the patients with brainstem gliomas received indeed the drug after the completion of their radiotherapy schedule. In a recent study of the 'Innovative Therapies in Children with Cancer' consortium, where imatinib was only given to patients with proven PDGFRA, PDGFRB or KIT over expression determined by immunochemistry [20], one child with recurrent DIPG harboring PDGFRA expression in 50% of the cells in the biopsy showed a sustained objective response (minus 31% for tumor size) for a period of ten months. Identifying the key predictive markers for efficacy of targeted agents will be a vital step in translating genomic data to the clinic, particularly where specific activating mutations are identified. The literature [70,71] indicates however that the effect of imatinib as single agent is limited and that combination with other agents such as irradiation should be considered [72,73]. In addition, insufficient drug penetration in the brain and in some part of the tumor may explain these disappointing results. Enhanced delivery would then need either blood to brain barrier opening [74] or P-gp and ABCG2 inhibition [75]. In this respect the DIPG orthotopic models newly described [63,64] will be valuable tools to study the appropriate way to deliver these drugs in addition to help our understanding of the disease. Combinatorial targeted approaches may also be valid given the observation of multiple oncogenic alterations activating the same downstream signaling cascades [76]. Our finding of simultaneous amplification of PDGFRA and MET in a subset of DIPG, for example, may justify the use of multikinase inhibitors or combinations of TKI, as has been demonstrated for pediatric glioblastoma cells in vitro [77].

Our integrated genetic profiling of diagnostic DIPG has identified two biologically and clinically distinct groups of DIPG, with clear differences from hemispheric HGG, and with likely differential treatment strategies warranted. These data highlight the importance of biologically driven guidance for novel therapeutic intervention in these currently untreatable tumors, and argue for the systematic biopsy of these lesions in order to facilitate this, in addition suggesting that some supratentorial deep-seated infiltrating HGG of the deep grey nuclei may deserve a similar approach.

Materials and Methods

Tumor and Nucleic Acids extraction

Tumor samples and clinical information were collected with written informed consent (see Supporting Information S1) of the parents/guardians before inclusion into protocols approved by the Internal Review Board of the Necker Sick Children's Hospital in Paris and the Gustave Roussy Cancer Institute in Villejuif [corresponding to two phase I/II trials, see references 20 and 21]. Only patient with classical diagnostic features of DIPG were included: 1) short clinical history of less than three months, 2) infiltrating neoplasm centered on the pons and involving at least 50% of the anatomical structure, 3) histology excluding a pilocytic astrocytoma or ganglioglioma.

Tumor biopsies were snap frozen in liquid nitrogen in the operating room to ensure preservation of high quality RNA, ground to powder and then RNA and DNA were extracted following two different protocols according to their respective efficiency: Rneasy Micro Kit (Qiagen) and/or TRIzol reagent (Invitrogen).

Microarray Analyses

DNA and RNA microarray hybridizations were carried out by the Functional Genomics Platform of the Integrated Research Cancer Institute in Villejuif (http://www.igr.fr/en/page/integrated-biology_1529) using the Agilent 44 K Whole Human Genome Array G4410B and G4112F, respectively (http://www.agilent.com). The microarray data related to this paper are MIAME compliant and the raw data have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-TABM-1107.

Bioinformactic Analyses

Raw copy number ratio data were transferred to the CGH Analytics v3.4.40 software for further analysis with the ADM-2 algorithm (http://www.agilent.com). A low-level copy number gain was defined as a log 2(ratio) .0.3 and a copy number loss was defined as a log 2(ratio) ,20.3. A high-level gain or amplification was defined as a log 2(ratio) .1.5. Minimum common regions (MCR) were defined as chromosome regions that show maximal overlapping aberrations across multiple samples with the STAC v1.2 software [78]. Probe-level measurement MCRs do not include all genes that are altered within a given aberrant region in a particular tumor but define the recurrent abnormalities that span the region.

Raw gene expression data using normal brainstem as reference were transferred into R software for statistical analysis. In order to discover groups in GE data set, the k-means algorithm from R software has been run for two to five groups on the entire dataset. Then for each clustering the BIC value was calculated, according to Guillemot et al [22], in order to determine the best one, which was the one with two groups. GSEA analysis [23] was performed with the pre-ranked tool on gene list ranked by increasing FDR adjusted p-values, for each contrast of interest, with default parameter values. A nominal False Discovery Rate (FDR) of ,0.25 was considered statistically significant for GSEA. We ran GSEA analysis with t-test option as metric parameter.

For integrative genomics analysis, we used the DR-Integrator package for R [79].

Fluorescent In Situ Hybridization

FISH was performed from formalin-fixed-paraffin-embedded (FFPE) tumor samples or frozen tumor touch slides for the

xenografts. The FIP1L1/PDGFRA (Q-biogen/MP Medicals) and LSI EGFR (Vysis/Abbot) were used according to the manufacturer's instructions. PDGFRA and MET probes were labelled from BAC-clones RP11-58C6 and RP11-819D11 (PDGFRA) and RP11-165C4 and RP11-951I21 (MET) using the Bioprime kit (Invitrogen) and DIG-6-dUTP (Roche). Slides were pre-treated in 0.2 M HCl, 8% sodium thiocyanate and 0.025% pepsin. Probes were hybridised overnight at 37C. Slides were washed and incubated with conjugates streptavidin-Cy3 (Invitrogen) and anti-DIG-FITC (Roche).

Mutation screening of selected genes

For direct sequencing, the exon 15 of BRAF and all the individual exons of PDGFRA were PCR amplified using Taq DNA polymerase (Invitrogen) and primers that can be provided upon request. PCR products were sequenced with BigDye v3.1 and run on an AB3730 genetic analyser (Applied Biosystems). Traces were analysed using Mutation Surveyor software (Softgenetics). The effect of the mutations on the protein structure was predicted using Polyphen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/) databases.

Histology and Immunohistochemistry on Primary Tumor Material

Tumor histology was reviewed by PV. Tumors were classified and graded according to the 2007 WHO classification. Representative formalin-zinc (formol 5%; Zinc 3 g/L; sodium chloride 8 g/L) fixed sections were deparaffinized and subjected to a Ventana autostainer (BenchMark XT, Ventana Medical system, Tucson, USA) with a standard pretraitement protocol included CC1 buffer for MIB (KI-67) and P53. A semi-automatised system using a microwave antigen retrieval (MicroMED T/T Mega; Hacker Instruments & Industries, Inc., Winnsboro, SC) for 30 minutes at 98uC (manufacturer recommendations) and the RTU Vectastain Universal detection system (Vector laboratories, Burligame, CA, USA) for Olig2. Sections were then incubated with various commercial monoclonal primary antibodies against Olig2 (AF 2418, 1/150, R/D system, CA, USA), P53 (DO-1, 1/1, Ventana) and MIB-1 (1/100; Dako, Glostrup, Denmark). Diaminobenzidine was used as the chromogen. A minimal threshold at 10% of the total stained tumor cells served as a cut-off for defining the p53-positive status. A MIB-1 labeling index (MIB-1 LI) was obtained by counting the number of MIB-1-positive tumor cells in regions with the maximum number of labeled tumor cells. Ten microscopic high-power field sets were counted, and the MIB-1 LI was computed as a percentage of immunopositive cells from the total cells counted in selected fields. Light microscopic images were digitally captured using a Nikon eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with Nikon DXM 1200 Digital camera. Photomicrographs were assembled for illustrations using the Adobe Photoshop version 7.0.1 software (Adobe, San Jose, California, USA).

Supporting Information

Figure S1 DIPG are different from supratentorial high-grade gliomas in children. Panel A: example of a biopsy sampling in a patient with DIPG. A maximum of 8 core biopsy samples can be obtained per patient. Panel B: heatmap of the unsupervised hierarchical clustering of 29 DIPG. From the 32 available samples, two had a completely flat profile and one was of unsufficient quality. The analaysis was then run on 29 samples. Gains are represented in green (the intensity being correlated to the log2ratio) and amplifications as blue dots. Losses are represented in red (the intensity being correlated to the log2ratio). The lower panel indicate the general profile of genomic imbalances encountered in the 32 samples, y axis scale being the frequency of the aberrations. The colored right panel shows the profile of each individual sample and the black & white right panel shows the percentage of the genome with imbalances. C: overall survival of the patients with CGHarray data according to the loss or the persistence of the TP53 locus. Overall survival was significantly lower in patients with TP53 gene loss ($p = 0.01$, log-rank test). D: principal component analysis (PCA) of pediatric high-grade gliomas (HGG) CGHarray data irrespective of their location. Hemispheric HGG are indicated in yellow, midline HGG are indicated in grey and brainstem HGG or DIPG are indicated in pink. All the probes passing the quality control were used for the analysis. E: box-plots comparing the expression of some of the key regulators of brainstem embryogenesis in DIPG (pink) and supratentorial HGG (yellow). The adjusted p-value of the comparison is given in the upper left corner of each panel. All values are given relative to the expression found in normal adult brainstem.
(TIFF)

Figure S2 DIPG comprises two biological subgroups with distinct survival and pathological characteristics. A: Identification of the most optimal Bayesian Information Criterion (BIC) value. The most optimal BIC value was obtained using the class prediction algorithm of Guillemot et al. (BIOTECHNO'08). The graphs show that the accuracy of class prediction did not improve with increasing number of groups. B: Integrative analysis of genomic and gene expression data. When considering all DIPG samples from whom both GE and CGHarray data were available, the expression of 1460 genes (ie 6% of the genome) was significantly correlated with copy numbers. The cheese-plots of the 20 genes with the highest correlation are provided. Complete data set is available upon request.
(TIFF)

Figure S3 Mesenchymal transition and a pro-angiogenic switch define a subset of DIPG. A: The master epithelial to mesenchymal transition regulators, SNAI1 and SNAI2/Slug are upregulated in a subset of DIPG. The box-plots of the two DIPG subgroups identified are shown in purple and brown respectively. Gene expression are given compared to normal adult brainstem. The p-value is indicated for each gene in the upper right corner of the panel. B: Angiogenic markers are overexpressed in a subgroup of DIPG. The two different subgroups of DIPG are represented in purple and light green. The p-value is indicated for each gene in the upper right corner of the panel. Gene expression are given compared to normal adult brainstem. C: Stem cell markers are overexpressed in a subgroup of DIPG. The two different subgroups of DIPG are represented in purple and cyan. The p-value is indicated for each gene in the upper left corner of the panel. Gene expression are given compared to normal adult brainstem. D: Gene expression profiling of one of the DIPG stem cell cultures. Principal component analysis of one of the DIPG stem cell cultures together with all the primary DIPG samples.
(TIFF)

Figure S4 Amplification of multiple RTK in the same tumor. Example of a DIPG sample for which simultaneous amplification of PDGFRA and EGFR could be observed by FISH.
(TIFF)

Table S1
(XLSX)

Table S2
(XLS)

Table S3
(XLS)

Supporting Information S1
(PDF)

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Author Contributions

Conceived and designed the experiments: JG BG CJ VL PD CP. Performed the experiments: SP CR LG-R FA PV DC RR DB M-PJ. Analyzed the data: GLT BJCP PV SP. Contributed reagents/materials/analysis tools: CS-R TR SP BG. Wrote the paper: JG SP CP CJ BG.

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Conclusion

Paul Valery used to say, "What is too simple is wrong and what is too complicated is not understandable". In the complex world of cancer biology (culminating in the notion of personalized medicine), we need to have a method to simplify matters. Pathology has been extremely useful to delineate coherent ensembles to direct diagnosis and adapt treatment. It has shown obvious limitations at the end of the last century when traditional diagnostic approach became insufficient to determine different behaviours within one same entity. This is especially true when targeted treatment is considered, even if the real benefit from such approach is still limited for most patients. High-throughput technologies have initially brought the illusion that they could replace accurately pathology. However, these genomic signatures are usually far too complex to be reproducible and the efficacy of the attempts for simplification to a limited set of genes (e.g. ONCOTYPE Dx.) has yet to be proven. Furthermore, the quantification and standardization of “genomic alterations” is not as straightforward as it might seem, and often one comes across findings of unknown significance.

This evolution of cancer medicine prompted pathologists:

- to refine their classifications with tumor class biomarkers,
- to detect relevant drug targets and surrogates of their efficacy with the simplest procedure
- to delineate complex biological process (e.g. epithelial-to-mesenchymal transition, neuronal differentiation…) representing the context within treatment and in which evolution of disease would take place.

In the present work, our aim was to discover and develop histopathological biomarkers for the classification, prognostication and management of pediatric ependymoma and HGG. The definition of a biomarker for us is therefore larger than usual since it is considered as a general simplified indicator of tumor biology.

A translational approach, integrating observation from our group’s experience in diagnostic pathology and molecular studies from our team and from the literature has allowed us to establish and develop biomarkers that, besides bringing new information concerning the biological mechanisms in these diseases, can be useful for predicting their prognosis. In pediatric ependymomas this is the case of neuronal markers (NEFL), which seems to indicate a better prognosis for supratentorial lesions and TNC, which is strongly associated with a worse outcome. The establishment of a multi-institutional collaboration program was fundamental for the validation and the success of the cross-validation of these biomarkers. Indeed, large cohorts of homogenously treated patients are needed to define and validate prognostic biomarkers.

On the other hand, a limited number of samples could be sufficient to discover new therapeutic targets or predictive biomarkers of efficacy of a given treatment. The finding of mutations in *PI3KCA*, constituting the first identification of oncogene mutations reported in DIPG is not only interesting as a scientific novelty, but also further highlights the heterogeneity of such lesions, largely unknown from a pathological and molecular point of view until very recently. The integrated approach was also fundamental for the analysis of the putative predictive markers for the response to targeted therapies in HGG in the setting of a clinical trial with the anti-EGFR agent erlotinib. Information drawn from this study, such as the frequent loss of PTEN in DIPG and the confirmation of the biological singularity of the subgroups expressing EGFR or displaying oligodendroglial differentiation with respect to prognosis in this setting has helped our group to establish the design of the next Phase II protocol for this disease, within the framework of the National Program of Clinical Research. Indeed, in this coming study, patients with DIPG will be allocated to the treatment arm corresponding:

- either to the activation of the EGFR pathway,
- either to the activation of the PDGFRA pathway,
- or to the activation of the mTOR pathway by PTEN loss or PI3KCA mutation. Molecular pathology (immunohistochemistry and FISH) will therefore have a major role in treatment guidance.

In addition, molecular pathology is the simplest and cheapest way to approach tumor heterogeneity (see for example the paper of Puget et al, in the appendix 1.2 of the present thesis, where FISH could prove that *PDGFRA* and *MET* gains were not present in the same tumor cells). Finally, molecular pathology can also help us to appreciate the role of microenvironnement in the evolution of the tumors (as shown with TNC in some gliomas, but not in our study of ependymomas). Tumors cells are able to modify the neighboring cells (eg Tp53 abnormalities in the stroma of breast cancer cells (Patocs et al., 2007) in one hand, and neighboring cells being able to favor tumor development (e.g. NF1 haploinsufficient mastocytes in neurofibroma (Yang et al., 2008) or PTEN dosages and breast cancer susceptibility in transgenic mice (Alimonti et al., 2010).

Molecular Pathology has a central role in the analysis of cancers, and from a pathologist's perspective, as it takes into account the enormous heterogeneity among and within different patients and their tissues. Molecular Pathology seems to us at the cornerstone of clinical medicine and biology of disease, a formidable doorway towards personalized medicine for the next decades. It is rewarding to have the opportunity to integrate these new data, which should be incorporated in our daily practice.

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POSTER:

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Abstract

Biomarkers for the classification, clinical management and prognosis of pediatric brain tumors (ependymoma and high grade glioma, (HGG)) are lacking. To address this, biomarkers were developed and explored in view of classification, prognostication, target identification and prediction of the efficacy of treatment for patients with such tumors.

We show that overexpression of neuronal markers distinguishes supratentorial from infratentorial ependymoma, and among the former higher immunoexpression of neurofilament 70 (NEFL) is correlated with better progression free survival (PFS). Tenascin-C (TNC) is significantly overexpressed in infratentorial ependymoma. A multi-institutional European ependymoma collaboration group was established and analyses were performed in a pediatric cohort of 250 patients, where immunohistochemistry (IHC) for TNC showed to be a robust marker of poor overall survival (OS) and PFS, particularly among children under 3 years, this being further validated in an independent cohort. Techniques and scoring performed in different laboratories were highly reproducible. IHC for NEFL and TNC could be used for prognostication of pediatric ependymoma.

The analysis of putative predictive markers for the response to targeted therapies in pediatric HGG in the setting of a clinical trial with the anti-EGFR agent erlotinib was performed by IHC and fluorescent in situ hybridization. The frequent loss of PTEN in diffuse intrinsic pontine glioma (DIPG) and the confirmation of the biological singularity of the certain subgroups (expressing EGFR, displaying oligodendroglial differentiation) which seem to be associated with better response to erlotinib have helped our group to establish the design of the next Phase III protocol for this disease at our institution. We report mutations in *PI3KCA* constituting the first identification of oncogene mutations in some DIPG, which further highlight their biological heterogeneity. Further studies are needed to define the interaction between *PTEN* loss, EGFR overexpression, oligodendroglial differentiation, *PI3KCA* mutations and other recent findings such as *PDGFRA/MET* gains/amplification and *TP53* mutations in these heterogeneous lesions and their relationship to the outcome of patients under new targeted therapies for this largely fatal disease.

This thesis has allowed us to explore the molecular pathology in the context of biology and clinical setting of pediatric brain tumors.

Key-words: ependymoma, child glioma, diffuse intrinsic pontine glioma, tenascin c, biomarker

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Frais de reprographie pris en charge par la taxe d'apprentissage collectée par l 'IGR