Cancer bronchique primitif, voies de signalisation intra-cellulaires et modèles précliniques

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Résumé

Contexte. Le cancer bronchopulmonaire (CBP) demeure la première cause de mortalité par cancer dans le monde. Malgré l'espoir suscité par le développement des thérapies ciblées, son pronostic demeure sombre, particulièrement dans les cas de CBP à petites cellules (CBP-PC) et de CBP non à petites cellules (CBP-NPC) présentant une activation de l'oncogène *KRAS*.

Matériel et Méthodes. Nous avons mené 3 études successives, visant à (i) radiosensibiliser des modèles de CBP-PC par l'ajout d'un inhibiteur de BCL2, (ii) cibler des modèles de CBP-NPC mutés *KRAS* par l'association d'un inhibiteur de mTOR et d'un inhibiteur de RAF, et (iii) créer un modèle préclinique orthotopique murin de CBP reproduisant la progression tumorale observée en clinique.

Résultats. Dans la première étude, l'inhibiteur de BCL2 oblimersen a présenté un effet radiosensibilisant sur des modèles de CBP-PC, *in vitro* et *in vivo*. Dans la seconde étude, l'association de l'inhibiteur de mTOR everolimus et de l'inhibiteur de RAF/VEGFR RAF265 a présenté un effet synergique sur des lignées cellulaires de cancers présentant la double mutation de *KRAS* et de *PIK3CA*, *in vitro* et *in vivo*. Dans la troisième étude, l'injection orthotopique d'une lignée bioluminescente de CBP-NPC chez des souris *nude* a permis d'établir des tumeurs intra pulmonaires évoluant vers une extension métastatique ganglionnaire et hématogène, et de détecter la présence de cellules tumorales circulantes.

Conclusion. L'association d'un inhibiteur de BCL2 à la radiothérapie est une stratégie intéressante dans le CBP-PC, l'association d'un inhibiteur de mTOR et d'un inhibiteur de RAF/VEGFR est une stratégie intéressante dans le CBP-NPC présentant une double mutation *KRAS-PIK3CA*, mais ces données doivent être confirmées sur des modèles orthotopiques afin de gagner en pertinence avant d'envisager un transfert en clinique.

Sommaire

1 Introduction 9

1.1 Cancer bronchique primitif (CBP) 9

1.2 Cancer bronchopulmonaire à petites cellules (CBP-PC) 10

1.2.1 Généralités 10

1.2.2 Chimiorésistance 11

1.2.3 Radiorésistance 13

1.2.4 But de l'étude n°1 « Radiosensibilisation de lignées de CBP-PC par l'oblimersen » 14

1.3 Cancer broncho pulmonaire non à petites cellules (CBP-NPC) 15

1.3.1 Généralités 15

1.3.2 KRAS 17

1.3.3 Chimiorésistance 19

1.3.4 Stratégies thérapeutiques 20

1.3.5 But de l'étude n°2 « Effet de l'association RAF 265 – RAD001 sur des lignées de CBP-NPC » 22

1.4 Modèles animaux de CBP 23

1.4.1 Généralités 23

1.4.2 Modèles existants 24

1.4.3 But de l'étude n°3 « Mise au point d'un modèle animal orthotopique bioluminescent » 25

2 Matériels et méthodes, Résultats 26

2.1 Etude n°1 « Radiosensibilisation de lignées de CBP-PC par l'oblimersen » 26

2.2 Etude n°2 « Effet de l'association RAF 265–RAD001 sur des lignées de CBP-NPC» 37

2.3 Etude n°3 « Mise au point d'un modèle animal orthotopique bioluminescent » 49

3 Discussion 59

3.1 Mutations oncogéniques de *KRAS* 59

3.2 Traitements pharmacologiques ciblés des tumeurs mutées KRAS 61

3.2.1 Pan-inhibition des récepteurs HER 61

3.2.2 Inhibition de HSP90 62
3.2.3 Inhibition de AKT 64
3.2.4 Association d'inhibiteurs de tyrosine kinase 66
3.3 Association d'inhibiteurs des voies PI3K-AKT-mTOR et RAF-MEK-ERK 68
3.3.1 Résultats précliniques 68
3.3.2 Résultats cliniques 70
3.4 Doubles mutations des voies PI3K-AKT-mTOR et RAF-MEK-ERK 73
3.4.1 Dans les tumeurs solides en général 73
3.4.2 Dans les CBP-NPC en particulier 74
3.5 Situations potentiellement favorables aux doubles mutations des voies PI3K-AKT-mTOR et RAF-MEK-ERK. 77
3.5.1 Variation selon les populations 77
3.5.2 Hétérogénéité tumorale 78
3.5.3 Discordance mutationnelle entre primitif et métastases 80
3.5.4 Maladie résiduelle 81
3.6 Modèles animaux 85
3.6.1 Modèles actuels 85
3.6.2 Modèle d'injection orthotopique transpleurale 88
3.7 Perspectives 91
4 Conclusion 92
5 Références 94
5. Annexes 104
5.1 Annexe 1: Bases moléculaires de l'oncogenèse bronchique 104
5.2 Annexe 2: Mutation de RAS et efficacité de la chimiothérapie 117
5.3 Annexe 3: Oligonucléotide anti Bcl-2 125
5.4 Annexe 4: Discordance mutationnelle entre primitif et métastases 128
5.5 Annexe 5: Maladie résiduelle en oncologie 131

Abréviations

Cancer bronchopulmonaire à petites cellules (CBP-PC)

Cancer bronchopulmonaire non à petites cellules (CBP-NPC)

Epidermal Growth Factor (EGFR)

Anaplasic Lymphoma Kinase (ALK)

Echinoderm Microtubule Associated protein Like 4 (EML4)

Phosphatidylinositol 3'-kinase (PI3K)

Mammalian target of rapamycin (mTOR)

Extracellular signal related kinase (ERK)

Mitogen-activated protein kinase kinase (MEK)

Homologue de l'oncogène du virus du sarcome de Harvey du rat (*H-RAS)*

Homologue de l'oncogène du sarcome de Kirsten du rat (*K-RAS)*

Homologue de l'oncogène du neuroblastome humain (*N-RAS)*

Multi-drug resistance-1-encoded P-glycoprotein (Pgp)

Metallothionein (MT)

Nucleotide excision repair (NER)

Mismatch repair (MMR)

Xeroderma Pigmentosum Complementation group D (XPD)

Excision Repair Cross Complementation 1 (ERCC1)

Time to progression (TTP)

Intervalle de confiance à 95% (IC95%)

RAS association domain family 1A (*RASSF1A)*

p70 ribosomal S6 kinase 1 (S6K1)

Eukaryotic initiation factor 4E (eIF-4E)

eIF-4E binding protein 1 (4EBP1)

FK506 binding protein 12 (FKBP12)

Surveillance Epidemiology and End Results (SEER)

Cancer stem cells (CSCs)

Circulating tumor cells (CTCs)

Disseminated tumor cells (DTCs)

Leucémie aigue myéloblastique (LAM)

ATP-binding cassette (ABC)

Aldehyde dehydrogenase (ALDH)

Multi Drug Resistance (MDR)

Hypoxia-inducible factors (HIFs)

Myelocytomatosis viral oncogene homolog (MYC)

Isolation by Size of Epithelial Tumor cells (ISET)

Food and Drug Administration (FDA)

Polymerase Chain Reaction (PCR)

Reverse Transcriptase (RT)

Hybridation In Situ par Fluorescence (FISH)

Response Evaluation Criteria in Solid Tumors (RECIST)

Bone morphogenic protein 4 (BMP4)

Sous cutanée (SC)

Percutaneous orthotopic injection (POI)

Surgical orthotopic implantation (SOI)

Transpleural orthotopic injection (TOI)

American Type Culture Collection (ATCC)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Heat Shock Protein 90 (HSP90)

Figures

Figure 1. Pourcentage de mutations de différents oncogènes dans le CBP-NPC, selon le type histologique.

Figure 2: Modèle de carcinogenèse multi étapes des CBP-NPC.

Figure 3 : Voies de signalisation RAS-RAF-MEK-ERK et PI3K-AKT-mTOR.

Figure 4 (Etude 1 Figure 1) : incorporation de l'oblimersen dans la lignée H69.

Figure 5 (Etude 1 Figure 2) : effet de l'oblimersen sur l'expression de BCL2.

Figure 6 (Etude 1 Figure 3) : effet de l'oblimersen sur l'apoptose des cellules H69.

Figure 7 (Etude 1 Figure 4) : effet radiosensibilisant de l'oblimersen *in vivo*.

Figure 8 (Etude 1 Figure 5) : effet radiosensibilisant de l'oblimersen sur les tumeurs H69.

Figure 9 (Etude 1 Figure 6) : effet de l'irradiation sur l'incorporation de l'oblimersen.

Figure 10 (Etude 2 Figure 1) : effet des inhibiteurs RAD001 et RAF265 sur les voies de signalisation intra cellulaires *in vitro*.

Figure 11 (Etude 2 Figure 2) : efficacité des inhibiteurs RAD001 et RAF265 *in vitro*.

Figure 12 (Etude 2 Figure 3) : efficacité de l'association RAD001-RAF265 *in vivo*.

Figure 13 (Etude 2 Figure 4) : effet de l'association RAD001-RAF265 sur les voies de signalisation intra cellulaires.

Figure 14 (Etude 2 Figure 5) : étude de confirmation sur la lignée H460.

Figure 15 (Etude 3 Figure 1) : déroulement de l'étude sur les modèles animaux.

Figure 16 (Etude 3 Figure 2) : suivi des animaux en bioluminescence.

Figure 17 (Etude 3 Figure 3) : examen anatomopathologique des tumeurs.

Figure 18 (Etude 3 Figure 4) : recherche de cellules tumorales circulantes (CTCs).

Figure 19. Cascade de transmission du signal dépendant d'AKT.

Tableaux

Tableau 1 : exemple d'anomalies moléculaires impliquées dans l'oncogenèse des CBP.

Tableau 2 (Etude 2 Tableau 1) : statut mutationnel des lignées A549, H460, HCT116, HT29, et MDAMB231.

Tableau 3 (Etude 3 Tableau 1) : mortalité opératoire et devenir des différents modèles.

Tableau 4 (Etude 3 Tableau 2) : examen anatomopathologie des différents modèles et recherche de cellules tumorales circulantes (CTCs).

Tableau 5. Comparaison des doubles mutations dans les carcinomes colo rectaux et bronchiques.

1 Introduction

1.1 Cancer bronchique primitif (CBP)

Classé en cancer bronchopulmonaire à petites cellules (CBP-PC) et cancer bronchopulmonaire non à petites cellules (CBP-NPC), le cancer bronchique primitif (CBP) reste la première cause de mortalité par cancer dans le monde. Malgré l'importance de la recherche clinique et le volume de son traitement médiatique, aucune révolution n'a eu lieu dans le traitement du CBP au cours des 20 dernières années.

Ainsi, une étude exhaustive des registres néerlandais a récemment montré qu'entre 1989 et 2008, la survie des patients atteints de CBP-PC ne s'est améliorée que pour les patients agés de 45 à 59 ans, et une survie supérieure à 5 ans reste exceptionnelle (Janssen-Heijnen *et al*, 2012). Dans le même temps, la survie des patients atteints de CBP-NPC ne s'est améliorée que pour les femmes, avec une survie à 5 ans estimée à 17% (van der Drift *et al*, 2012). La même modestie d'amélioration est retrouvée dans les registres américains, quoique l'annonce en fut plus triomphante (Morgensztern *et al*, 2009).

Actuellement, le taux de mortalité annuel par cancer bronchique reste proche de son taux d'incidence. Quelque soient les voies thérapeutiques explorées plus loin, il paraît donc indispensable de rappeler l'importance de la prévention du cancer bronchique, prévention primaire par augmentation du prix du tabac (Hill, 2012), et prévention secondaire par inclusion des patients à risque dans un programme de dépistage (National Lung Screening Trial Research Team, 2011).

1.2 Cancer bronchopulmonaire à petites cellules (CBP-PC)

1.2.1 Généralités

Le cancer bronchopulmonaire à petites cellules (CBP-PC) représente 15% des cancers bronchiques primtifs, et se caractérise par une morphologie cellulaire typique, une prolifération rapide, et une dissémination métastatique précoce. Au niveau moléculaire, le CBP-PC exprime des marqueurs neuro endocrines, tels que la neuron-specific eldolase (NSE), la chromogranine, la synaptophysine, et occasionnellement la sérotonine. Les CBP-PC sont également souvent positifs pour les cytokératines (CK 8 et 18). On retrouve le plus souvent une perte d'allèle au bras court du chromosome 3, fréquemment une expression du récepteur c-KIT et de son ligand Stem Cell Factor (SCF), et un rôle clé du récepteur à l'Insulin Growth Factor (IGFR) de type 1 (Govindan *et al*, 2006).

Sur les voies de signalisation de l'apoptose, une délétion de gènes pro-apoptotiques et une amplification de gènes anti-apoptotiques, dont l'amplification de *BCL2L1* et de *BCL2L2*, sont souvent rapportés dans le CBP-PC. Une augmentation de l'expression de BCL2 a été rapportée dans près de 90% des CBP-PC (Kim *et al*, 2006).

Tableau 1. Exemple d'anomalies moléculaires impliquées dans l'oncogenèse des CBP (Loriot *et al*, 2009, Annexe 1).

Anomalies moléculaires	Rôle	CBNPC	CBPC
EGFR	Prolifération	+	–
HER-2	Prolifération	+	–
RAS	Prolifération	+	–
PI3K/AKt	Prolifération/apoptose	+	+
c-myc	Prolifération	+	–
IGF	Prolifération/apoptose	+	+
Bcl-2	Apoptose	+	+
p53	Apoptose	+	+
Cycline D1/E	Cycle cellulaire	+	–
Rb	Cycle cellulaire	+	+
p16	Cycle cellulaire	+	+
p21	Cycle cellulaire	+	–
Télomérase	Immortalisation	+	+
VEGFR	Angiogenèse	+	+

Le traitement de référence des patients en bon état général atteints de CBP-PC de stade limité est une association d'irradiation thoracique et de chimiothérapie par sel de platine et étoposide. Ce protocole conduit initialement à des taux de réponse complète compris entre 50 et 80%. Cependant, si les patients atteints de CBP-PC sont initialement chimio et radio sensibles, ils acquièrent une chimio et radio résistance dans les 12 premiers mois de leur traitement, conduisant à une mortalité importante et à une survie à 5 ans anecdotique (Spira *et al*, 2004).

1.2.2 Chimiorésistance

La chimiothérapie des CBP-PC est basée sur l'association d'un sel de platine et d'une seconde molécule, qui est le plus souvent l'étoposide, mais peut également être le docetaxel, la gemcitabine, le pemetrexed, ou la vinorelbine. Les doublets à base de sels de platine sont

associés au développement rapide d'une chimiorésistance, qui peut être médiée par (i) l'inhibition de l'entrée de la drogue dans la cellule ; (ii) l'inactivation de la drogue par liaison avec les metallothionines ou le système glutathione, associée à un export du composé cisplatine-glutathione par la protéine multi drug resistance 2 (MDR2) ; ou (iii) l'accélération de la réparation de l'ADN ou la création de sauts réplicatifs évitant les adduits cisplatine-ADN (Wang *et al*, 2005).

Au niveau cellulaire, les sels de platine induisent au sein de la molécule d'ADN la création de ponts moléculaires inter- ou intra-brin puis de cassures double brins. Ces volumineux adduits contenant de l'ADN sont repérés par les kinases ADN dépendantes phosphoinositide-3 kinase (PI3K), ataxia telangiectasia mutated (ATM), et ataxia telangiectasia and Rad3-related (ATR). Le signal intra-cellulaire est ensuite transmis aux cascades PI3K/AKT, protein 53 (p53), et mitogen-activated protein kinases (MAPKs), dont extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), et p38α/β/γ MAPKs. En aval de p38 MAPK ou de JNK, la forme phosphorylée de p53 active les caspases et induit la mort cellulaire par apoptose. D'une façon plus générale, p53 contrôle les checkpoints G2/M et G1 du cycle cellulaire, son activation induit un arrêt de croissance permettant l'activation de protéines de réparation de l'ADN. Si l'altération de l'ADN n'est pas réparable, la mort cellulaire programmée est également initiée par p53 (Olszewski *et al*, 2012). Troisième possibilité, la survie cellulaire malgré une aberration chromosomique non ou mal réparée peut conduire à la transformation cellulaire.

Les mécanismes d'apoptose jouent donc un rôle primordial dans la mort cellulaire induite par les sels de platine. De plus, *in vitro*, la surexpression de BCL2 et l'inhibition de BAX sont corrélées à une résistance des lignées cellulaires de CBP-PC à la chimiothérapie. Ces données

suggèrent que l'utilisation d'agents anti-apoptotiques pourrait accroitre ou péreniser la chimio sensibilité des cellules de CBP-PC (Sartorius *et al*, 2002).

1.2.3 Radiorésistance

Les mécanismes apoptotiques ont également un rôle majeur dans l'efficacité de la radiothérapie. La sur-expression de BCL2 apparaît comme un facteur de radiorésistance dans plusieurs types tumoraux. En effet, l'inhibition de l'apoptose radio-induite par BCL2 a été démontrée dans plusieurs modèles de tumeurs *in vitro* et *in vivo* (Sirzén *et al*, 1998).

De nombreuses études ont montré que la résistance des CBP-PC aux traitements conventionnels par chimio et/ou radiothérapie était liée à la diminution de la réponse apoptotique constatée dans un sous groupe de cellules tumorales. La délétion de gènes pro apoptotiques et l'amplification de gènes anti-apoptotiques, dont *BCL2L1* et *BCL2L2*, sont souvent observées (Kim *et al*, 2006). Au niveau protéique, une augmentation de l'expression de BCL2 a été rapportée dans 73 à 90% des CBP-PC. La sur-expression de BCL2, la sous expression de BAX, et un ratio BCL2/BAX supérieur à 1 sont corrélés à une diminution de l'activité apoptotique, à une chimio résistance et à une radio résistance (Brambilla *et al*, 1996 ; Sartorius *et al*, 2002).

De plus, la diminution de l'expression de BCL2 par l'oblimersen, un oligonucléotide phosphorothioate ciblant BCL2, a permis d'améliorer la radiosensibilité de plusieurs tumeurs sur-exprimant cette protéine, en particulier de la prostate ou du nasopharynx, *in vitro* et *in vivo*.

1.2.4 But de l'étude n°1 « Radiosensibilisation de lignées de CBP-PC par l'oblimersen »

Compte-tenu de la sur-expression de la protéine BCL2 dans plus de 60% des CBP-PC et de sa corrélation avec un mauvais pronostic, nous avons émis l'hypothèse que la diminution de l'expression de BCL2 par l'oblimersen pourrait être associée à une augmentation significative de la radiosensibilité de lignées de CBP-PC *in vitro* et *in vivo*. Dans cette première étude, nous avons donc étudié le composé oblimersen, un nouvel inhibiteur de BCL2, comme radiosensibilisant dans le traitement de lignées cellulaires de CBP-PC.

1.3 Cancer broncho pulmonaire non à petites cellules (CBP-NPC)

1.3.1 Généralités

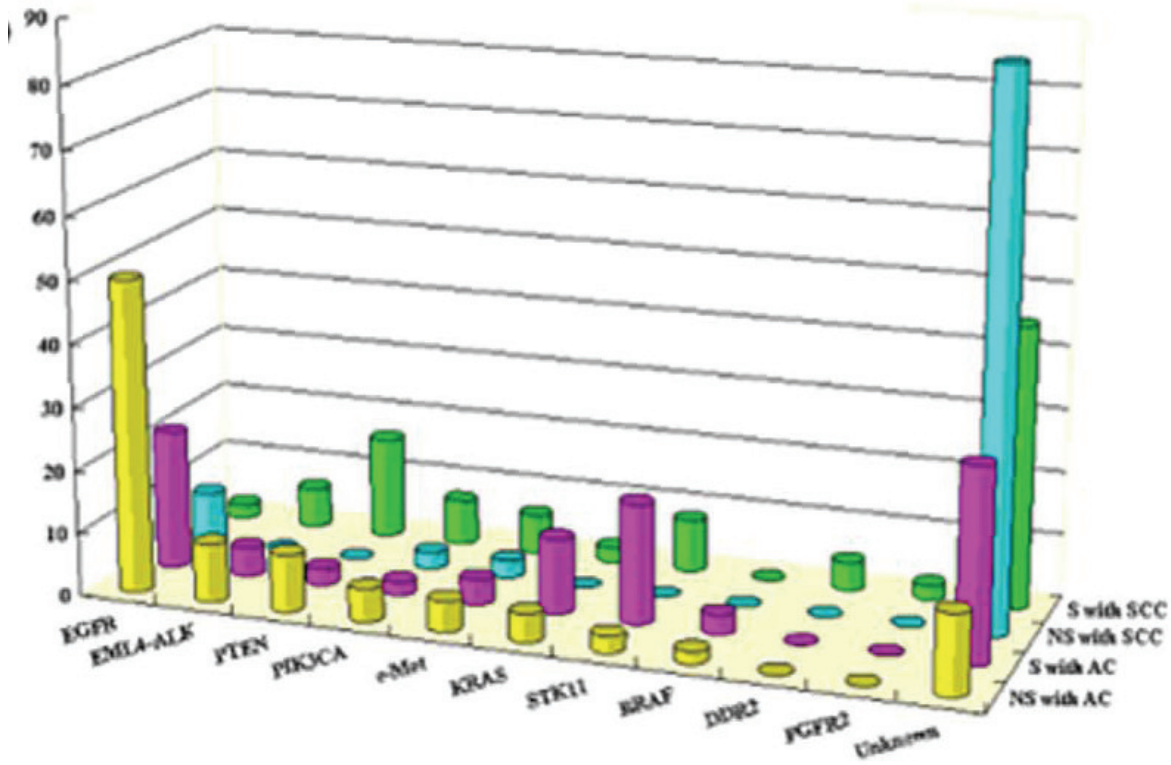
Le CBP-NPC représente 85% des cancers bronchiques. Son traitement est basé, selon son extension, sur un traitement local chirurgical ou par radiothérapie, éventuellement associé à un traitement systémique par chimiothérapie. Depuis la fin des années 1970, les sels de platine constituent la base des protocoles de chimiothérapie dans le traitement du CBP-NPC. Durant les années 1990, ces protocoles ont été complétés par l'arrivée de nouvelles drogues cytotoxiques : vinorelbine, gemcitabine, taxanes … (Giaccone, 1995).

Plus récemment, les thérapies ciblées ont soulevé des espoirs importants, laissant penser que leur capacité à modifier les mécanismes spécifiques de la survie cellulaire pourrait conduire à des guérisons cliniques. Loin de la solidité du rationnel biologique et de l'importance des espoirs précliniques, les résultats cliniques de ces nouveaux traitements sont globalement décevants, car les thérapies ciblées n'ont apporté qu'un gain de survie marginal si l'on considère la population de patients atteints de CBP-NPC dans son ensemble (Shepherd *et al*, 2005 ; Thatcher *et al*, 2005, Herbst *et al*, 2005, Reck *et al*, 2010).

Après ces premiers essais cliniques de thérapies ciblées, l'analyse rétrospective des prélèvements tumoraux a permis de définir des sous groupes de patients qui répondent de façon importante et prolongée aux traitements ciblés, et la prise en charge de ces patients spécifiques a été profondément modifié par les nouveaux traitements (Soria *et al*, 2011). Ainsi, parmi les patients atteints de CBP-NPC traités par erlotinib, la présence d'une mutation

de l'Epidermal Growth Factor Receptor (EGFR) augmente la réponse au traitement (Tsao *et al*, 2005 ; Eberhard *et al*, 2005) mais ne concerne que 16% des patients (Rosell *et al*, 2009). Plus récemment, l'inhibition de l'Anaplasic Lymphoma Kinase (ALK) a permis d'obtenir des réponses tumorales importantes ou des stabilisations de la maladie chez la plupart des patients atteints de CBP-NPC présentant un gène de fusion Echinoderm Microtubule Associated protein Like 4 (*EML4*) – *ALK* (Kwak *et al*, 2010). Cette nouvelle cible ne concerne que 2 à 7% des patients, mais la rapidité du développement de la nouvelle molécule, de l'identification de la cible à l'enregistrement du médicament, en fait un véritable changement de paradigme dans la recherche clinique en cancérologie (Scagliotti *et al*, 2012).

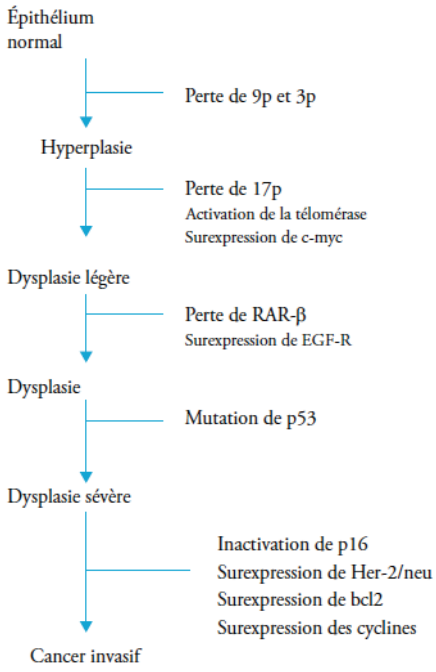
Figure 1. Pourcentage des différentes mutations oncogéniques dans le CBP-NPC, selon le type histologique (adénocarcinome AC ou carcinome épidermoïde SCC) et le tabagisme (fumeurs S ou non fumeurs NS), à Honk-Kong (An *et al*, 2012).



1.3.2 KRAS

Les facteurs de croissance jouent un rôle primordial dans la croissance cellulaire et la prolifération via des récepteurs membranaires spécifiques et leurs voies de signalisation intra cellulaires adjacentes. Les voies de signalisation PI3K (phosphatidylinositol 3'-kinase) – AKT – mTOR (mammalian target of rapamycin) et RAS – RAF – MEK (Mitogen-activated protein kinase kinase) – ERK (extracellular signal related kinase) sont deux voies de signalisation intra-cellulaires importantes. Ces voies de signalisation régulent des facteurs de transcription et d'autres protéines impliquées dans la prolifération, la survie, la mobilité et la différenciation. Les principales protéines des voies RAS et AKT sont dérégulées par des mécanismes génétiques et épigénétiques dans de nombreux cancers humains (Legrier *et al*, 2007), et constituent la base du modèle de carcinogenèse multi étape dans le CBP-NCP (Figure 2)

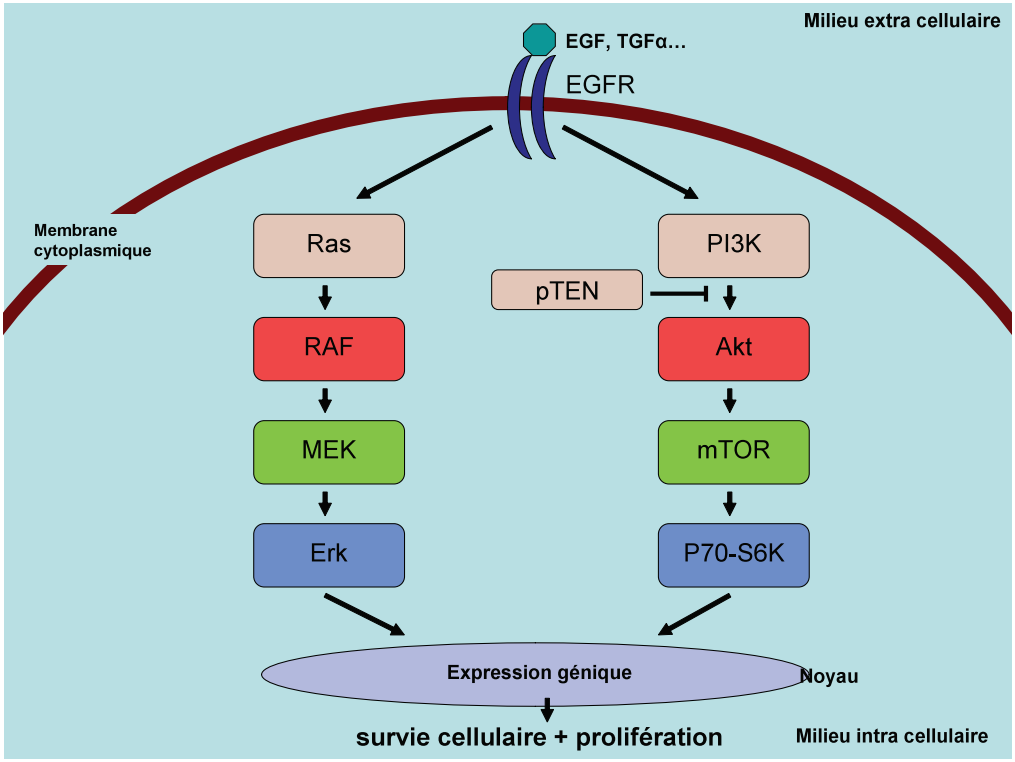
Figure 2. Modèle de carcinogenèse multi étapes des CBP-NPC (Loriot *et al*, 2009, Annexe 1).



RAS est une des principales protéines des voies de signalisation intra cellulaires. RAS peut activer les kinases RAF, ERK 1 et 2, et un grand nombre de protéines nucléaires pour promouvoir la prolifération cellulaire. Trois gènes *RAS* ont été identifiés chez l'homme: *HRAS* (homologue de l'oncogène du virus du sarcome de Harvey du rat), *KRAS* (homologue de l'oncogène du sarcome de Kirsten du rat) et *NRAS* (initialement isolé à partir d'un neuroblastome humain) (Schubbert *et al*, 2007).

Les gènes de la famille *RAS*, et particulièrement *KRAS*, ont été impliqués dans la pathogenèse et le pronostic de nombreuses tumeurs. Dans ces tumeurs, une mutation ponctuelle conduisant à la perte de l'activité GTPase conduit à une activation constante de la protéine, un signal de prolifération constant, et une action transformante de la protéine (Figure 2). Des mutations de *KRAS* sont retrouvées dans 30% des cancers humains, particulièrement dans les localisations pancréatiques, colorectales, endométriales, utérines cervicales, pulmonaires, et des voies biliaires (Schubbert *et al,* 2007).

Figure 2. Voies de signalisation RAS-RAF-MEK-ERK et PI3K-AKT-mTOR



Une activation oncogénique des gènes *RAS* est retrouvée dans 15 à 30% des adénocarcinomes pulmonaires (Rodenhuis *et al*, 1988). Les patients présentant une tumeur mutée *KRAS* ont plus de chance d'être fumeurs sevrés ou actifs, et d'avoir une maladie localement avancée (Marks *et al*, 2008). Malgré des études parfois contradictoires, les mutations de *RAS* seraient associées à un mauvais pronostic dans une méta-analyse (Mascaux *et al*, 2005). Enfin, les mutations de *KRAS* et d'*EGFR* sont fonctionnellement redondantes et mutuellement exclusives (Eberhard *et al*, 2005).

Actuellement, l'activation oncogénique de *KRAS* est donc à la fois la mutation la plus fréquemment retrouvée dans les CBP-NPC, et un facteur de mauvais pronostic, constituant un défi thérapeutique majeur.

1.3.3 Chimiorésistance

La plupart des patients atteints de cancer bronchopulmonaire sont traités avec des protocoles de chimiothérapie standard, adjuvante à la chirurgie en cas de maladie localisée, associée à la radiothérapie en cas de maladie avancée localement ou sur le plan ganglionnaire, et exclusive en cas de maladie métastatique. Avant de nous intéresser à la réponse aux thérapies ciblées et aux nouvelles stratégies thérapeutiques possibles, nous avons donc mené une revue de la littérature pour savoir si la présence d'une mutation *KRAS* était prédictive de la réponse des CBP-NPC à la chimiothérapie standard (Annexe 2). L'ensemble de ces études ne retrouve pas de rôle prédictif du statut *KRAS* sur la réponse à la chimiothérapie. Des études complémentaires, notamment prospectives et de puissance adaptée, doivent être menées. Dans l'intervalle, le statut *KRAS* est intéressant pour guider le choix d'une thérapie ciblée seule, ou d'une association de thérapies ciblées.

1.3.4 Stratégies thérapeutiques

L'activation oncogénique de *KRAS* joue en elle même un rôle essentiel dans la survie tumorale, et constitue donc une cible intéressante pour de nouveaux traitements anticancéreux. De nombreuses stratégies pharmacologiques ont été utilisées pour inhiber l'activation oncogénique de la protéine KRAS, dont l'inhibition de ses contacts avec la membrane plasmatique (inhibiteurs de la prenylation et de la post-prenylation), l'inhibition des signaux d'aval (inhibiteur de kinase), l'inhibition des signaux d'amont (inhibiteur de kinase et anticorps monoclonal), et l'inhibition de l'expression protéique de RAS et d'autres composants de la voie de signalisation (siRNA et oligonucleotide antisense) (Saxena *et al*, 2008).

Beaucoup de ces stratégies ont eu des résultats décevants. Les inhibiteurs de la farnesyl transferase ont montré à la fois une efficacité limitée dans les essais cliniques et des problèmes de toxicité (Blum *et al*, 2005), et sont actuellement développés dans le cadre d'associations avec des agents immunologiques (Caraglia *et al*, 2007), hormonaux (Liu *et al*, 2007 ; Martin *et al*, 2007 ; Li *et al*, 2009), cytotoxiques (Chow *et al*, 2008, Taylor *et al*, 2008) ou ciblés (Copland *et al*, 2008 ; Hong *et al*, 2008).

L'inhibition d'amont de KRAS est basée sur l'inhibition de l'EGFR, qui a montré son absence d'efficacité en monothérapie chez les patients atteints de cancers mutés *KRAS*, qu'il s'agisse de carcinomes colorectaux (Lievre *et al*, 2008 ; Di Fiore *et al*, 2007 ; Karapetis *et al*, 2008, Amado *et al*, 2008) ou pulmonaires (Zhu CQ *et al*, 2008 ; Miller *et al*, 2008). Les thérapies ciblant l'EGFR sont actuellement testées en association avec d'autres traitements afin de dépasser la résistance liée à la mutation *KRAS* (Johnson *et al*, 2007).

L'utilisation thérapeutique potentielle d'oligonucléotides antisense et siRNA ont également été largement étudiée. Malgré des résultats encourageants dans les études précliniques et cliniques, ces molécules ne sont pas encore utilisées en pratique courante. Parmi les obstacles restant à franchir, on retrouve des problèmes d'efficacité, d'effet de voisinage (off-target), de pharmacocinétique et d'effets secondaires (Rayburn *et al*, 2008 ; Loriot *et al*, 2009, Annexe3).

Enfin, l'inhibition pharmacologique des voies de signalisation d'aval de KRAS à l'aide d'inhibiteurs de kinase reste prometteuse. Parmi les molécules situées en aval de KRAS, mTOR est une serine-threonine kinase conservée à travers les espèces, qui détecte les signaux de stress (privation de nutriments et d'énergie, stress oxidant et hypoxique, signaux de prolifération et de survie) via la voie de signalisation PI3K-AKT. Le signal effecteur de mTOR passe par la phosphorylation de ses substrats p70 ribosomal S6 kinase 1 (S6K1) et eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4EBP1). La phosphorylation de 4EBP1 libère eIF-4E, permettant l'initiation de la traduction protéique dépendant de la coiffe. En effet, chez les eucaryotes comme dans la cellule cancéreuse, la traduction des ARNm en séquences d'acides aminés est initiée par un mécanisme dépendant de la coiffe (cap) des ARNm. Les facteurs d'initiation dont eIF-4E recrutent la sous unité ribosomale 40S et la maintiennent en contact avec la coiffe protéique positionnée sur l'extrémité 5' des ARNm, permettant l'initiation de la traduction (Mukherjee *et al*, 2012). La voie de signalisation de mTOR est dérégulée dans de nombreux cancers humains, faisant de mTOR une cible intéressante pour de nouveaux traitements anti cancéreux (Faivre *et al*, 2006). L'everolimus (RAD001) est un inhibiteur de mTOR administré par voie orale, se fixant avec une haute affinité au récepteur intra cellulaire FK506 binding protein 12 (FKBP12), et réalisant un complexe qui interagit avec mTOR pour inhiber les signaux d'aval de mTOR (O'Donnell *et al*, 2008).

Les voies de signalisation RAS et AKT se croisent en de nombreux points, rendant ces réseaux redondants (Legrier *et al*, 2007), avec des rétrocontrôles positifs et négatifs complexes, et des bifurcations et croisements à plusieurs niveaux (Dougherty *et al*, 2005 ; Zimmermann *et al*, 1999 ; Roux *et al*, 2004 ; Ma *et al*, 2005). Parmi ces intersections, la phosphorylation de RAF par AKT modifie la réponse cellulaire de lignées tumorale mammaires humaines vers la prolifération (Zimmermann *et al*, 1999).

1.3.5 But de l'étude n°2 « Effet de l'association RAF 265 – RAD001 sur des lignées de CBP-NPC »

Ces observations constituent la base moléculaire de dialogues entre les voies de signalisation PI3K - AKT - mTOR et RAS - RAF - MEK - ERK au niveau de RAF et d'AKT, suggérant que la double inhibition de RAF et de mTOR pourrait être un moyen efficace de lutter contre la plasticité de ces voies de signalisation.

Afin de tester cette hypothèse, nous avons étudié l'association d'un nouvel inhibiteur de RAF et de VEGFR, le RAF265, et d'un dérivé de la rapamycine, l'everolimus ou RAD001, sur des lignées cellulaires présentants différents status mutationnels pour *KRAS*, *PI3K* et *RAF*.

1.4 Modèles animaux de CBP

1.4.1 Généralités

Certains échecs d'essais cliniques proviennent d'erreurs dans la sélection des nouvelles drogues, à cause d'une mauvaise définition de l'activité de la molécule, d'un manque d'intégration des données moléculaires des tumeurs répondeuses *vs* non répondeuses, et/ou d'une inadéquation des modèles précliniques utilisés, tant du point de vue de la progression tumorale, que de l'évaluation de la maladie résiduelle après traitement. Dans le cas des thérapies ciblées, la discordance entre la quantité de données précliniques (plus de 10.000 références sur la base de données pubmed) et le faible rendement de leur transfert en clinique (un taux de réponse médian de 10% sur les 143 essais de phase 2 publiés entre 2000 et 2009) impose une remise en cause des modèles précliniques actuels (Janku *et al*, 2012).

Il apparait désormais primordial que ces modèles aient de meilleures valeurs prédictives positive et négative, afin d'éviter le transfert en clinique de thérapies inutiles et/ou dangereuses. Cela permettrait de limiter le nombre de nouvelles drogues à tester en clinique, et de n'envisager des essais cliniques que pour les couples tumeur – drogue qui resteraient des candidats à haut potentiel (Sausville *et al*, 2006).

Le modèle préclinique idéal devrait reproduire l'histoire naturelle du cancer humain, afin d'améliorer notre compréhension de la cancérogenèse, prédire l'efficacité des traitements en cours d'investigation, et identifier quel traitement va bénéficier à quel patient avant même de concevoir les essais cliniques.

1.4.2 Modèles existants

Dans ce contexte, les modèles de xénogreffes restent la pierre angulaire de l'investigation préclinique et bénéficieront certainement d'améliorations techniques, tout comme la conception médicamenteuse assistée par ordinateur, la modélisation informatique, et les animaux génétiquement modifiés (Dolgin, 2010).

Les xénogreffes humaines de CBP-NPC peuvent être implantées à l'animal immunodéficient en position sous cutanée (SC) ou en position orthotopique. Les xénogreffes SC sont faciles à réaliser et à suivre, mais manquent de pertinence quant à (i) l'histoire naturelle de la maladie tumorale à cause de l'absence d'extension lymphatique ou métastatique, et (ii) la capacité à prédire l'efficacité d'une nouvelle molécule en clinique, comme en témoigne la forte proportion d'essais cliniques négatifs (Sausville *et al*, 2006 ; Janku *et al*, 2012).

Les xénogreffes orthotopiques sont plus difficiles à réaliser, ne reproduisent pas toujours l'histoire naturelle de la maladie, mais soulèvent beaucoup d'espoirs sur leur capacité à discriminer les nouvelles molécules (Kubota, 1994). Notre compréhension récente des dialogues entre la tumeur et son environnement (Karnoub *et al*, 2007 ; Räsänen *et al*, 2010 ; Pietras *et al*, 2010) et son implication dans la résistance aux traitements, le processus métastatique, l'échappement immunitaire, et l'angiogenèse permet de penser que les modèles orthotopiques pourraient être plus informatifs que les modèles SC.

Dans le cas des tumeurs thoraciques, le défi technique est de faire pousser des cellules tumorales pulmonaires dans le thorax. Plusieurs techniques d'administration ont été rapportées, dont l'administration intra-trachéale de cellules tumorales en solution (Buckle *et*

al, 2010), l'injection percutanée de cellules tumorales en solution (Onn *et al,* 2003) ou encore l'implantation chirurgicale d'un morceau de tumeur SC provenant d'une lignée cellulaire (Wang *et al*, 1992). L'administration intra-trachéale est associée à une diffusion des cellules tumorales dans les deux poumons, ce qui interfère avec la progression tumoral et le processus métastatique, et plaide en faveur des modèles percutanés et chirurgicaux.

1.4.3 But de l'étude n°3 « Mise au point d'un modèle animal orthotopique bioluminescent »

La faisabilité et l'histoire naturelle des modèles orthotopiques percutanés et chirurgicaux n'ont jamais été comparées. Nous avons donc cherché à comparer les modèles orthotopiques existants, avec le but de reproduire la progression des CBP-NPC humains, depuis une tumeur initialement intra-parenchymateuse et localisée, vers un développement métastatique entraînant des conséquences systémiques.

Pour cela, nous avons comparé 4 modèles de xénogreffes, sous-cutanées (SC) comme contrôle, par injection percutanée orthotopique, par implantation chirurgicale orthotopique, et par injection orthotopique transpleurale. Afin de suivre l'évolution dans le temps, nous avons utilisé une lignée transfectée avec de la luciférase et effectué une imagerie bioluminescente *in vivo*.

2 Matériels et méthodes, Résultats

2.1 Etude n°1 « Radiosensibilisation de lignées de CBP-PC par l'oblimersen »

Loriot Y, Mordant P, Brown BD, Bourhis J, Soria JC, Deutsch E. Inhibition of BCL-2 in small cell lung cancer cell lines with oblimersen, an antisense BCL-2 oligodeoxynucleotide (ODN): in vitro and in vivo enhancement of radiation response. Anticancer Res 2010 ;30(10):3869-78.

ANTICANCER RESEARCH 30: 3869-3878 (2010)

Inhibition of BCL-2 in Small Cell Lung Cancer Cell Lines with Oblimersen, an Antisense BCL-2 Oligodeoxynucleotide (ODN): *In Vitro* and *In Vivo* Enhancement of Radiation Response

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Abstract. *Background: Oblimersen, an ODN targeting BCL-2 RNA, has been shown to be effective in reducing BCL-2 expression in vitro and in in vivo models engineered to overexpress BCL-2. The present study evaluated the efficacy of combining BCL-2 ODN and radiation in small-cell lung cancers (SCLC) cell lines. Materials and Methods: The in vitro effect was determined using short term (cell viability) and long term (clonogenic) assays. Apoptosis, BCL-2 expression and intratumoural uptake of the FAM-ODN with or without prior radiation treatment were also evaluated. Combination of ODN and RT was also assessed in vivo. Results: Radiation was shown to increase intracellular and intratumoural penetration of oblimersen, confirming previous results obtained in prostate cancer xenograft models. Oblimersen decreased BCL-2 protein expression in vitro and in vivo. BCL-2 ODN sensitised H69 cells to radiation in vitro and in vivo. Oblimersen increased radiation-induced apoptosis and decreased in vivo tumoural vascularisation. Conclusion: Oblimersen was shown to increase in vitro and in vivo effect of RT on SCLC cell lines. Radiation increases intracellular and intratumoural penetration of ODN. This pre-clinical study argues in favour of clinical development in localised SCLC.*

Defects in the ability to appropriately regulate apoptotic processes are among the fundamental occurrences that underlie cancer (1). BCL-2 was the first identified member of a family of apoptotic regulators which have at least one BCL-2 homology domain in common. BCL-2 family members include antiapoptotic (prosurvival) proteins (*e.g.* BCL-2, BCL-xL and MCL-1), multidomain proapoptotic proteins (*e.g.* BAX and BAK) and BH3-only proapoptotic proteins (*e.g.* BIM, BID, NOXA and PUMA) (2). Interactions between and relative ratios of proapoptotic and antiapoptotic BCL-2 family members are key determinants of cellular sensitivity to multiple cell death triggers, including many standard chemotherapeutic agents and ionising radiation (3-4). Overexpression of BCL-2 is known to increase clonogenic survival and inhibit radiation-induced apoptosis (3-4), but there is limited information on the *in vivo* efficacy of combining radiation with BCL-2 family therapeutics for cancer therapy.

Lung cancer is the leading cause of cancer death for both men and women in western countries (5). Small cell lung cancer (SCLC) accounts for 15% of all lung cancer cases and is distinguished from non-SCLC by its characteristic cellular appearance, rapid proliferation, and early dissemination to metastatic sites (6). The standard treatment strategy for patients with limited stage SCLC and good performance status is a combination of chest radiotherapy and chemotherapy using etoposide and cisplatin, which results in complete response rates of 50-80% and 12-25% 5-year survival rates (7-8). While SCLC tumours are primarily chemo- and radio-sensitive, the development of acquired chemo- and radio-resistance occurs within the first 12 months and the overall 5-year survival rate is about 10% for patients in this setting (9). Increased expression of BCL-2 has been reported in 73% to 90% of SCLC. BCL-2 overexpression, BAX down-regulation, and BCL-2 to BAX ratio >1 are correlated with low values of apoptotic index (10) and are associated with chemotherapeutic resistance in SCLC cell lines (11). Overexpression of BCL-2 can abrogate chemotherapy-induced apoptosis in lung cancer cell lines (11). Apoptosis is one the key mechanism that causes SCLC

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cells to die when given radiotherapy (12, 13). Hence, defects in apoptosis could be correlated with radioresistance in SCLC (12).

BCL-2-targeted antisense oligodeoxynucleotides are members of the therapeutic family of pro-apoptotic agents. Anti-BCL-2 oligonucleotide therapy was first introduced by Reed *et al.* (14) and has been further evaluated in the form of G3139 (oblimersen sodium, Genasense; Genta, Inc., Berkeley Heights, NJ, USA). G3139 is a phosphorothioate-modified antisense sequence complementary to the first six codons of human BCL-2 mRNA. Although clinical trials have shown that G3139 has a favourable safety profile, clinical efficacy is limited when given as a single agent (15). Oblimersen has been previously shown to increase radiation response in *in vivo* prostate cancer models engineered to overexpress BCL-2, especially by inducing apoptosis and reduction of angiogenesis (16).

This study investigated the role of phosphorothioate-modified BCL-2 oligodeoxynucleotide antisense (BCL-2 ASO) therapy (oblimersen), in combination with radiation for small-cell lung cancer. It intended to evaluate the relationship between pharmacodynamic parameters (oblimersen uptake, BCL-2 inhibition) in a naturally BCL-2 overexpressing model and the potential of oblimersen as an enhancer of radiation response.

Materials and Methods

Cells and culture conditions. Human small-cell lung cancer cell lines H69 (characterised by marked BCL-2 overexpression, and p53 mutated) were purchased from ATCC (Manassas, VA, USA). These cells were maintained in RPMI-1640 supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Gibco, Inc., France), 100 units/ml of penicillin and 100 μg/ml of streptomycin. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Antisense oligonucleotides. All oligodeoxynucleotides were twice high-performance liquid chromatography-purified phosphorothioates obtained from Genta, Inc; Berkeley Heights, U.S.A. BCL-2 antisense oligodeoxynucleotide (ODN) G3139 (oblimersen sodium) is a fully phosphorothioated 18mer ODN directed against the first six codons of the human BCL-2 mRNA translation initiation site (5'-TCTCCCAGCGTGCGCCAT-3'). G3622 is a fully phosphorothioated 18mer ODN of reversed polarity G3139 sequence (5'-TACCGCGTGCGACCCTCT-3') and was used as control ODN in all experiments. FITC-ASO oligonucleotides consisted of a FITC molecule labeled on the 5' end of an ASO oligonucleotide.

For *in vitro* experiments, lyophilized antisense oligonucleotides were diluted in PBS (PBS without Ca^{2+} and Mg^{2+} was used throughout this study). For *in vivo* experiments, injections of ODNs were given *i.p.* at a dose of 10 mg/kg in a volume of 100 μl PBS.

X-ray irradiation. For *in vitro* experiments, cells were irradiated at room temperature using a 200-kV X-ray irradiator at a dose rate of 0.85 Gy/min. For *in vivo* irradiation, radiation was given using mouse jigs designed to expose only the tumour bed to radiation at a dose rate of 1.1 Gy/min.

Antisense uptake assays. H69 cells were seeded in T-25 flasks ($5x10^5$ per flask). First, H69 cells were incubated with ODN-FAM with or without lipofectamine (Invitrogen, France) according to the manufacturer's recommendation and cellular uptake was determined 24 hours later. In a second experiment, 24 hours after cell seeding, experimental and control cells were given radiation at a dose of 6 Gy either 3 hours before, after or concomitantly with the administration of ODN-FAM. Twenty-four hours after antisense administration, the cells were examined under the Axiovert S 100 microscope (Zeiss, Gottigen, Germany) at 488 nm. Subsequently, the cells were pelleted and resuspended in PBS and analyzed using CellQuest software (Becton Dickinson, Mountain View, CA, USA) with a FACSCalibur flow cytometer (Becton Dickinson). For *in vivo* experiments, xenograft tumour-bearing mice were injected with FAM-ASO (10 mg/kg for 3 days, 100 μl, *i.p.*) with or without radiation administered at different times. Tumours were harvested 2 hours following the last injection. Tumours were imaged using a Zeiss Axiovert 200M (Zeiss) for widefield microscopy (FITC: excitation, 480 nm; beam splitter, 505 nm; emission, 535 nm; Hoechst 33342: excitation, 360 nm; beam splitter, 395 nm; emission, 460 nm).

Measurement of cell growth. Cells were seeded in 96-well plates, 24 h before transfection with various concentrations of oligonucleotides for 72 h. The number of viable cells was determined by using WST-1 assay according to the manufacturer's instructions (Roche, France). The absorbance was measured at 450 nm with a 96-well plate reader (Biorad, France). Absorbance values were normalized to the values obtained from untreated cells to determine survival rates. Each assay was performed in triplicate.

Clonogenic survival assays. To investigate the effect of G3139 cell response to radiation, a standard clonogenic assay was performed. Survival following radiation exposure was defined as the ability of the cells to maintain clonogenic capacity and form colonies. Briefly, after exposure with G3139 or G3622 at doses ranging from 50 nmol/l to 500 nmol/l for 3 days, cells were exposed to radiation at doses ranging from 2 Gy to 6 Gy using 200 kV X-rays; they were then separated by gently pipeting, counted and seeded for colony formation in 35 mm dishes at 500 to 2,000 cells/dish. Upon incubation intervals of 21 days, colonies were stained with crystal violet and manually counted. All colonies of 50 cells or more were then counted. The survival fraction (SF) was estimated according to the formula: SF=number of colonies formed/number of cells seeded/plating efficiency of the control group. Experiments were performed in triplicate.

Western blot analysis. Cells were seeded ($3x10^6$ per T-75 flask), incubated with G3139 or G3622 (500 nmol/l) and harvested at different times. Cells extracts were prepared using a lysis buffer, and protein concentration was determined using the Bio-Rad Detergent-compatible protein assay. Immunoblotting was performed as previously described (17). Briefly 45 μg of protein were loaded into Tris-glycine (4-12%) protein gels (Invitrogen) for electrophoresis; semidry transfer was done using nitrocellulose membranes and a Trans-Blot SD semidry transfer Cell (Bio-Rad) and blocking was

done using PBST (0.1 % Tween 20 in PBS) containing 5% low-fat milk. The monoclonal mouse anti-human BCL-2 oncoprotein clone 100 (1:200 dilution; Santa Cruz technology) and monoclonal mouse anti-β-actin clone AC-15 (1:10,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) antibodies were used.

Cell cycle analysis. Cells were harvested after 72 hours exposure to either G3139 or G3622, or 24 hours after exposure to 6 Gy radiation, or the combination of G3139 or G3622 and irradiation. Cells were harvested by trypsinization, washed with PBS, fixed and stored at 4°C before DNA analysis. After removal of ethanol by centrifugation, cells were incubated with phosphate-citric acid buffer at room temperature for 45 minutes. After centrifugation, cells were then stained with a propidium iodide solution for 24 hours. Stained nuclei were analysed for DNA-propidium iodide fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analysed by Modfit (Verity Software House, Inc., Topsham, ME, USA) for the proportion of cells in sub-G_0, G_1, S, and G_2M phases of the cell cycle.

Assay for tumour growth in athymic nude mice. The *in vivo* experiments were carried out at the Institut Gustave Roussy under the Animal Care license n°C94-076-11 (French Ministry of Agriculture). Female athymic nude mice (6-8 weeks old) obtained from Janvier CERT (Le Genest St. Isle, France) were used. H69 and H209 cells were harvested in exponential phase growth and 5×10^6 cells were injected subcutaneously into the flank area of 6- to 8-week-old female athymic nude mice on day 0. When tumours reached appropriate size, mice were randomised into 6 mice per group and treated with G3139 or RC or saline solution 10 mg/kg *i.p.* q.d.x6, x1 week, or irradiation with 6 Gy or the combination of G 3139 or RC, and irradiation (administered on day 3). Mice were weighed, and the tumour size was measured twice a week with an electronic caliper. Individual mouse follow-up was performed over the 30 days following the beginning of the treatment. The tumour volume was estimated from two-dimensional tumour measurements by the formula:

$$\text{Tumour volume=length (mm)} \times \text{width}^2\,(\text{mm}^2)/2$$

In each group (six mice per group), the relative tumour volume was expressed as the Vt/Vo ratio (Vt is the mean tumour volume on a given day during the treatment and Vo is the mean tumour volume at the beginning of the treatment).

Immunohistochemistry. Animals were euthanised 24 hours after the 6-day treatment with ASO. Tumours were excised and were both frozen and fixed in Finefix (Milestone, Italy) to prepare paraffin sections (4 µm thick). For terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assays (R&D Systems, Minneapolis, MN, USA), frozen tissue sections (10 Amold thick) were treated with 1:500 proteinase K solution (20 µg/ml) for 30 min, and endogenous peroxidase was blocked by using 3% hydrogen peroxide for 5 min. The samples were incubated for 1 hat 37°C with terminal deoxynucleotidyl transferase buffer and for 1 h at 37°C with antibromodeoxyuridine. After counterstaining with peroxidase-conjugated streptavidin, the slides were incubated with diaminobenzidine, counterstained with methyl green, and then mounted.

For CD34 staining, immunohistochemistry anti-CD34-positive murine endothelial cells (HyCult biotechnology b.v., The Netherlands) was performed after xylene treatment and rehydration. Heat-induced epitope retrieval was achieved with pH8 Tris-EDTA at 98°C for 40 min. Endogenous peroxidase activity was quenched by 3% H_2O_2 for 10 min. The sections were placed in coverplates (Shandon, USA) and incubated with blocking serum Biogenex wash buffer 1:10 (San Ramon, CA, USA) for 10 min. This step was followed by incubation with anti-mouse CD34 1:20, diluted in blocking serum 1:10 during 1 hr. Slides were then incubated with a rabbit anti-rat 1:400 (Southern Biotech., AL, USA), diluted in blocking serum 1:10. The revelation step used a Rabbit PowerVision kit (ImmunoVision Technologies, CA) 20 min and DAB 10 min. Slides were counterstained with Mayer's haematoxylin and mounted (Pertex). Tumour necrosis was assessed by light microscopy.

Statistical analysis. Results are expressed as mean±standard deviation (S.D.). All statistical analyses were made with a two-sided Student's *t*-test, where $p<0.05$ was considered statistically significant. SPPS software version 17.0 (Boix-Colombes, France) was used for all statistical analysis.

Results

H69 cell uptake of antisense oligonucleotides. To determine the efficiency of antisense oligonucleotide uptake *in vitro*, H69 cells were incubated with 500 nmol/l FAM-G3139 for 24 hours. Using flow cytometry, H69 cells were found to have high levels of uptake, with 98% of cells having a fluorescent signal of >100.0% of untreated cells (Figures 1a and 1b). When 6 Gy of irradiation was combined with ASO (3 hours before, during or after FAM-G3139 administration), antisense uptake varied greatly (Figure 1c). ASO followed by 6 Gy irradiation was the most efficient combination for improving uptake of antisense oligonucleotides.

BCL-2 ASO effectively decreases BCL-2 expression in SCLC. The effect of BCL-2 ASO on H69 cell BCL-2 expression was determined *in vitro* using Western blotting. Relative to RC-exposed cells, treatment with 500 nmol/l BCL-2 ASO for 3 days decreased BCL-2 protein levels (Figure 2a).

BCL-2 ASO does not reduce cell viability in combination with radiation. The cytotoxic effects of BCL-2 ASO and radiation were assessed using the WST-1 inner salt assay and compared with the effects of RC or no treatment (Figure 2b). A slight reduction in cell viability was observed when BCL-2 ASO was given alone. The fraction of viable cells decreased to 0.7 (500 nmol/l BCL-2 ASO) after 3 days of incubation. In contrast, no dose-dependent and time-dependent reduction in cell viability 24 hours or 48 hours after increasing the dose of radiation was observed when BCL-2 ASO was given in combination (data not shown)

BCL-2 ASO decreases clonogenic survival in combination with radiation. H69 cells were incubated with mock, RC or BCL-2 ASO for 3 days and then were given radiation at

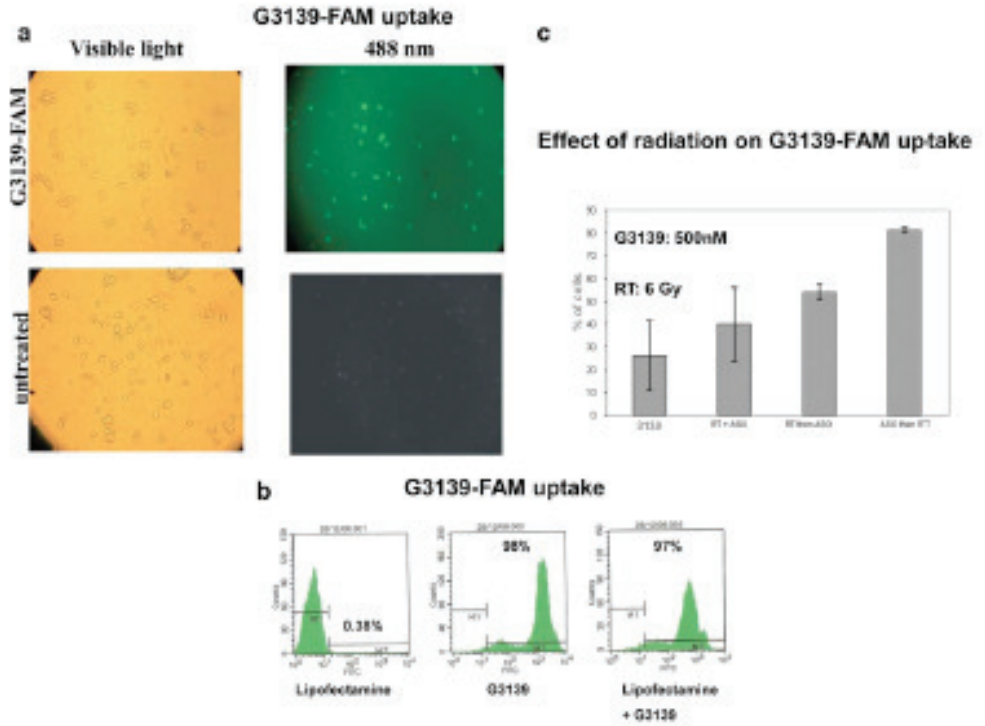


Figure 1. H69 cell uptake antisense oligonucleotides. a: H69 cells were incubated with 500 nmol/l of FAM-ASO for 24 hours and compared with an untreated control. b: Treated cells were analyzed by flow cytometry. c: Cells were treated as in (a), but antisense was administered during radiation or 3 hours before or after radiation (6 Gy). Columns, mean; bars, standard error. % FAM-positive cells. Each experiment was performed twice in duplicate.

increasing doses (0, 2, 4 or 6 Gy). At 4 Gy, SF fraction decreased from 0.3 with mock or MM to 0.2 with ASO ($p<0.05$) and at 6 Gy, SF fraction decreased from 0.1 with mock to 0.05 with ASO ($p<0.05$) (Figure 2c).

BCL-2 ASO slightly increase apoptosis. The presence of a sub-G1 DNA peak/population detected by flow cytometry suggested apoptosis-related cell death. BCL-2 ASO increased the apoptotic fraction over untreated or RC cells ($p<0.05$) (Figure 3).

BCL-2 ASO and radiation inhibit in vivo tumour growth. To determine the efficacy of the combination of BCL-2 ASO and radiation in SCLC, H69 xenograft tumours were established in nude mice and then were given radiation (6 Gy on day 3) with or without ASO (10 mg/kg, one injection daily for 6 days). Combination of radiation with G3139 in sequential schedule decreased tumour growth. RTV5 was obtained on day 10 for untreated mice and day 11 for mice RC-treated. In contrast, BCL-2 ASO extended RTV5 until day 17 and radiation combined with RC alone extended RTV5 until day 16. BCL-2 ASO plus radiation extended survival until day 21 ($p<0.05$) (Figure 4a). Western blot analysis on tumour specimens excised on day 7 showed BCL-2 down-regulation in mice treated with BCL-2 ASO compared to mice treated with RC or mock (Figure 4b).

BCL-2 oligodeoxynucleotides increases in vivo radiation-induced apoptosis. To determine whether BCL-2 oligodeoxynucleotide could improve *in vivo* radiation-induced apoptosis, nude mice bearing H69 xenografts were given mock, RC or G3139 for 6 days alone or combined with radiation (RT) on day 3. Tumours were excised on day

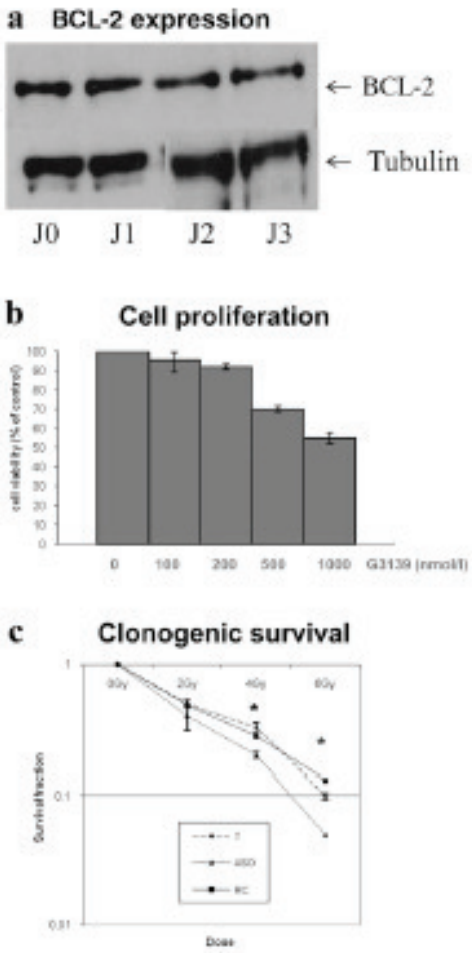


Figure 2. a: BCL-2 ASO decreases BCL-2 expression. H69 cells were incubated with 500 nmol/l BCL-2 ASO. Cells were harvested for Western blot analysis at different time after treatment. A representative blot is shown. Each experiment was performed twice independently. b: BCL-2 ASO slightly decreases cell viability alone but does not decrease cell viability in combination with radiation. H69 cells were incubated with RC or BCL-2 ASO for 5 days at growing concentration. Cell viability was assessed by WST-1 assay. Columns, mean three independent experiments; bars, standard error. c: BCL-2 ASO decreases clonogenic survival following radiation. H69 cells were incubated with vehicle, RC or BCL-2 ASO for 5 days and then were given radiation at increasing dose (0, 2, 4 or 6 Gy). Cells were then seeded for colony formation in 35 mm dishes at 200 to 1,000 cells/dish in methylcellulose-based media. After an incubation interval of 21 days, all colonies of 50 cells or more were counted using light microscopy. Experiments were performed twice in triplicate. Bars, standard deviation.

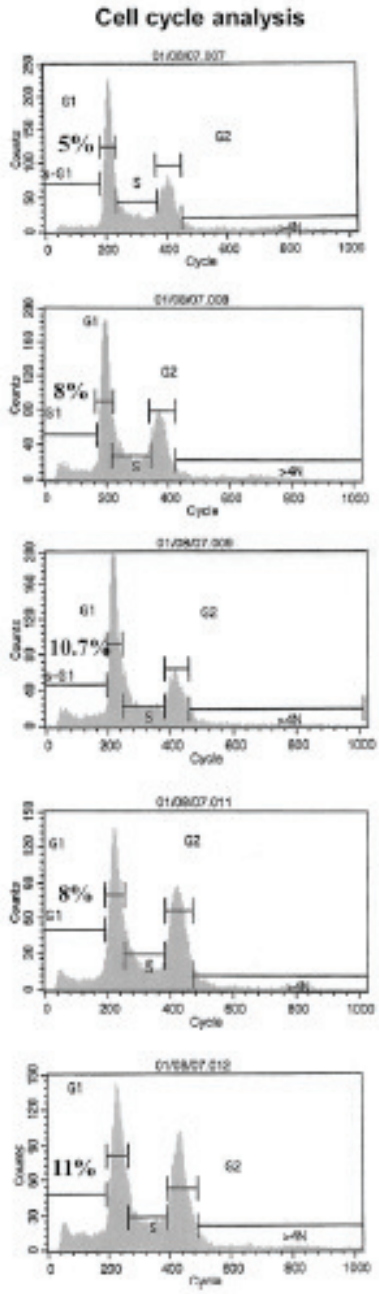
7 and analysed for TUNEL assay (Figure 5a). G3139 alone increased TUNEL-positive H69 cells (from 2.6% with mock plus RT to 12.4 % with ASO plus RT, $p<0.05$) suggesting activation of apoptosis. When combined with radiation, the fraction of apoptotic cells increased significantly (Figure 5b).

BCL-2 oligodeoxynucleotides decreases in vivo tumoural vascularisation. As BCL-2 exhibits pro-angiogenic properties, this study evaluated whether G3139 targeting BCL-2 mRNA could induce antiangiogenic effect *via* staining of endothelial cells using the CD34 expression assay. As shown in Figure 5c, G3139 decreased vascularisation and this effect was more pronounced in the combination of G3139 with radiation.

Antisense oligonucleotides display systemic tumour penetration effects. Some systemically administered molecules have limited penetration into tumours. This study evaluated whether radiation could improve intratumoural penetration of ASO. As shown previously *in vitro*, mice treated with ASO and then radiation displayed better intratumoural penetration of ASO compared with other combinations or ASO alone (Figure 6). Hence, the antisense was observed to distribute throughout the tumour, with at least some fluorescence evident throughout all areas of the tumour, whereas a lower apparent distribution was observed in mice treated with ASO alone or other combinations of radiation and ASO.

Discussion

The current treatment strategy for localised SCLC combines radiation and chemotherapy either sequentially or concurrently. However, only 10% of patients with localised small-cell lung cancer are disease free at 5 years. Therefore, new radiotherapeutic techniques or new radiation-sensitising strategies must be developed if the outcome of patients treated with radiation therapy is to improve (18). Failure of cells to undergo apoptosis could lead to resistance to treatment of SCLC (19). Reducing the anti-apoptotic BCL-2 protein may induce an increase of apoptotic response to anti-tumoural therapies (11). This study demonstrated a sensitisation of SCLC to RT by down-regulating BCL-2 expression with antisens oligonucleotides. This down-regulation resulted in a slight inhibition of cell viability but in a marked inhibition of clonogenic survival with an increase of RT-induced apoptosis. Previous reports have shown that clonogenic survival is more sensitive than viability assay such as WST-1 tetrazolium assay that relies on metabolic active cells only (20). Cells that have lost their reproductive capacity immediately but are still viable after the combination of radiation and ASO, have been scored in WST-1 assay but not in clonogenic assay (21). In accordance



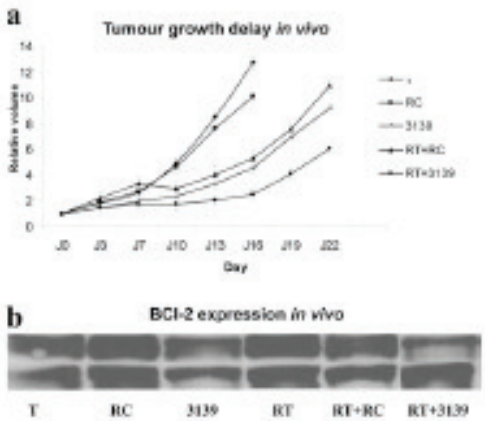


Figure 4. a: BCL-2 ASO and local radiation produce an in vivo therapeutic benefit. H69 xenografts were established in nude mice and randomised into the following groups: no treatment, RC, G3139, radiation (RT), RC plus RT or G3139 plus RT. Six injections of antisense (10 mg/kg) from day 1 through day 6 with or without one dose of radiation (6 Gy on day 5) were given. Two independent experiments were conducted, with 5 mice per group in each experiment. Bars, standard deviation. b: BCL-2 ASO decreases in vivo BCL-2 expression. Mice bearing H69 xenograft were treated as described in (a). Tumours were excised on day 7 and BCL-2 expression was analyzed by Western blot. Representative blot of two experiments.

with these results, combination of ASO targeting BCL-2 mRNA with radiation significantly induced tumour growth delay in nude mice.

BCL-2 proteins, such as BCL-2, BCL-xL, and MCL-1, have been found to be essential in the resistance to radiotherapy in many cancers including prostate cancer (22- 23), lymphomas (24) and head and neck cancers (25). BCL-2 proteins have been found to be involved in the development and progression of SCLC. Indeed, some studies have underscored the role of altered apoptotic balance in the pathogenesis of SCLC with the involvement of the amplification of anti-apoptotic genes including *BCL2* gene and a deletion of proapoptotic genes such as *MAPK10* and *TNFRSF6*, leading the apoptotic balance towards survival (26). Up-regulation of BCL-2 has been shown to be involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines (11).

Figure 3. Bcl-2 ASO induces apoptosis in H9 cells. H69 cells were incubated with RC or BCL-2ASO for 5 days before the administration of 6 Gy radiation (where indicated). The cells were harvested for sub-G_1 flow cytometry 1 day later. Representative experiment from two independent experiments. % Apoptotic cells: sub-G_1 fraction, G_0-G_1 fraction, S fraction and G_2-M fraction.

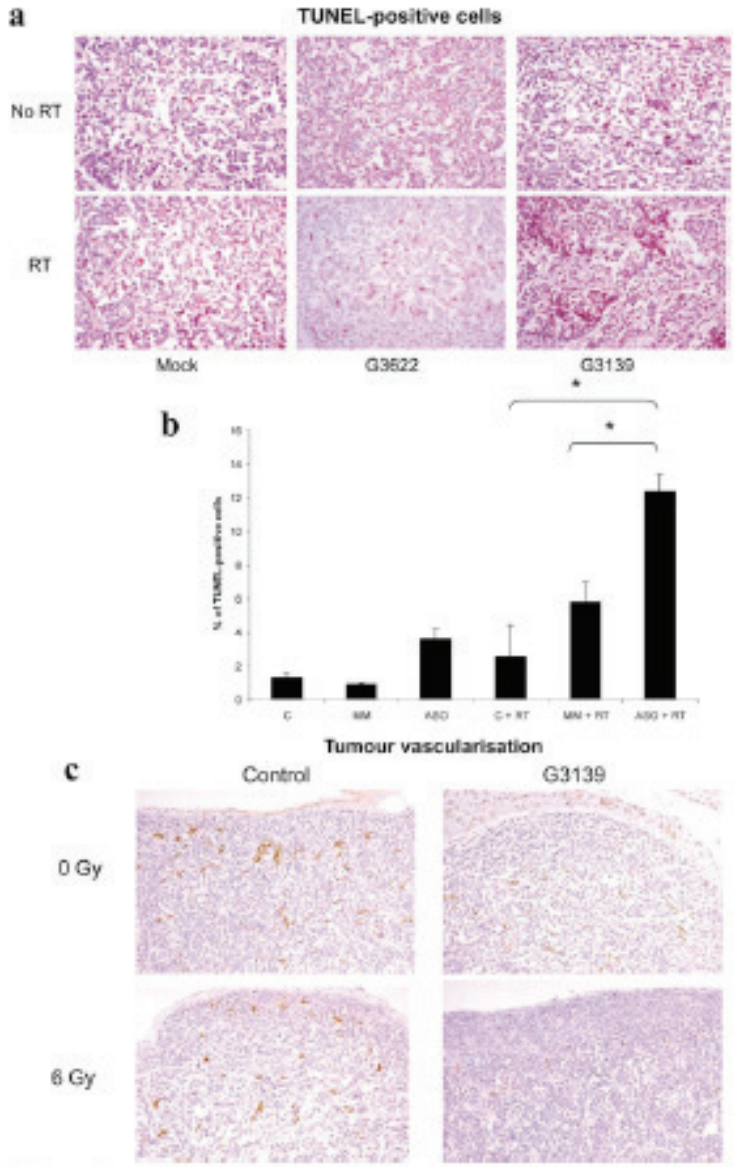


*Figure 5. a: H69 xenografts were established in nude mice and randomized into the following groups: no treatment, BC, BCL-2 ASO, radiation (RT), BC plus RT or G3139 plus RT. Six injections of antisense (10 mg/kg) from day 1 to day 6 with or without one dose of radiation (6 Gy on day 5) were given. Tumours were then excised on day 7 and stained for TUNEL assay. b: Quantification of apoptotic staining. At least 1,000 cells were counted for each sample, with two mice in each group. Columns, means; bars, standard deviation; *Statistically significant difference (p<0.05). c: Quantification of microvessel density. Mice bearing H69 xenografts were treated with G3139 (10 mg/kg for 6 days) with or without RT (6 Gy on day 5). Tumours were excised on day 7 and tissue sections were stained for CD34 (as described in the Materials and Methods).*

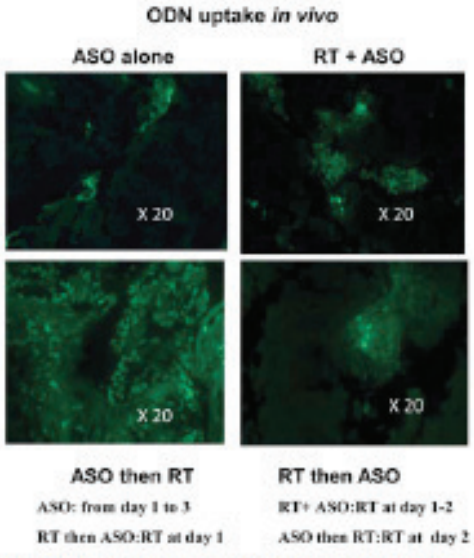


Figure 6. More antisense was observed in tumours treated with ASO than radiation. Xenograft tumour-bearing mice were injected with FAM-ASO (10 mg/kg for 5 days, 100 μl, i.p.) with or without radiation (RT) administered at different time. Tumours were harvested 2 hours following the last injection. Tumours were imaged using a Zeiss Axiovert 200M (Zeiss) for widefield microscopy (FITC: excitation, 480 nm; beam splitter, 505 nm; emission, 535 nm; Hoechst 33342: excitation, 360 nm; beam splitter, 395 nm; emission, 460 nm). Wide-field microscopy was used to demonstrate antisense (green) tumour penetration.

One mechanism explaining this positive association consists of an induction of apoptosis, which occured with both the BCL-2 ASO alone and when combined with radiation, as indicated *in vivo* by terminal deoxynucleotidyl transferase-mediated nick-end labelling staining (Figure 4a and 4b) The efficacy of oblimersen seems to be more potent *in vivo* than *in vitro*, suggesting that others mechanisms may be involved in this interaction. Others studies assessing the potential value of the combination of oblimersen and radiation reported an increase of radiation-induced apoptosis by oblimersen (27). Moreover, some studies have shown that oblimersen exhibits an antiangiogenic effect provided that BCL-2 has been shown to play role in angiogenesis (28). In this study oblimersen decreased CD34 cells alone and in combination with radiation contributing at least partially to the antitumoural effect (Figure 4C). Other studies have reported similar data. Ami *et al.* reported that antisense BCL-2 ODN and irradiation decreases VEGF expression and MVD in PC-3-BCL-2 xenografts (16). They showed that in PC-3-BCL-2 xenografts, the proportion of CD31-expressing endothelial cells undergoing apoptosis was highest in tumours treated with the combination of antisense BCL-2 ODN and irradiation resulting in a reduction of microvessel density, CD 31 and VEGF expression.

Recently, the antisense field has experienced some difficulties due to oblimersen not receiving approval from the U.S. Food and Drug Administration after disappointing results from phase III clinical trials in advanced melanoma, multiple myeloma and diffuse SCLC (29-30). The main reason explaining these failures was the inability of oligonucleotides to decrease *in vivo* BCL-2 protein levels, resulting in early degradation, poor intratumoural penetration and poor intracellular uptake (31-32) . The currently used phosphorothioate antisense molecules, such as G3139, represent the first generation of chemical oligonucleotide modifications. Newer 2V-*O*-methyl and locked nucleic acid modifications, as well as the use of chimeric antisense molecules, provide increased target binding affinity and improved pharmacokinetics (33). Another mechanism involved in this positive interaction between radiation and BCL-2 ODN may be a better tumoural penetration of ODN following irradiation. Drug distribution in tumours is often a challenge for systemic therapies, whether using small molecules, chemotherapies or antibodies (34). Fluorescent ODN was slightly visible at the tumour when administered alone and these data underscore impaired pharmacokinetics with antisense explaining partially the moderate antitumoural effects. However, the present study showed the potential effect of radiation to increase oligonucleotide uptake both *in vitro* and *in vivo*. These results are not consistent with others reporting that there is not enhancement of ODN uptake by radiation in nasopharyngeal cancer cell line C666-1 (27). However, Ami *et al.* have recently reported that the uptake of fluorescent ODN was increased in prostate cancer cells exposed to low doses of irradiation both *in vitro* and *in vivo* (16). Irradiation before fluorescent ODN treatment resulted in increased fluorescent signal intensity in xenograft tumours compared with those irradiated after fluorescent ODN treatment. This phenomenon was seen in other cell lines (bladder, kidney and fibroblast). Low-dose irradiation may perturb the cellular membrane enough to allow increased cellular uptake of ODN (16).

More stable drugs such as oral inhibitors may contribute to better BCL-2 family protein inhibition leading to improved anti-tumoural efficacy. Recently, several studies have reported the antitumoural efficacy of ABT 737, a BCL-2 and BCL-XL inhibitor in lymphoma, myeloma and SCLC (35-37). Increasing the antitumoural efficacy of radiation by BCL-2 protein down-regulation with ODN represents proof-of-concept of combining BCL-2 family protein and radiation in SCLC, warranting confirmatory studies with these more potent compounds.

In summary, this preclinical study showed that antisense BCL-2 enhances both *in vitro* and *in vivo* the antitumour effect of radiation SCLC which overexpresses BCL-2. The improved response to this sensitisation strategy seems to be the result of enhanced induction of apoptosis and antiangiogenic effects. These moderate effects were explained in part by an impaired oligonucleotide uptake in tumour. However, a strong uptake was observed when oligonucleotides were combined with radiation. This study represents a proof-of-concept of combining radiation and BCL-2 protein inhibitors with more potent drugs such as BCL-2/BCL-XL inhibitors.

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2.2 Etude n°2 « Effet de l'association RAF 265–RAD001 sur des lignées de CBP-NPC»

Mordant P, Loriot Y, Leteur C, Calderaro J, Bourhis J, Wislez M, Soria JC, Deutsch E. Dependence on phosphoinositide 3-kinase and RAS-RAF pathways drive the activity of RAF265, a novel RAF/VEGFR2 inhibitor, and RAD001 (Everolimus) in combination. Mol Cancer Ther 2010;9(2):358-68.

Research Article

Molecular Cancer Therapeutics

Dependence on Phosphoinositide 3-Kinase and RAS-RAF Pathways Drive the Activity of RAF265, a Novel RAF/VEGFR2 Inhibitor, and RAD001 (Everolimus) in Combination

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Abstract

Activation of phosphatidylinositol-3-kinase (*PI3K*)-AKT and Kirsten rat sarcoma viral oncogene homologue (*KRAS*) can induce cellular immortalization, proliferation, and resistance to anticancer therapeutics such as epidermal growth factor receptor inhibitors or chemotherapy. This study assessed the consequences of inhibiting these two pathways in tumor cells with activation of *KRAS*, *PI3K*-AKT, or both. We investigated whether the combination of a novel RAF/vascular endothelial growth factor receptor inhibitor, RAF265, with a mammalian target of rapamycin (mTOR) inhibitor, RAD001 (everolimus), could lead to enhanced antitumoral effects *in vitro* and *in vivo*. To address this question, we used cell lines with different status regarding *KRAS*, *PIK3CA*, and *BRAF* mutations, using immunoblotting to evaluate the inhibitors, and MTT and clonogenic assays for effects on cell viability and proliferation. Subcutaneous xenografts were used to assess the activity of the combination *in vivo*. RAD001 inhibited mTOR downstream signaling in all cell lines, whereas RAF265 inhibited RAF downstream signaling only in *BRAF* mutant cells. *In vitro*, addition of RAF265 to RAD001 led to decreased AKT, S6, and Eukaryotic translation initiation factor 4E binding protein 1 phosphorylation in HCT116 cells. *In vitro* and *in vivo*, RAD001 addition enhanced the antitumoral effect of RAF265 in HCT116 and H460 cells (both *KRAS* mut, *PIK3CA* mut); in contrast, the combination of RAF265 and RAD001 yielded no additional activity in A549 and MDAMB231 cells. The combination of RAF and mTOR inhibitors is effective for enhancing antitumoral effects in cells with deregulation of both RAS–RAF and PI3K, possibly through the cross-inhibition of 4E binding protein 1 and S6 protein. *Mol Cancer Ther; 9(2); 358–68. ©2010 AACR.*

Introduction

Growth factor receptors play a major role in cell survival and proliferation. Two important downstream pathways are the phosphatidylinositol 3′-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway and the RAS-RAF-mitogen-activated protein (MAP)/extracellular signal-related kinase (ERK) kinase (MEK)-ERK pathway. These pathways regulate transcription factors and other proteins involved in cell proliferation, survival, motility, and differentiation. In many human cancers, genetic and epigenetic mechanisms have deregulated major components of the RAS and AKT signaling network (1).

RAS is one of the most important molecules in the growth factor downstream signaling pathway. It can activate the serine/threonine kinase RAF, ERK1 and ERK2, and several nuclear proteins to promote cell proliferation. *RAS* genes, especially *KRAS* (homologous to the oncogene of the Kirsten rat sarcoma virus), have been implicated in the pathogenesis and prognosis of several cancers. In many tumors, a point mutation leading to the loss of GTPase activity is associated with transforming activation of the protein, resulting in continued proliferation signal. Overall, *KRAS* mutations are found in ~30% of human cancers, mainly in pancreatic, colorectal, endometrial, biliary tract, lung, and cervical cancers (2).

RAS plays an essential role in tumor maintenance and is therefore an appropriate target for anticancer therapy. Different pharmacologic strategies have been developed to inhibit *KRAS* oncogenic activation, including inhibition of its association with the plasma membrane (prenylation and postprenylation inhibitors), downstream signaling (kinase inhibitor), upstream pathways (kinase inhibitor and monoclonal antibody), and protein expression of RAS or other components of the pathway (small interfering RNA and antisense oligonucleotides; ref. 3). However,

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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several of these therapeutic agents have yielded disappointing results (4). RAS upstream pathway inhibition is based mostly on epidermal growth factor receptor (EGFR) inhibition, which is ineffective when administered alone in patients with colorectal (5–8) or lung cancer (9) harboring *KRAS* mutations. EGFR-targeted therapies are now being tested in combination with other therapies to overcome this *KRAS*-related resistance (10). Finally, pharmacologic inhibition of RAS downstream pathways with kinase inhibitors remains promising.

mTOR is a highly conserved serine-threonine kinase that recognizes stress signals (e.g., nutrients and energy depletion, oxidative and hypoxic stress, proliferative and survival signals) through the PI3K-AKT pathway. mTOR signaling occurs through the phosphorylation of substrates p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Phosphorylation of 4EBP1 releases eukaryotic initiation factor 4E, permitting the initiation of cap-dependent protein translation. mTOR activation also results in glucose transporter 1 (GLUT1) mRNA translation and protein expression. mTOR signaling pathways are dysregulated in a variety of human malignancies, making mTOR an attractive target for anticancer therapy (11). RAD001 (everolimus) is an orally bioavailable mTOR inhibitor that binds with high affinity to its intracellular receptor FKBP12, resulting in a complex that interacts with mTOR to inhibit downstream signaling events, including phosphorylation of S6 protein, phosphorylation of 4EBP1, and expression of GLUT1 protein (12, 13).

The RAS and AKT signaling network intersects at various points, conferring high redundancy on this network (1), with complex positive and negative feedback controls and bifurcations at several levels (14–17). For example, AKT-mediated phosphorylation of RAF shifts the cellular response in a human breast cancer cell line from cell cycle arrest to proliferation (15). Such observations suggest a molecular basis for cross-talk between the two signaling pathways at the level of RAF and AKT, and that combined RAF and mTOR inhibition may be promising for overcoming the intrinsic plasticity of the RAS-AKT network. We tested this hypothesis by investigating the effect of the combination of a novel RAF/vascular EGFR (VEGFR) inhibitor, RAF265, with the rapamycin derivative RAD001, in cell lines with different *KRAS*, *PI3K*, and *RAF* mutational status.

Materials and Methods

Compounds

Novartis Pharma AG synthesized and provided RAF265 and RAD001. Stock solutions (20 mmol/L) of these compounds were made in DMSO (Sigma) and diluted in PBS (Invitrogen) shortly before use. Solutions were then diluted in the culture medium of each cell line to achieve the indicated final concentrations.

Cell Lines

We used human adenocarcinoma and large cell carcinoma cell lines with different mutations of the RAS-RAF and PI3K-AKT pathways (Table 1). Human A549 and H460 lung, HT29 and HCT 116 colon, and MDAMB231 breast cancer cell lines were purchased from the American Type Culture Collection. Cell lines were cultured in complete medium, consisting of 10% (v/v) fetal bovine serum, 2 mmol L-glutamine, and 50 units/mL penicillin-streptomycin in the medium recommended by the American Type Culture Collection (all from Invitrogen). Cells were grown at 37°C and 5% CO_2 in an incubator. All cell lines were tested as mycoplasm free using the 4′,6-diamidino-2-phenylindole assay.

Immunoblotting

Immunoblotting was done to identify inhibitor activity and the upstream and downstream consequences of inhibition. After plating of 1.5×10^6 cells in six-well plates for 24 h, cells were treated with RAD001 (1 nmol/L), RAF265 (concentrations of 1–10 μmol/L), or concurrently with both at a fixed dose of RAD001 and increasing doses of RAF265 to achieve final concentrations of 1 nmol/L and 10 μmol/L, respectively. After 24 h, cell extracts were prepared by detergent lysis. The soluble protein concentration was determined by the microbovine serum albumin assay. Protein immunodetection was achieved by electrophoretic transfer of SDS-PAGE–separated proteins to nitrocellulose, incubation with the appropriate antibody,

Table 1. RAS, RAF, and PI3K mutational status of the selected cell lines

	RAS-RAF pathway		PI3K-Akt pathway	
	KRAS	*BRAF*	*PIK3CA*	*PTEN*
A549 (lung adk)	G12S mutant	Wt	Wt	Wt
H460 (lung LC)	Q61H mutant	Wt	E545K mutant	Wt
HCT116 (colon adk)	G13D mutant	Wt	H1047R mutant	Wt
HT29 (colon adk)	Wt	V599E mutant	Wt	Wt
MDAMB231 (breast adk)	G13D mutant	G469V mutant	Wt	Wt

Abbreviations: Adk, adenocarcinoma; LC, large cell carcinoma; wt, wild-type.

and chemoluminescent second-step detection. All antibodies were obtained from Cell Signaling Technology.

Cell Viability Assay

The MTT assay and Bliss additivism model were used to assess the effect of the combination on cell viability. In each well of a 96-well plate, 1×10^4 cells were grown in 200 μL of medium. After 24 h, RAD001, RAF265, or the combination was added to achieve a final concentration of 0.1 to 10 nmol/L and 0.1 to 10 μmol/L, respectively. After 48 h of treatment, 20 μL of 5 mg mL^{-1} MTT (Organics Research, Inc.) solution in PBS was added to each well. After 4 h, supernatant was removed and formazan crystals were discarded in 200 μL of DMSO. Absorbance was then measured at 595 nm using an absorbance plate reader (Bio-Rad Microplate Reader). Data are expressed as the percentage of viable cells in treated relative to nontreated conditions.

Analysis of Additivity and Synergy

The Bliss additivism model was used to classify the effect of combining RAD001 and RAF265 as synergistic, additive, or antagonistic. A theoretical curve was calculated for combined inhibition using the equation $E_{Bliss} = EA + EB - E_A \times E_B$, in which E_A and E_B are the fractional inhibitions obtained by drug A alone or drug B alone at specific concentrations. Here, E_{Bliss} is the fractional inhibition that would be expected if the combination of the two drugs were exactly additive. If $E_{observed}$, the experimentally measured fractional inhibition, is less than E_{Bliss}, the combination is said to be antagonistic, and $\delta = E_{observed} - E_{Bliss}$ is a negative value. If the experimentally measured fractional inhibition is greater than E_{Bliss}, the combination is said to be synergistic, and δ is a positive value. The data are expressed as the percentage decrease in cell viability above what would be expected if the combination were strictly additive (18, 19).

Clonogenic Assay

Cell proliferation was assessed by clonogenic assay. Cells were seeded in six-well plates at a density of 200 to 800 (A549, H460, HT29, and HCT116) or 1,600 to 6,400 (MDAMB231) cells in 2 mL of medium per well. After 4 h, they were treated with vehicle, RAD001, RAF265, or the combination to achieve a final concentration of 1 and 5 μmol/L, respectively. After 24 h, the medium was changed to stop the treatment. After 10 to 14 d, cells were stained with crystal violet, and colonies up to 50 cells were counted. Plating efficiency, surviving fraction, and colony mean size were determined for each condition.

In vivo Experiments

We also tested the efficacy of the combination *in vivo*. A total of 3×10^6 A549, H460, HCT116, or MDAMB231 cells were injected s.c. into the flank region of 6-wk-old female athymic mice (Janvier). When tumors reached 50 mm^3, the mice were randomized into four groups ($n = 7$/group) for the following treatment: vehicle, RAF265 (12 mg/kg daily), RAD001 (12 mg/kg daily), or both. All drug were administered over 14 d (6 d on, 2 d off, 6 d on), and the drug combination was administered concurrently. Control mice received the respective vehicles of both drugs. Animal weight and tumor volumes were taken twice weekly and expressed relative to initial tumor volume. Tumors were measured until achieving a relative volume of 10 times the initial volume, and the time to this end point was noted. Drug efficacy was assessed based on the tumor growth curve, growth delay, and tumor volume inhibition percentage. The tumor growth curve was designed to depict the evolution of the relative tumor size over time. For statistical comparison of tumor volumes in treated versus control mice, the Student's *t* test (two sided) and ANOVA were used. Growth delay was defined as the time to achieve a relative volume of 10 times the initial volume (T10) in experimental groups compared with control group. The tumor volume inhibition percentage (TVI%) was calculated as: TVI% = 100 − (mean TV treated/mean TV control × 100) on day 15 after the last day of treatment. Toxic effects of the drug treatment were assessed as body weight loss percentage and lethal toxicity, assessed as deaths occurring in treated mice before the death of the first control mouse (20).

Immunohistochemistry

During the *in vivo* experiment, one animal of each group was dedicated to immunohistochemistry experiments and sacrificed after 5 d of treatment. Tumors were resected, fixed in a formalin-free alcoholic-based fixative (Finefix), and embedded in paraffin. CC1 antigen retrieval buffer (Ventana Medical Systems) was used for anti–cleaved caspase- 3, phospho-EGFR, phospho-AKT, mouse CD34, and Glut1 staining, and CC2 antigen retrieval buffer (Ventana Medical Systems) was used for anti–phospho-VEGFR2 staining. All immunohistochemical stainings were done on an automated immunostainer (Benchmark; Ventana) using the Ventana I-view 3,3′-diaminobenzidine detection kit with the following antibodies: anti-mouse CD34 (rat monoclonal; MEC14.7; Hycult Biotechnology; dilution, 1:50), anti–phospho-AKT (rabbit monoclonal; 4060; Cell Signaling Technology; dilution, 1:50) anti-glut1 (rabbit polyclonal; A3586; DAKO Corp.; dilution, 1:400), anti–phospho-EGFR (rabbit polyclonal; 4404; Cell Signaling Technology; dilution, 1:25), anti–phospho-VEGFR2 (rabbit monoclonal; 2478; Cell Signaling Technology; dilution, 1:300), and anti-cleaved caspase-3 (rabbit polyclonal; 9661; Cell Signaling Technology; dilution, 1:400). Slides were counterstained with hematoxylin. Immunostaining was evaluated on whole standard tissue sections of mouse subcutaneous xenografts. Five medium-power fields per section were viewed, and tumors were given a semiquantitative score of 0, <50% (+), >50% (++), or 100% (+++) positive tumor cells. The staining positivity of cells was defined as a membranous staining (anti-CD34, anti–phospho-EGFR, anti–phospho-VEGFR2, anti-glut1), a cytoplasmic staining (anti-cleaved caspase- 3), or both nuclear and cytoplasmic stainings (anti–phospho-Akt). A qualified

pathologist with no knowledge of the clinicopathologic variables evaluated the specimens.

Results

Addition of RAD001 Enhanced the Cytotoxic Effect of RAF265 in the HCT116 Cell Line *In vitro*

We investigated the inhibition of targeted kinase by RAD001 and RAF265 in the context of selected *KRAS*, *BRAF*, and phosphoinositide-3-kinase, catalytic α polypeptide (*PIK3CA*) mutations, assessing phosphorylation of downstream proteins by immunoblotting in selected cell lines (Fig. 1). Exposure to RAD001 at a concentration of 1 nmol/L led to a decrease in phosphorylation of S6 ribosomal protein at serines 235 and 236 in all tested cell lines. Exposure to RAD001 was not linked to any modification in MEK phosphorylation at serines 217 and 221. Exposure to RAF265 at concentrations ranging from 1 to 10 μmol/L decreased MEK phosphorylation in *BRAF*-mutated cell lines. Interestingly, exposure of non-*BRAF*–mutated cell lines to 1 μmol/L of RAF265 paradoxically increased the phosphorylation of MEK, but this change was reversible when RAF265 concentration reached 5 μmol/L. In the HCT116, HT29, and MDAMB231 cell lines, increasing concentrations of RAF265 were associated with decreased phosphorylation of S6 ribosomal protein, an mTOR downstream effector. This unexpected effect has not been explained yet, and several hypotheses should be tested, including a transient effect linked to the antiproliferative activity of the combination, a downstream effect following VEGFR inhibition by RAF265, or a cross-inhibition of S6 protein by the RAF-MEK pathway.

We evaluated drug efficacy *in vitro* using the MTT viability assay and clonogenic proliferation assay. The MTT assay revealed that in HCT116, HT-29, and MDAMB231 cells, RAD001 alone (0.1–10 nmol/L) failed to decrease cell viability while achieving a decrease of 30% in cell viability in A549 cells. In HT29 and MDAMB231 cells, RAF265 alone showed significant activity with IC_{20} values of 1 to 3 μmol/L and IC_{50} values of 5 to 10 μmol/L. In A549 and HCT116 cells, IC_{20} values were 1 μmol/L for both, but RAF265 concentrations up to 10 μmol/L did not reach IC_{50} values. However, in the presence of 1 nmol/L RAD001, the IC_{50} for RAF265 was 5 μmol/L in A549 cells and 10 μmol/L in HCT116 cells (Fig. 2A).

We used the Bliss additivism model to classify the effect of combining RAD001 and RAF265 as synergistic, additive, or antagonistic. Δ was positive in HCT116 cells, signaling a synergy between RAD001 and RAF265. Δ was negative or close to zero in A549, HT29, and MDAMB231 cells, indicating no synergy between the drugs in these cell lines. Therefore, the effect of the combination was found to be synergistic in HCT116 (*KRAS* mut, *PIK3CA* mut) cells but not in the other selected cell lines (Supplementary Fig. S1A).

Notably, exposure to RAD001 led to a small decrease in clonogenic survival in only the HCT116, HT29, and MDAMB231 cell lines. In the same experiments, RAF265 led to a significant decrease in clonogenic survival in all tested cell lines. When compared with the MTT assay findings, these results indicate that RAF265 induces a dominant effect on clonogenic survival compared with the MTT assay. This finding is consistent with the dominant role of the RAF-ERK-MEK pathway in cell proliferation. Interestingly, addition of RAF265 to RAD001 induced a small but significant decrease in cell proliferation only in

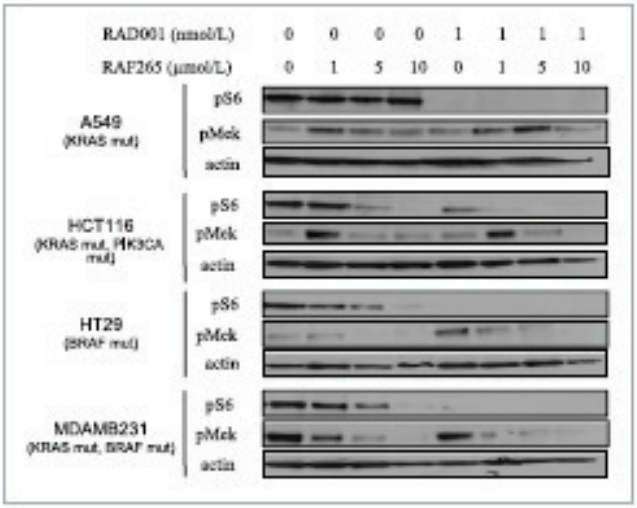

Figure 1. RAF265 and RAD001 decreased the phosphorylation of downstream components of the RAS-RAF and AKT-mTOR pathways. Immunoblotting showed suppression of protein phosphorylation directly downstream of mTOR after exposure of all cell lines to RAD001 and downstream of RAF after exposure of *BRAF*-mutated cells to RAF265. Exposure to RAF265 also led to a slight inhibition of S6 phosphorylation in HCT116, HT29, and MDAMB231 cells.

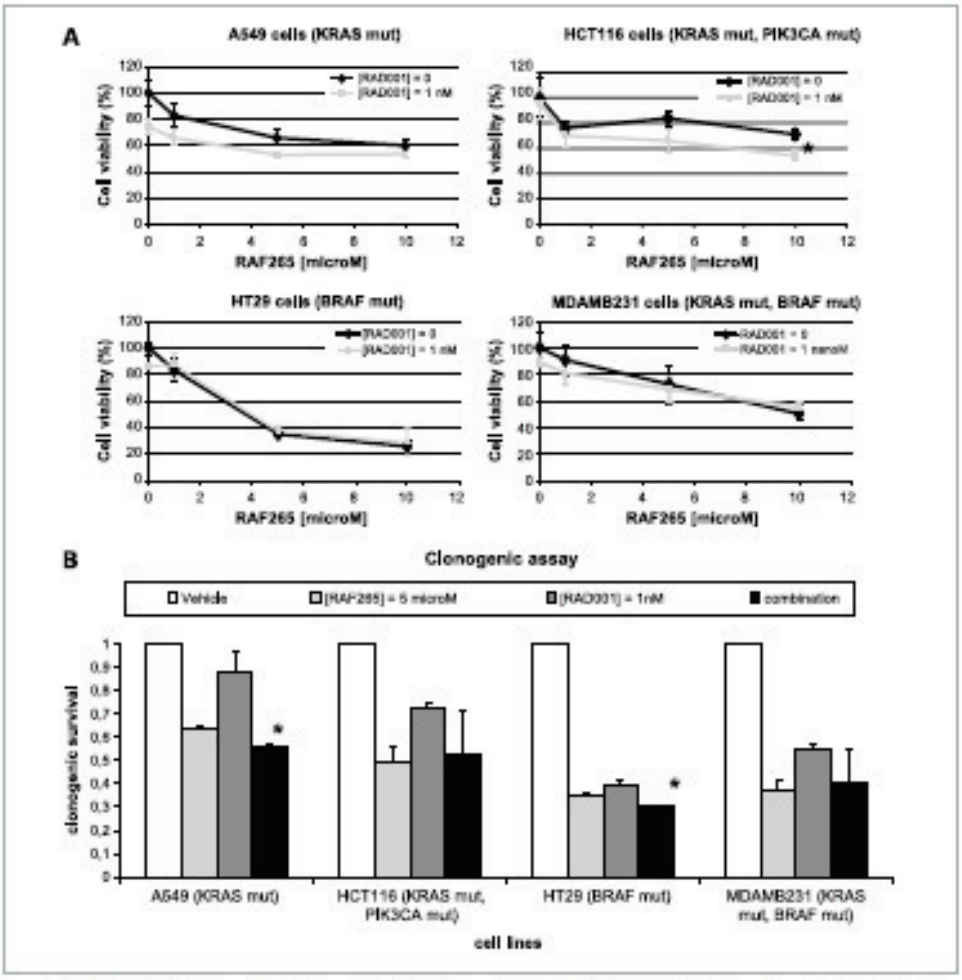

Figure 2. RAF265 and RAD001 showed limited efficacy in vitro. A, MTT assay. Addition of RAD001 to RAF265 led to an enhanced cytotoxic effect in HCT116 cells, as assessed by the MTT assay (*t* test, $P = 0.01$). This effect was identified as synergistic using the Bliss additivism model. B, clonogenic assay. Compared with vehicle, RAD001 induced a decrease in clonogenic survival in all cell lines except HCT116, whereas RAF265 induced a decrease in clonogenic survival in all cell lines. Addition of RAD001 to RAF265 enhanced the antiproliferative effect of RAF265 in A549 cells (combination versus RAD001 or RAF265 alone, $P = 0.022$ and $P = 0.006$, respectively) and HT29 cells ($P = 0.036$ and $P = 0.039$, respectively).

the A549 and HT29 cell lines (Fig. 2B). No significant difference was found in the size of the colonies between cells exposed to each compound alone or to the combination (Supplementary Fig. S1A and B). The different mechanisms involved in each assay likely explain the discrepancy between the MTT and clonogenic assay results, i.e., short-term cytotoxicity in the MTT assay and long-term proliferation and clonogenic survival in the clonogenic assay.

Combination of RAD001 and RAF265 Is Well Tolerated *In vivo* and Has an Additive Effect in the HCT116 Cell Line

In single-compound efficacy studies, optimal dosing of RAD001 and RAF265 was 5 to 12 mg/kg daily (21) and 30 mg/kg every two days (22), respectively. However, combination tolerability studies in nontumor-bearing mice defined dose-limiting toxicity as a 10% weight loss

with the combination of RAD001 at a dose of 12 mg/kg daily and RAF265 at a dose of 20 mg/kg every two days. Therefore, the combination of RAF265 at a dose of 12 mg/kg qd and RAD001 at a dose of 12 mg/kg qd seemed to be the maximal tolerated dose. In our studies, RAD001 and RAF265 were both given at a dose of 12 mg/kg qd, alone or concurrently, over 6 days. After a 2-day stop, the compounds were given for another 6 days, and the treatment was then stopped. Under these conditions, no death or major toxicity occurred, and no animal lost >10% of its initial body weight (data not shown).

To confirm the potential of the combination of RAF265 and RAD001, we chose to test *in vivo* the antitumor effect of the combination in HCT116 xenografts (*KRAS* mut, *PIK3CA* mut). We then compared HCT116 sensitivity with that of A549 (*KRAS* mut) and MDAMB231 (*KRAS* mut, *BRAF* mut). In HCT116 xenografts, RAD001 or RAF265 given alone showed 60% to 65% and 71% to 72% TVI%, respectively. Time to achieve a relative tumor volume of 10 times the initial tumor volume (T10) was 20 days in the control group, 25 days in the RAD001 group, 25 days in the RAF265 group, and 35 days in the combination group. Therefore, the tumor growth delay was 5 days with each compound alone, and 15 days with the combination, indicating an additive effect of the combination *in vivo* (Supplementary Fig. S1D and E; Fig. 3). Conversely, the combination of RAF265 and RAD001 showed no additional activity in A549 and MDAMB231 cells.

Addition of RAF265 to RAD001 in HCT116 Cells Leads to Moderately Decreased AKT, S6 Protein, and 4EBP1 Phosphorylation

We performed immunoblotting to investigate the cellular effects of the RAD001/RAF265 combination *in vitro*. The efficacy of this combination may be linked to the inhibition of upstream and/or downstream signaling, coding for survival, and/or proliferative information. Regarding upstream signaling, mTOR inhibition leads to enhanced AKT phosphorylation in mammalian cells (11), a negative feedback loop that may involve growth factor receptors and PI3K signalization. In addition, AKT phosphorylation may lead to cell survival and ionizing radiation resistance. Therefore, we investigated whether addition of RAF-MAPK inhibition to mTOR inhibition

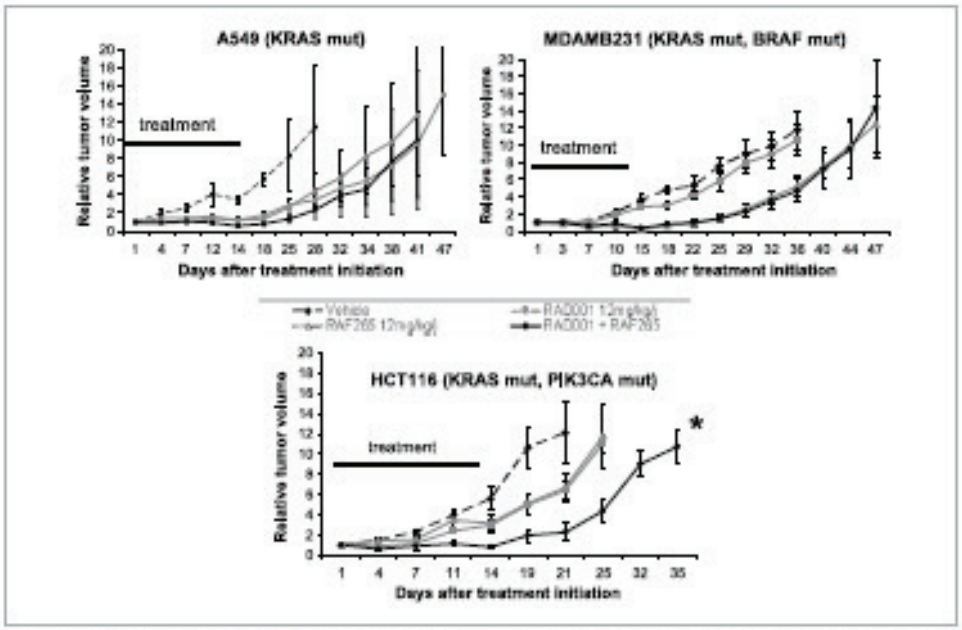

Figure 3. Combination of RAF265 and RAD001 showed synergistic efficacy in HCT116 xenografts. Data are expressed as percent change in initial tumor volume (T0). Horizontal black line, initial treatment length. Combination of RAF265 and RAD001 induced tumor growth retardation compared with each compound alone in HCT116 but not in A549 or MDAMB231 xenografts. In HCT116 xenografts, RAD001 and RAF265 given alone exhibited limited effect. However, the combination of RAD001 and RAF265 significantly delayed tumor growth (ANOVA, $P < 0.001$) compared with vehicle or to each compound given alone. In A549 xenografts, each compound given alone exhibited limited efficacy, without any improvement when given together. MDAMB231 xenografts showed high sensitivity to RAF265 but showed resistance to RAD001 without any benefit from the addition of RAD001 to RAF265.

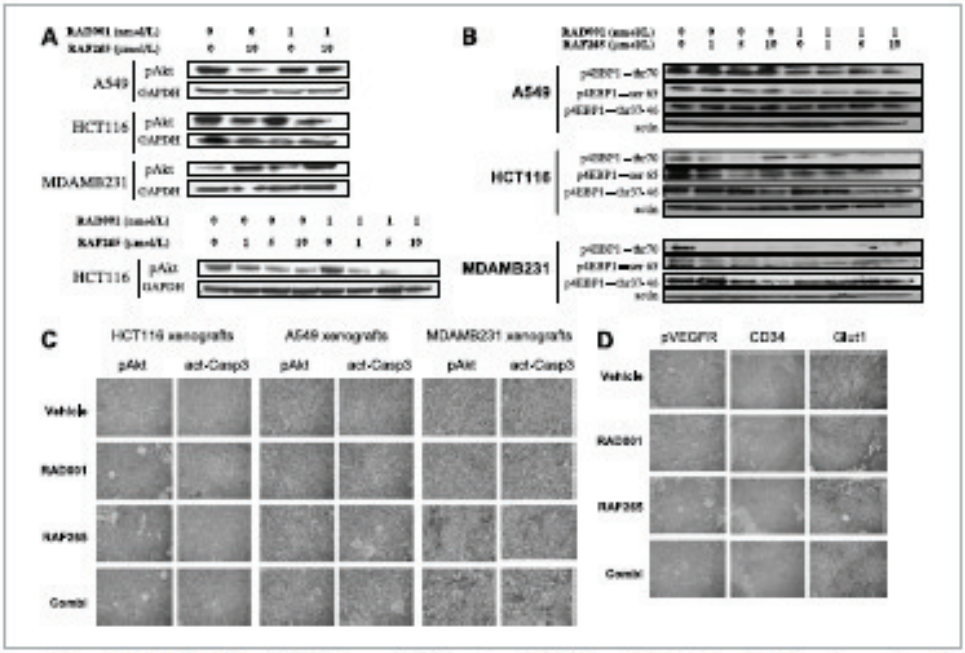

Figure 4. The combination of RAF265 and RAD001 decreased AKT, S6 protein, and 4EBP1 phosphorylation *in vitro* without decreasing vasculogenesis *in vivo*. A, AKT phosphorylation. AKT phosphorylation is inhibited after the addition of RAF265 to RAD001 in HCT116 cells, but not in A549 and MDAMB231 cells. B, 4EBP1 phosphorylation. In HCT116 cells, exposure to RAF265 induced the cross-inhibition of p86 (Fig. 1) and p-4EBP1, mainly in ser 65 and thr 70 phosphorylation sites. The exposure of A549 cells to RAD001 was associated with the decreased phosphorylation of 4EBP1, as expected. RAF265 addition did not modify this decrease. The exposure of MDAMB231 cells to RAF265 decreased 4EBP1 phosphorylation, without changes after RAD001 addition. C, caspase-3 activation and AKT phosphorylation in HCT116 xenografts. Combination of RAD001 and RAF265 significantly enhanced caspase-3 activation in HCT116 and MDAMB231 but not in A549 xenografts. *In vivo*, RAD001 alone or combined with RAF265 did not lead to a significant increase in AKT phosphorylation in HCT116 xenografts. D, vasculogenesis in HCT116 xenografts. Phospho-VEGFR remained undetectable in all four groups. CD34 staining remained stable after exposure to each compound alone or in combination. GLUT1 expression decreased after exposure to RAF265 or to the combination of RAF265 and RAD001.

interferes with AKT phosphorylation. Exposure to RAD001 was associated with an increase in AKT phosphorylation in MDAMB231 (*KRAS* mut, *BRAF* mut) but not in the A549 or HCT116 cell lines. Exposure to RAF265 was associated with decreased AKT phosphorylation in A549 and HCT116 cells but not in MDAMB231 cells. Exposure to the combination of RAD001 and RAF265 in HCT116 cells led to a slight inhibition of AKT phosphorylation, compared with each drug given alone, but not in A549 or MDAMB231 cells. Dose-effect immunoblotting confirmed these findings with a RAD001 concentration of 1 nmol/L and RAF265 concentrations of 5 or 10 μmol/L (Fig. 4A).

We have already noted that RAF265 exposure led to the decreased phosphorylation of the mTOR downstream effector S6 in HCT116, HT29, and MDAMB231 cells (Fig. 1). To investigate the mechanism of the RAD001/RAF265 combination efficacy, we studied the phosphorylation of another downstream protein, 4EBP1. This binding protein plays a key role in translation initiation because its phosphorylation releases eukaryotic initiation factor 4E, permitting initiation of cap-dependent protein translation. In HCT116 cells, phosphorylation of 4EBP1 at four different phosphorylation sites (thr 37, thr 46, ser 65, and thr 70) seemed to be decreased more notably after exposure to the combination rather than after exposure to each compound alone. A similar outcome was not observed in either A549 (*KRAS* mut) or MDAMB231 (*KRAS* mut, *BRAF* mut) cell lines (Fig. 4B).

Combination of RAF265 and RAD001 in HCT116 Xenografts Led to Increased Caspase-3 Activation and Decreased GLUT1 Expression without Change in CD34 Staining

The combination of RAD001 and RAF265 significantly enhanced the activation of caspase-3 in HCT116 and MDAMB231 but not in A549 xenografts. Interestingly,

RAD001 alone did not lead to an increase in AKT phosphorylation in A549, HCT116, or MDAMB231 xenografts. The combination of RAD001 and RAF265 significantly increased AKT phosphorylation in MDAMB231 xenografts but not in A549 or HCT116 xenografts. In these cell lines, the level of AKT phosphorylation remained too low to be detected by immunohistochemistry (Fig. 4C).

Vasculogenesis-targeted immunoblotting was done to dissect the mechanism of the effects of combined RAD001/RAF265 *in vivo*. Phosphorylation of VEGFR was not detected in HCT116 xenografts in any group. CD34 staining remained stable after the administration of RAF265, RAD001, or the combination. Interestingly, GLUT1 expression, as assessed by immunoblotting, decreased in the RAF265 and combination groups (Fig. 4D). Decreased GLUT1 expression may be linked to hypoxia, nonspecific glucose starvation, an antitumoral effect, or intracellular inhibition of the mTOR pathway. The lack of decrease in CD34 or phospho-VEGFR2 expression suggests that the decreased GLUT1 expression is not attributable to oxygen starvation. The lack of decreased GLUT1 expression after the administration of RAD001 alone, together with no additional decrease in GLUT1 expression after addition of RAF265 to RAD001, argues against the role of nonspecific glucose starvation or antitumoral effects. Therefore, the decrease in GLUT1 expression after exposure to RAF265 may be linked to the cross-inhibition of mTOR downstream effectors, rather than to nonspecific or VEGFR-driven antiangiogenic effects.

Confirmation of the Efficacy of the Combination of RAF265 and RAD001 in a Cell Line with both *KRAS* and *PIK3CA* Mutational Background

To confirm the need for concomitant mutations of *KRAS* and *PIK3CA* for optimal efficacy of the combination, we selected H460, a lung cancer cell line with specific mutation of oncogenic *KRAS* and concomitant mutation of *PIK3CA*. In the MTT assay, addition of RAD001 to RAF265 led to decreased H460 cell viability compared with each drug given alone (Fig. 5A). Using the Bliss additivism model, we found that the combination was not synergistic (Supplementary Fig. S1A). In the clonogenic assay, fewer colonies were found with the combination than with the drug alone or the predictive sum of both drugs, suggesting synergistic inhibition of cell proliferation and clonogenic survival in this cell line (Supplementary Fig. S2A; Fig. 5B).

In vivo, the combination of RAD001 and RAF265 had an enhanced effect compared with each compound given alone to mice harboring H460 xenografts. The tumor growth curve showed a limited effect of RAD001 or RAF265 given alone, but the combination of RAD001 and RAF265 induced a significantly enhanced and sustained cytostatic effect in H460 xenografts, confirmed by tumor growth curves, increased TVI%, and delayed T10 (Supplementary Fig. S2B and C; Fig. 5C). Therefore, the combination of RAF265 and RAD001 is efficient in xenografts with mutations of both *KRAS* and *PIK3CA*. Together, these data may suggest that concomitant mutations of *KRAS* and *PIK3CA* underlie the effectiveness of the RAD001/RAF265 combination, both *in vitro* and *in vivo*.

Discussion

KRAS mutations remain a major therapeutic challenge in oncology, and specific treatments require further investigation. Tyrosine kinase inhibitor combinations may be an interesting option in the setting of *KRAS*-mutated tumors, with different combinations being available. Legrier et al. (1) have shown synergism of the combination of concurrent low-dose MEK and mTOR inhibitor therapy *in vivo* in A549 (*KRAS* G12S) but not in H157 (*KRAS* G12R and *PTEN* G251C) xenografts. In a genetically engineered mouse model of lung adenocarcinomas, Engelman et al. (23) found that cancers driven by mutant *KRAS* (G12D) did not substantially respond to dual pan-PI3K and mTOR inhibitor NVP-BEZ235 used as a single agent, but did respond when NVP-BEZ235 was combined with a MEK inhibitor, ARRY-142886. In a third study, She et al. (24) tested concomitant MEK and PI3K inhibition *in vivo* in pTEN-deficient tumor cells. In tumor cells with PTEN deletion and significant activation of MAPK, inhibition of PI3K-AKT signaling alone was insufficient to induce marked apoptosis, but inhibition of both pathways had synergistic effects, probably through additive inhibition of BAD phosphorylation. In a fourth study, Yu et al. (25) tested the antiproliferative effects of the PI3K inhibitors WAY-266176 and WAY-266175 in different cancer cell lines and established that heightened PI3K-AKT-mTOR signaling was linked to a sensitive phenotype. In HCT116 cells, concomitant *PIK3CA* and *K-RAS* mutations correlated with a resistant phenotype. However, a profoundly augmented growth suppression and apoptosis were achieved in resistant cells by combination treatment with WAY-266176/WAY-266175 and a MEK1 kinase inhibitor, CI-1040 or UO126 (25). Furthermore, the combination of the dual RAF/VEGFR inhibitor sorafenib with the mTOR inhibitor rapamycin has been reported to synergistically inhibit melanoma cell growth and induce enhanced apoptosis *in vitro* (26).

We found that the addition of RAF265 to RAD001 did not decrease cell viability or cell proliferation in cell lines with mutant *KRAS* and wild-type *PIK3CA*. Therefore, our results do not confirm the previous findings of Legrier et al. (1) and Engelman et al. (23). This discrepancy may have several explanations, including different pathway intersections, intrinsic signal plasticity, and downstream signaling differences between RAF and MEK inhibitors on the one hand, and PIK3CA and mTOR inhibitors on the other hand. Conversely, we found that the combination of RAF265 and RAD001 was efficient in cells with concomitant *PIK3CA* and *KRAS* mutations, confirming the findings of She et al. (24) and Yu et al. (25). Compared with their results, we describe similar findings with the upstream inhibition of the RAF-MEK-ERK pathway and

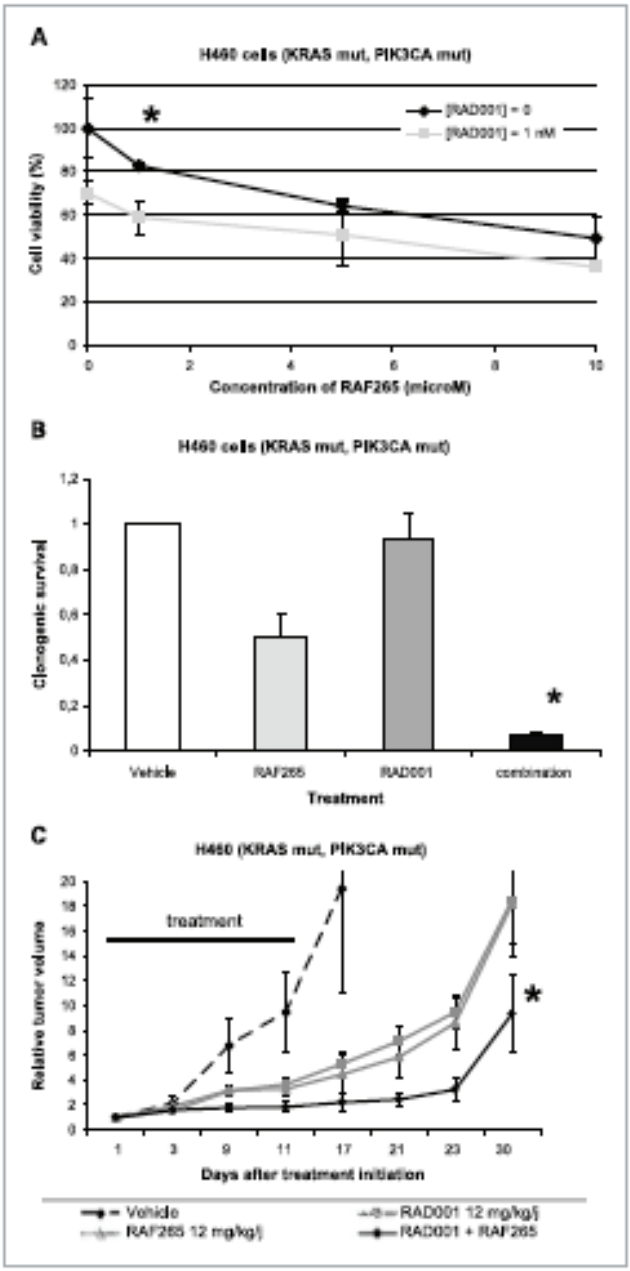

Figure 5. Confirmatory studies in H460 cells: the combination of RAF265 and RAD001 is additive in H460 cells. A, effect of RAF265 and RAD001 on H460 cell viability. Addition of RAD001 to RAF265 led to an enhanced cytotoxic effect in H460 cells (*t* test, $P = 0.02$). B, clonogenic assay in H460 cells. Compared with vehicle, RAD001 failed to decrease the clonogenic survival of H460 cells ($P = 0.4$), whereas RAF265 induced a significant decrease ($P = 0.01$). Addition of RAD001 to RAF265 increased its antiproliferative effect (combination versus RAD001 or RAF265 alone, $P = 0.003$ and $P = 0.016$, respectively). C, relative tumor growth curve. In H460 xenografts, RAD001 and RAF265 given alone exhibited limited effect. However, the combination of RAD001 and RAF265 significantly delayed tumor growth (ANOVA, $P < 0.001$) compared with vehicle or to each compound given alone.

the downstream inhibition of the PI3K-AKT-mTOR pathway, and we offer three important observations relevant to our findings. First, RAF inhibitors have a chemical structure that allows the cross-inhibition of VEGFR2 and may contribute antiangiogenic properties to intracellular signal inhibition. Second, mTOR inhibition occurs downstream of PI3K and may interfere with other growth factor receptor proliferation signals. Third, RAD001 is already available in the clinic and combines good tolerance profile with high potency, and RAF265 is currently being tested in a phase I study in patients with locally advanced or metastatic melanoma (ClinicalTrials.gov Identifier NCT00304525).

Our findings suggest that the combination of RAD001 and RAF265 is effective in the setting of concomitant mutations of both the RAS-RAF-MAPK and PI3K-AKT-mTOR pathways. Three different mechanisms may mediate this efficacy, inferred from our current results. First, cross-inhibition of 4EBP1 phosphorylation induces a shift toward the hypophosphorylated form of 4EBP1. This shift correlates with a reduced level of cap-dependent translation that permits the 4EBP1 sequestration of eIF4E (27). Second, the inhibition of the negative feedback loop by RAF265 decreases RAD001-induced AKT hyperphosphorylation and may interfere with AKT-associated survival signaling (28). Third, the combination of RAF265 and RAD001 may lead to decreased GLUT1 expression. However, these data require further investigation, including experiments involving genetic inhibition of AKT using small interfering RNA, and ongoing investigations should clarify the respective roles of AKT, S6 protein, 4EBP1, GLUT1, and VEGFR2 in the observed effects.

Together, our results identify the deregulation of the RAS-RAF-MEK-ERK pathway as a dominant determinant in cancer cell resistance to mTOR inhibitors. Recent studies have outlined the existence of oncogenic *PIK3CA* mutations concomitantly with *EGFR*, *KRAS*, or *BRAF* mutations. In non-small-cell lung cancer, mutation and amplification of *PI3K* have recently been reported to be involved in as much as 20% of lung tumors (29). Interestingly, if *EGFR*, *KRAS*, *RAF*, and *HER2* mutations are mutually exclusive, *PIK3CA* mutations can be associated with a mutation in one of these four proteins (29, 30). To date, the exact frequency of double mutations remains unknown either in primary lung tumors or in distant metastases, and significant discrepancies between both sites have already been suggested for *EGFR* (31) and *KRAS* mutations (32, 33). In colorectal cancer, a recent population-based study of 586 patients with colon adenocarcinomas found mutations in *KRAS*, *BRAF*, and/or *PIK3CA* in 316 (56%) of the 586 tumors studied. *KRAS* is the most commonly mutated gene in this pathway, with mutations in 35% to 45% of colorectal adenocarcinomas; mutations in *PIK3CA* (≤20%) and *BRAF* (<15%) are less common. Again, even if *KRAS* and *BRAF* mutations seem to be mutually exclusive, mutations in *PIK3CA* and *KRAS* may coexist within the same tumor (34).

Conclusion

A combination of the RAF/VEGFR2 inhibitor RAF265 with the mTOR inhibitor RAD001 is an effective strategy to enhance cytotoxic and antiproliferative effects on cells with deregulation of both the RAS-RAF and PI3K-PTEN pathways. Our results indicate that RAD001 and RAF265 inhibit their targets as well as downstream effectors thought to be in other pathways, providing evidence for cross-talk between the different signaling pathways studied. Therefore, concomitant PI3K-AKT-mTOR and RAF-MAPK inhibition is a promising strategy to overcome concomitant *KRAS* and *PIK3CA* oncogene activation. Together, these findings argue for the combination of targeted therapies in selected tumors. Further studies are needed to confirm the potential of this particular association in terms of tolerance and efficacy and to establish a translational project to select patients with both *PIK3CA*- and *KRAS*-mutated tumors who may benefit from combination therapy with RAF265 and RAD001.

Disclosure of Potential Conflicts of Interest

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2.3 Etude n°3 « Mise au point d'un modèle animal orthotopique bioluminescent »

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Bioluminescent Orthotopic Mouse Models of Human Localized Non-Small Cell Lung Cancer: Feasibility and Identification of Circulating Tumour Cells

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Abstract

Background: Preclinical models of non-small cell lung cancer (NSCLC) require better clinical relevance to study disease mechanisms and innovative therapeutics. We sought to compare and refine bioluminescent orthotopic mouse models of human localized NSCLC.

Methods: Athymic *nude* mice underwent subcutaneous injection (group 1-SC, n = 15, control), percutaneous orthotopic injection (group 2-POI, n = 30), surgical orthotopic implantation of subcutaneously grown tumours (group 3-SOI, n = 25), or transpleural orthotopic injection (group 4-TOI, n = 30) of A549-luciferase cells. Bioluminescent *in vivo* imaging was then performed weekly. Circulating tumour cells (CTCs) were searched using Cellsearch® system in SC and TOI models.

Results: Group 2-POI was associated with unexpected direct pleural spreading of the cellular solution in 53% of the cases, forbidding further evaluation of any localized lung tumour. Group 3-SOI was characterized by high perioperative mortality, initially localized lung tumours, and local evolution. Group 4-TOI was associated with low perioperative mortality, initially localized lung tumours, loco regional extension, and distant metastasis. CTCs were detected in 83% of *nude* mice bearing subcutaneous or orthotopic NSCLC tumours.

Conclusions: Transpleural orthotopic injection of A549-luc cells in *nude* mouse lung induces localized tumour, followed by lymphatic extension and specific mortality, and allowed the first time identification of CTCs in a NSCLC mice model.

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Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide. Despite numerous clinical trials of preclinically promising drugs, no major outbreak has been made in NSCLC management in the last decades. Although there is strong evidence based medicine supporting the clinical use of cytotoxic, around 3 types of agents have shown consistent but limited efficacy (platinum, taxanes, gemcitabine). More recently, targeted therapies have waived important hopes given their ability to directly hit cancer cell specific survival mechanisms. In sharp contrast with the robustness of the underlying biological rational, clinical translational of this knowledge into clinical benefit still remains challenging since targeted therapies showed only a marginal survival benefit when considering the whole NSCLC patients population [1–4]. After these initial trials, retrospective molecular analyses of tumor tissue led to the definition of subgroups of patients that favourably respond to investigational treatments, with management of certain cancers being revolutionized [5]. Among patients with NSCLC who receive erlotinib, the presence of an EGFR mutation increased responsiveness to the agent [6,7], but concerned only 16% of patients [8]. More recently, the inhibition of anaplastic lymphoma kinase (ALK) has been reported to result in tumour shrinkage or stable disease in most patients with NSCLC harbouring EML4-ALK fusion genes, but concerned only 2 to 7% of NSCLC [9]. However, in the meantime, considerable efforts have been spent in clinical trials testing active treatments on resistant tumors [2,3,10–12].

The discrepancy between the values of the clinical rationale, the amount of preclinical data on one hand and the low output of their transfer in clinical trials suggest that the relevance of preclinical models may be questioned. Especially, preclinical testing should

have a more clearcut positive versus negative predictive value [13]. Current in vivo preclinical models are not reflecting the steps of malignant progression from normal tissue to precancerous lesion and then to cancer (localized and then metastatic). Ideal model should mimic the natural history of human cancer, in order to improve our understanding of cancer pathogenesis, predict the efficacy of the investigated treatment and identify which treatment will fit to which patient before the design of clinical trials. In this context, xenografts models remain the cornerstones of preclinical experiments and will gain from which technical improvements, along with the recent development of computer-assisted drug design, computer modeling, and genetically engineered animals [14]. Human NSCLC xenografts may be implanted into immunodeficient animals either subcutaneously (SC) or orthotopically. In one hand, SC xenografts of lung cancer are easy to perform and to follow, but lack relevance regarding (i) the natural history of cancer due to absence of lymphatic or metastatic extension, and (ii) the prediction of drug efficacy in clinical trials as witnessed by the high proportion of negative clinical trials [13]. In the other hand, orthotopic models of lung cancer are technically more challenging, do not always mimic the natural history of lung cancer, but raise considerable hope regarding drug screening [15]. The recent increase in our understanding in the cross talk of the tumor stroma [16–18] and its involvement in drug resistance, metastasis, immune escape, angiogenesis strongly suggests that orthotopic models should provide valuable information that one could not obtain from sub-cutaneous models.

Considering thoracic tumors, the technical challenge is to grow "lung tumor cells within the chest". Several techniques may be used to this aim, including intra tracheal administration [19] or percutaneous orthotopic injection [20] of NSCLC cells in solution, and surgical orthotopic implantation of subcutaneously grown tumour originating from NSCLC cell line [21]. Intratracheal administration is associated with diffuse initial spreading of the cells to both lungs, interfering with progression and lung metastasis process, therefore favouring percutaneous and surgical models. However, technical feasibility and natural history of these two models have never been compared. This study sought to compare and refine these two orthotopic xenografts models of lung cancer, with the ultimate goal to reproduce human NSCLC progression, from initially localized intraparenchymatous tumor, to metastasis development and general worsening. For this purpose, we compared four xenografts models, including SC injection used as control, percutaneous orthotopic injection and surgical orthotopic implantation as previously described, and transpleural orthotopic injection as a new model. To allow longitudinal follow up of tumor progression and further evaluation of treatment efficacy, we used a cell line transfected with luciferase and performed in vivo bioluminescent imaging. Feasibility was assessed by perioperative mortality and engraftment rate. Clinical relevance was assessed by initial intra parenchymatous location, loco-regional extension, metastatic extension, median survival and histological aspect. In an exploratory analysis, identification of Circulating tumor cells (CTCs) was performed using the Cellsearch® assay (Veridex LLC, USA).

Materials and Methods

Cell line

Human A549 lung adenocarcinoma cell line stably transfected with luciferase (A549luc) was purchased from Caliper Lifesciences Corp. Cells were cultured in complete medium, consisting of 10% (v/v) fetal bovine serum, 2 mmol L-glutamine, and 50 units/mL penicillin-streptomycin in RPMI (all from Invitrogen). Cells were grown at 37°C and 5% CO2 in an incubator, tested as mycoplasm free using a PCR mycoplasma detection kit (MycoProbe, R&D Systems, MN, USA), and tested as luciferase positive using direct application of 2 mL of a 150 µg/ml solution of luciferine (Firefly Luciferin, Caliper Lifescience Corp, USA), followed by immediate bioluminescent imaging (IVIS system, Caliper Lifescience Corp, Figure 1a).

Animals

Six-week-old female athymic mice were purchased from Janvier (Elevage Janvier, Mayenne, France), and kept in appropriate conditions. Once arrived in our animal facility, mice were divided in 4 groups, underwent xenografts implantation following the protocols described below (Figure 1b), and subsequent bioluminescent imaging. Per procedure mortality rate, engraftment rate, locoregional and metastatic progression were determined. The end of the experiment was defined as cachexia, dyspnea, or clinical worsening. Animals were then humanely killed. Primary tumour, lungs, and mediastinal lymph nodes were surgically harvested, fixed using a formalin-free, water-based concentrate (Finefix, Milestone S.r.l., Sorisole, Italy), and embedded in paraffin. Histological examination was then performed. Cancer-related mortality was assessed. All animal experiments were approved by the local Ethics Committee (CEEA IRCIV/IGR n°26, registered with the French Ministry of Research), and were in compliance with the European Directive 86/609/CEE and French laws and regulation.

Subcutaneous (SC) injection (group 1 - SC)

A solution of 3×10^6 A549luc cells in 200 µL of culture medium was injected subcutaneously into the left flank region of the animals (n = 15), using 1-mL tuberculin syringes with 30 G hypodermic needles (Becton Dickinson, NJ, USA).

Percutaneous orthotopic injection (group 2 - POI)

Animals (n = 30) were anesthetized using isoflurane and putted in a position of dorsal decubitus. Under radiographic control, 3×10^6 A549luc cells in a solution containing 30 µL of culture medium, 10 µL of contrast medium (Omnipaque 300, GE Healthcare SA, France), and 10 µL of mouse sarcoma extracellular matrix (Matrigel, BD Biosciences, NJ, USA) were injected percutaneously into the left lung of the animals using 1-mL tuberculin syringes with 30 G hypodermic needles (Becton Dickinson, NJ, USA). Mice were then allow to rest on a heating carpet until fully recover.

Surgical orthotopic implantation (group 3 - SOI)

As a first step, 3×10^6 A549luc cells were injected subcutaneously into the flank region of 3 donor animals. Once SC tumours reached 50 mm3, tumours were harvested and subdivided into 1 mm^3 pieces constituting tumour grafts. Recipient animals (n = 25) were anesthetized using intraperitoneal injection of xylazine (20 mg/kg) and ketamine (100 mg/kg), intubated using a 22 G i.v catheter, mechanically ventilated (stroke volume 200 µL, respiration rate = 120 strokes/min, Ventilator Minivent Type 845, Harvard Apparatus, MA, USA) and putted in a position of right lateral decubitus. A 2-cm skin incision was made below the left scapula and a sharp dissection of the chest muscles was performed, in order to expose the costal layer. A 0.5 cm intercostals incision between the third and fourth costa on the chest wall was made, and the chest wall was opened. The left lung was taken up by a forceps, clamp with a carotid clamp (Scanlan International, MN, USA), incised with surgical knife, and the

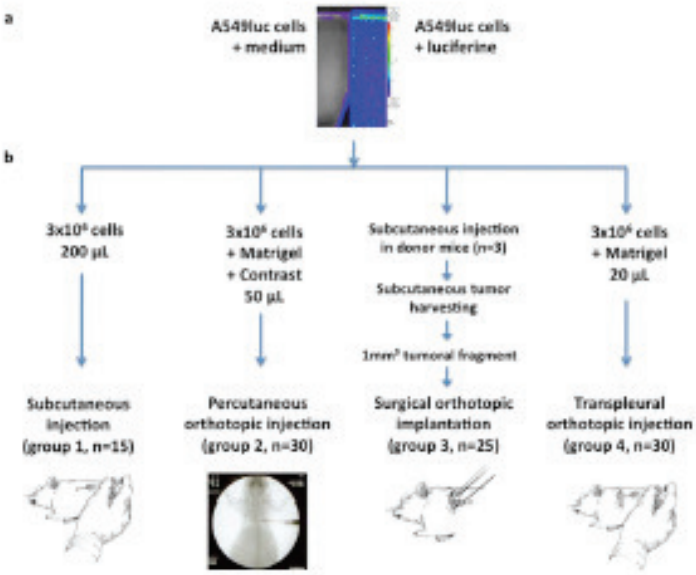

Figure 1. Study flowchart. a. A549 cells stably transfected with luciferase were tested by direct application of serum saline (left side, control) or luciferine (right side), and analysed after optical imaging (Time 1 min after addition of luciferine, exposure 1 min, bin medium). b. Animals were divided in 4 groups, subcutaneous injection (group 1-SC), percutaneous orthotopic injection under radiographic control (group 2-PO), surgical orthotopic implantation after orotracheal intubation and thoracotomy approach (group 3-SO), and transpleural orthotopic injection after costal layer surgical exposure (group 4-TO).
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tumour was introduced promptly into the lung parenchyma. The incision of the lung parenchyma was closed with surgical glue (Bioglue, Garnidu, France). The incision of the chest wall was closed by a 4-0 polypropylene suture (Prolene, Ethicon Inc, USA). Mice were then allow to rest on a heating carpet until fully recover.

Transpleural orthotopic injection (group 4 - TO)

Animals (n = 30) were anesthetized using intraperitoneal injection of xylazine (20 mg/kg) and ketamine (100 mg/kg), and putted in a position of right lateral decubitus. A 2-cm skin incision was performed below the left scapula, and a sharp dissection of the chest muscles was performed, in order to expose the costal layer. On observing the left lung motion through the pleura, 3×10^6 A549luc cells in a solution containing 10 µL of culture medium, and 10 µL of mouse sarcoma extracellular matrix (Matrigel, BD Biosciences, NJ, USA) was directly injected through the intercostal space into the lung to a depth of 3 mm using a 29 G needle permanently attached to a 0,5 mL insulin syringe (Becton Dickinson, NJ, USA). The skin incision was closed by a 4-0 polypropylene suture (Prolene, Ethicon Inc, USA). Mice were then allow to rest on a heating carpet until fully recover.

Bioluminescent imaging

Bioluminescent imaging was detected from luciferase expressing A549 cells (A549luc) after implantation of the xenografts into mice. Luciferin (Firefly Luciferin, Caliper Lifescience Corp, USA) was used as the substrate for the luciferase expressing tumour cells and injected intra peritoneally at a concentration of 150 mg/kg in PBS, 15 minutes before imaging. Mice were then anesthetized using 2% isoflurane and imaged using a cooled CCD camera (IVIS system, Caliper Lifesciences Corp, USA). Exposure times ranged from 1 minute to 1 second. Images were quantified as photons/s using the Living Image software (Caliper Lifesciences Corp, USA). Bioluminescent imaging was performed at day one, then weekly, and then immediately before sacrifice. Xenograft implantation rate was defined as the number of primary tumour on imaging 2 weeks after grafting divided by the number of animal alive after the procedure. Subsequent locoregional extension, lymphatic and haematogenous metastasis rates were determined during a 2-month follow up, and confirmed by pathologic examination at the end of the experiments.

Identification of CTCs

To further characterize SC and transpleural injection models, we sought to identify CTCs in these models, using 2 additional groups. In the CTC group 1, athymic nude mice underwent SC injection of A549luc cells in both flanks (n = 6). In the CTC group 2, animals underwent general anesthesia, chest wall incision, and transpleural injection of cells in the parenchyma of the left lung (n = 15). After 2 weeks, bioluminescent imaging was performed, and tumour-bearing animals were identified. During the third week, 6 tumour-bearing animals from each group were randomly chosen and anesthetized. A venous blood puncture of 600 µL was performed in the cavernous sinus and was tested for CTC using the CellSearch® system and a modified protocol based on the

CellSearch® Epithelial Cell kit (Veridex LLC, USA). Briefly, to identify human CTCs in mouse blood, each 600 µL mice blood samples was mixed with 7 mL of healthy human blood. Then, each sample was automated enriched for cells expressing the epithelial-cell adhesion molecule (EPCAM) with antibody-coated magnetic beads, and cells were labeled with the fluorescent nucleic acid dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescently labeled monoclonal antibodies specific for leukocytes (CD45-allophycocyan) and epithelial cells (cytokeratin 8,18,19-phycoerythrin) were used to distinguish epithelial cells from leukocytes. Cell Enrichment and labeling were performed using the CellSearch® Autoprep. The identification and enumeration of CTCs was performed using the CellSearch® Analyzer II. CTCs were defined as nucleated cells lacking CD45 and expressing cytokeratin 8, 18, 19. As a negative control, a solution containing 600 µL of non-tumor bearing mouse blood and healthy human blood was analyzed. As a positive control, 50 and 500 A549luc cells were analyzed in a solution containing 600 µL of medium and 7 mL of healthy human blood.

Statistical analysis

Normally distributed continuous variables were expressed as means ± standard deviation, and compared with unpaired t-tests. Categorical data were expressed as counts and proportions, and compared with Fisher exact tests. All tests were 2-sided and a p-value<.05 was considered significant. All data analyses were performed with the free R software (http://www.r-project.org, R Foundation for Statistical Computing, Vienna, Austria).

Results

Feasibility

Feasibility was assessed by determination of perioperative mortality and engraftment rate. The perioperative mortality was null in group 1-SC used as a control, and significantly higher in group 3-SOI (60%, p<0.001) but not in groups 2-POI (6%, p = .79) or 4-TOI (8%, p = .72). Assessing the effect of a learning curve on the perioperative mortality of group 3-SOI, we observed a non significant decrease in the mortality between the first 10 animals and the former 15 animals (mortality 80% vs 47%, respectively, p = .21). No significant difference was observed between groups 2-POI and 4-TOI regarding the perioperative mortality rate (p = .99, **Table 1**). The engraftment rate was 100% in group 1-SC used as a control, and significantly lower in group 2-POI (68%, p = .037), group 3-SOI (60%, p = .034) and group 4-TOI (65%, p = .027). No significant difference was observed between the 3 experimental groups regarding the engraftment rate (p = .90, **Table 1**).

Clinical relevance

Clinical relevance was assessed by location of the initial tumor, imaging of tumor extension, survival analysis and histological examination. The initial tumor was confined to the SC tissue in group 1-SC and to the lung parenchyma in group 3-SOI. In group 2-POI, 53% of the animals underwent pleural seeding during percutaneous orthotopic injection and developed initial loco regional extension to both pleurae. As the goal of this study was to obtain an intrapulmonary localized NSCLC model, this immediate pleural seeding disqualified the technique. In group 4-TOI, the tumour was initially confined to the lung parenchyma (**Table 1, Figure 2a and 2b**). Bioluminescent imaging found irregular curves in group 1-SC and 3-SOI, with high inter individual and chronological variations. No animal developed locoregional or metastatic extensions during follow up. In group 2-POI, no bioluminescence curve was plotted because of perioperative pleural seeding. One animal developed bone metastasis. In group 4-TOI, bioluminescent imaging found a regular, exponential curve after day 21. Seven percents of the animals developed locoregional extension to homolateral pleura as an evolution of the primary tumour. One animal developed bone metastasis (**Table 1, Figure 2a**). Survival analysis revealed that median survival was not achieved after a follow up of 2 months in group 1-SC and 3-SOI. In group 2-POI, no survival curve was plotted because of perioperative pleural seeding. In group 4-TOI, median survival was 40 days (**Table 1, Figure 2c**). Histological examination revealed SC round tumours with a regular shape, high proportion of undifferentiated carcinoma, few capsular ruptures, and few vascular or lymphatic emboli in group 1-SC. In group 2-POI, regular intraparenchymatous tumors were obtained, with a low proportion of undifferentiated carcinoma, a high number of capsular ruptures, and vascular or lymphatic emboli. In group 3-SOI, irregular intraparenchymatous tumors were obtained, with a low proportion of undifferentiated carcinoma (+), capsular ruptures (++) and a high number of vascular or lymphatic emboli. The findings were similar in group 4-TOI and group 2-POI (**Figure 3, Table 2**).

Identification of CTCs

Xenograft implantation rates were 100% in CTC group 1 (SC, n = 6) and 60% in CTC group 2 (TOI, n = 9). Three Cellsearch® assays were performed in each control group, and 6 Cellsearch® assays were performed in each experimental group. Negative controls demonstrated zero or one CD45−, DAPI+, CKPE+ cell per assay (data not shown). Positive controls demonstrated a correlation between the number of tumor cells in solution and the Cellsearch® CTC count (**Figure 4a and 4b**). In CTC group 1-SC, 5 assays (83%) were positive for CTCs detection (CTCs level

Table 1. Per-procedure mortality, tumor engraftment, and tumor extension rates.

	Number of animals (n)	Per-procedure mortality rate (n)	Implantation rate (n)	Loco-regional invasion (n)	Metastasis (n)
Subcutaneous injection (group 1)	15	0	100% (15)	-	-
Percutaneous orthotopic injection (group 2)	30	6% (2)	60% (19)	53% (15)	3% (1)
Surgical orthotopic implantation (group 3)	25	60% (15)	60% (6)**	-	-
Transpleural orthotopic injection (group 4)	30	3% (1)	65% (19)	7% (2)	3% (1)*

*Bone metastasis.
**n = 1 false positive (infection); n = 2 false negatives.
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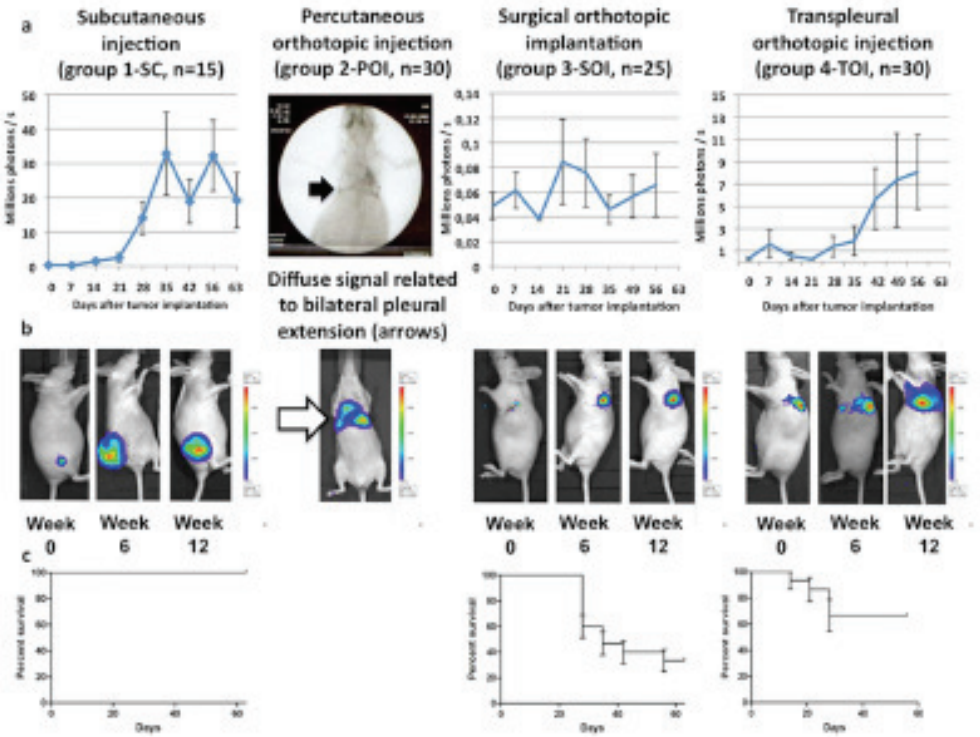

Figure 2. Follow-up using bioluminescent imaging and survival curves. a. Evolution of the photons count (expressed as 10^6 photons/s) over time, from day 0 (implantation) to day 63 (end of the bioluminescent follow up). For each time, the mean and standard deviation of the animals alive are reported. In group 2-POI, percutaneous injection led to pleural seeding in 53% of the cases, as postoperative X-ray showed contrast agent in either left pleural space, right pleural space (anatomical continuum), or both. b. Representative evolution of the bioluminescent signal, from day 14 to day 42. In group 2-POI, pleural seeding created a signal located at the right basis of the thorax, contradicting with the primary goal of this study. No subsequent follow-up as been performed for this group. c. Survival curve from the time of tumor implantation to 2 months using the Kaplan-Meier method and 95% confidence interval bars. The 2-month survival is 100% in group 1-SC, 40% in group 3-SOI, and 65% in group 4-TOI.
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range 2–5, mean 2.5±1.76). In CTC group 3-TOI, 5 assays were positive for CTCs detection (CTCs level range 2–21, mean 9.5±9.35). All together, 3 weeks after tumour implantation, CTCs were detected in 83% of nude mice bearing either SC or orthotopic NSCLC tumours (**Figure 4c and 4d**). When comparing groups, the difference in CTC levels showed a trend toward statistical significance ($p = .058$, **Figure 4c, Table 2**).

Discussion

Tumor implantation site and method impacts tumor development and kinetics

Comparing SC, percutaneous orthotopic injection and surgical orthotopic implantation models with intend to refine the follow up of tumour progression using bioluminescent imaging, we faced the limits of these models, including organ discrepancy (SC), frequent pleural seeding (percutaneous orthotopic injection) and high perioperative mortality (surgical orthotopic implantation). Furthermore, none of these models allow a reliable follow up of tumour progression using bioluminescent imaging. Therefore, we developed a transpleural orthotopic injection model, combining low perioperative mortality, reasonable engraftment rate, scarcity of pleural seeding, and tumour progression with metastasis for which tumor progression may be monitored by bioluminescent imaging. Furthermore, this study allows identification of CTCs in murine models of NSCLC for the first time to date. We believe that besides functional imaging, the application at the preclinical in vivo stage of CTCs will contribute to define biomarkers of tumour burden and treatment efficacy in subsequent clinical trials.

SC xenografts models

SC xenografts from human-derived cell lines to immunodeficient animals have been the gold standard of preclinical cancer experiments for decades [14]. This model presented several advantages, including technical feasibility (absence of anesthesia, easy-to-perform injection) and tumor accessibility (direct measurement of the SC tumor allowing longitudinal follow up). However, SC models are limited by discrepancy between xenograft origin

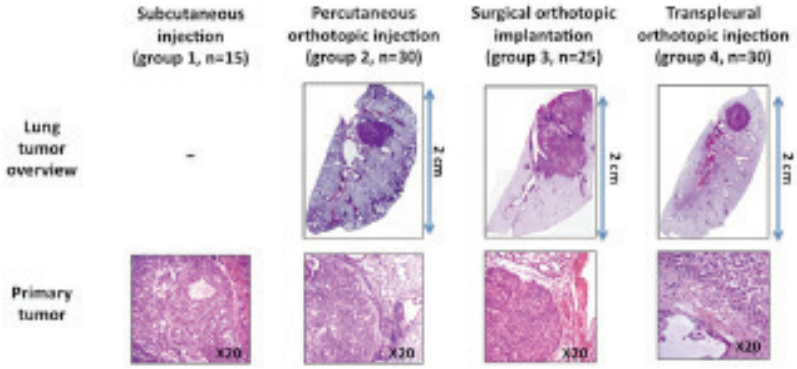

Figure 1. Pathological examination. Surgical orthotopic implantation (Group 3) resulted in localized infiltrating tumors, whereas percutaneous orthotopic injection (Group 2) and transpleural orthotopic injection (group 4) resulted in localized rounded nodules. Histological examination revealed a high proportion of undifferentiated carcinoma in group 1-SC tumor, but a low proportion in the 3 experimental groups. Furthermore, the 3 experimental groups were associated with more capsule rupture and vascular emboli than group 1-SC.
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and host microenvironnement, including extra cellular matrix, paracrine signals, and therefore do not model Paget's "seed and soil" theory [22]. This discrepancy has been found to impact tumour response to cytotoxic agents and radiation therapy [23,24] and certainly impact the response to novel anticancer agents. This discrepancy might be of critical importance in NSCLC, were hypoxia and neoangiogenesis play a major role in tumor progression [25], and occur differently in SC and intrathoracic xenografts [26]. Last, only a minority of SC models disseminate and progress to the metastatic stage, the absence of progression from localized to metastatic stage through loco-regional lymph node extension is therefore a major limitation of SC models while the major cause (2/3) of cancer death is metastatic disease.

These differences have major implications regarding tumor progression, with few circulating tumor cells and no metastasis in our experiments, pointing a major limit to drug evaluation, with two risks. The first risk is to push into the clinic a molecule or a drug combination with little or no efficacy. As an example, the combination of chemotherapy and EGFR inhibitors has proved efficacy in SC models of NSCLC [27,28], but has never reached significant efficacy in clinical trials including unselected NSCLC patients upon EGFR status [3,10,29]. The second risk is to stop the development of promising drugs. As an example, HIF-1alpha antagonist PX-478 showed no measurable activity against NSCLC SC xenografts, but inhibited progression and spread of orthotopic human small cell lung cancer and lung adenocarcinoma in mice, and is finally tested in phase 1 trial [30].

Altogether, these data suggest that SC xenografts are not fully adequate for the preclinical study of NSCLC, and should only be used and analyzed carefully. Similar conclusions have been drawn from SCLC preclinical drug testing [31].

Previous orthotopic models

To take the influence of organ specificity and tumor micro environnement into account, orthotopic models have been progressively developed over the last 20 years, with the ultimate goal to obtain a single intraparenchymatous lung tumor that mimic the clinical situation and allow longitudinal follow up. Intravenous [32], intrabronchial [33–35] and intrapleural administration [36,37] of lung cancer cell lines resulted in pleural, locally advanced, or multiple synchronous tumors. Therefore, these models may be interesting to study these particular situations, but may not be extended to localized intrapulmonary NSCLC, constituting the majority of NSCLC patients and the basis of further tumor progression.

Genetically engineered mouse models (GEMMs) have also been developed to study NSCLC in mice. These animals are usually p53 mutated, favoring genetic instability and tumor formation, and develop lung tumors driven by the EGFR [38], KRAS [39], PIK3CA [40], BRAF [41] oncogenic activation or the presence of

Table 2. Pathological examination and CTC levels.

	Primary tumor	Undifferenciated population	Capsular rupture	Emboli	CTC
Subcutaneous injection (group 1)	SC	+++	+	+	
Percutaneous orthotopic injection (group 2)	lung+pleura	+	+++	+++	2.5±1.76/animal
Surgical orthotopic implantation (group 3)	lung	+	++	+	
Transpleural orthotopic injection (group 4)	lung	+	+++	+++	9.5±9.25/animal

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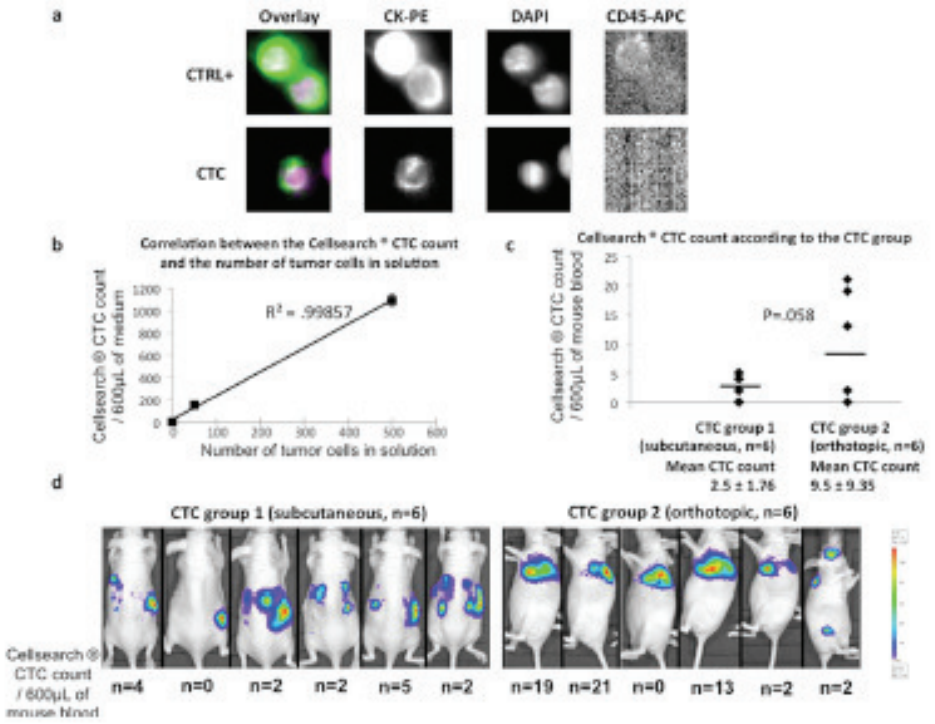

Figure 4. Identification of circulating tumor cells. a. Representation of the staining of circulating cells using the Cellsearch® assay. CTC are defined as CD45−, DAPI+, and CKPE+ cells. - Upper line: as a positive control, 50 and 500 A549luc cells were analysed in a solution containing 600 µL of medium and 7 mL of healthy human blood. Here, as an example, both CD45−, DAPI+, CKPE+ cells are tumor cells. - Bottom line: representation of the results of the experimental groups. Here, as an example, this CD45−, DAPI+, CKPE+ cell is a CTC. b. Correlation between the Cellsearch ® CTC count and the number of tumor cells in solution. As controls, 0 (negative control), 50 and 500 (positive controls) A549luc cells were analysed in a solution containing 600 µL of medium and 7 mL of healthy human blood. This experiment demonstrated a correlation between the Cellsearch® CTC count and the number of tumor cells in solution (R^2 = .99857). c. Number of CTC according to the experimental group, CTC group 1 (SC) or CTC group 2 (orthotopic). The overall positivity of the Cellsearch ® assay is comparable in both groups (83%), with a trend toward more CTC in the orthotopic rather than in the subcutaneous model. d. Representation of the bioluminescent signal and number of CTC detected in each of the 12 animals.
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EML4-ALK fusion oncogene [42]. KRAS mutated lung cancer may be observed in mouse strains carrying oncogenic alleles of KRAS that can be activated only on a spontaneous recombination event in the whole animal [43], or after KRAS sporadic activation through Cre-lox mediated somatic recombination after adenoviral mediated delivery of Cre recombinase in lung epithelia [39]. Both models allow the development of KRAS-mutated lung cancer in a short period of time. However, these models are limited by their distance to clinical situation, as mutations of one or two oncogenes or tumor suppressor genes are far from the multi step progression of lung cancer occurring on underlying chronic inflammation. Furthermore, GEMM models result in hundreds of primary NSCLC in the same period of time those are difficult to follow and to quantify during preclinical testing and non-invasive imaging. Despite these drawbacks, GEMM have the advantage to refine the definition of oncogenes and to take immune response into account. Recent studies have reported that GEMM may be of interest when studying the efficacy of common NSCLC treatments [44].

Surgical implantation of NSCLC fragment into the lung of immunodeficient mice via thoracotomy was first reported in 1992 [21,45,46]. Tumor fragment came from primary fresh tumors or subcutaneously-grown tumors developed after NSCLC cells injection. Tumor fragment were inserted into the lung parenchyma, on the visceral pleura, or on the parietal pleura, according to the disease extension aimed for. Per procedure mortality was below 10%, engraftment rates ranged between 60 and 100%, but not systemic metastasis was observed. The same model was then developed in rat [47] and confirmed using primary cultured cells from surgical-resected NSCLC in SCID mice [48]. Addition of GFP to implanted tumor allow in vivo imaging of orthotopic xenografts development, and metastatic extension to mediastinal lymph nodes, contralateral lung, and bone [49–52]. However, these experiments are technically difficult, require specialized laboratory and dedicated manpower, and despite encouraging preliminary results [53], have not been generalized in preclinical NSCLC research to date.

Orthotopic transpleural injection combined to bioluminescent imaging

Orthotopic injection of NSCLC cells in nude mice has been developed to overcome the technical difficulties of thoracotomy [20,54,55]. This technique is technically feasible with a low mortality rate and a high engraftment rate, with 2 limits. The first limit is the impossibility to predict perioperatively if the tumor will be localized to the lung parenchyma or if there is a pleural seeding leading to a locally advanced tumor. Therefore, to obtain a single tumor limited to the lung parenchyma, we used a high number of cells in a small volume of medium and add some matrix of mouse sarcoma, improving the solution anchorage to the lung parenchyma. We injected this solution trans pleurally, under direct vision of the lung motion through the intercostal space, combining technical feasibility (absence of orotracheal intubation and mechanical ventilation) and high precision.

The second limit is the requirement of a longitudinal follow up using micro CT in the absence of fluorescent or bioluminescent imaging. MicroCT has limited spatial resolution and is available only in a limited number of facilities. Therefore, we used luciferase-transfected NSCLC cell line to allow in vivo bioluminescent imaging. This technique has already been reported in a percutaneous orthotopic model with pleural seeding [56], and has now been applied successfully to transpleural model with localized intra parenchymateous NSCLC model.

This xenogenic, orthotopic and bioluminescent model allowed the first time identification of CTCs in murine models of NSCLC. CTCs are released into the bloodstream from tumors of epithelial origin [57].

As haematogenous dissemination of tumor cells is the main mechanism for distant metastasis, the assessment of cancer patients' blood is a highly desirable approach for detecting systemic tumor cell spreading [58] and residual disease. CTCs may be identified directly after automatic enrichment and immunocytochemical detection (CellSearch® System) [59], magnetic bead enrichment and laser scanning cytometry (Maintrac System) [60], microfluidic harvesting and molecular characterization (CTC-Chip) [61], or membrane filter device (Isolation by Size of Epithelial Tumor cells – ISET) [62]. The CellSearch® system is the most available, and is now FDA-approved in breast, colorectal, and prostate cancers. This new application to NSCLC preclinical model could be of significant interest to study the role of CTCs in NSCLC progression and response to new therapeutic strategies.

Study limits and future developments

Our study has important limitations regarding the use of immunodeficient animals and the heterogeneous genetic background of the xenografts, as the validity of xenograft models remains disputed by the compromised of anti tumour immunity and the absence of clearly characterized molecular abnormalities in comparison with GEMM. Therefore, we believe that the next step refinement in preclinical models in vivo could take advantage of the *in situ* implantation of tumour fragments or cells into the lung as well as the advantage of murine tumour models (immune response and GEMM derived tumours).

The application of current NSCLC treatments to both models would ultimately determine the most relevant model for preclinical studies. The value and the additional benefit of these novel approaches will have to be compared not only to other techniques available but also in terms of go/no go decision making value toward the clinical stage. Our vision is that functional imaging and biological pre and per treatment parameters such as CTCs may also contribute to demonstrate the improvement in clinical value of these approaches. In particular, we propose that orthotopic implantation of GEMM derived lung tumours into the lung could be an optimal strategy to evaluate tumour immunity and to monitor the efficacy of therapies at the non metastatic, locally advanced tumour stage (i.e. surgery and/or radiotherapy combined to novel agents).

Conclusion

The tumour microenvironment plays a major role in promoting tumour growth [63]. In xenografts models, tumour localization is important regarding sensitivity to chemotherapy [23], and will be critical regarding sensitivity to targeted therapies, as suggested by the involvement of integrin in the interactions between the tumour and its environment, and subsequent cross talks with targeted pathways. For these reasons, we have developed a murine intrapulmonary model of human non-small cell lung cancer, associated with low peri operative mortality, high engraftment rate, locoregional grow, and development of metastasis. This model allows the first time identification of a high number of circulating lung tumour cells in mice bearing human xenografts of NSCLC.

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Author Contributions

Conceived and designed the experiments: PM YL J-CS M-CV CD ED. Performed the experiments: PM BL. Analyzed the data: PM YL J-CS M-CV CD ED. Contributed reagents/materials/analysis tools: PM YL J-CS M-CV CD ED. Wrote the paper: PM YC GL ED.

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3 Discussion

3.1 Mutations oncogéniques de *KRAS*

Des mutations de *KRAS* sont retrouvées dans 20 à 30% des tumeurs solides humaines, et particulièrement dans les localisations pancréatiques, colorectales, endométriales, bilaires, cervicales utérines, et pulmonaires (Schubbert *et al*, 2007). Dans le cas du CBP-NPC, les mutations de *KRAS* sont un facteur de mauvais pronostic (Mascaux *et al*, 2005). De plus, si l'influence du statut *KRAS* sur la réponse aux chimiothérapies conventionnelles n'est pas prouvée (Loriot *et al*, 2009 ; Annexe 2), l'activation oncogénique de *KRAS* est un facteur de résistance aux inhibiteurs des récepteurs membranaires aux facteurs de croissance.

Dans le cancer bronchique, les mutations de *KRAS* sont associées à une absence de réponse des inhibiteurs de l'activité tyrosine kinase de l'EGFR tels que le gefitinib et l'erlotinib (Eberhard *et al*, 2005). Dans le cancer colorectal, plusieurs études incluant des patients sous cetuximab (Lievre *et al*, 2006 ; Di Fiore *et al*, 2007 ; Karapetis *et al*, 2008) ou panitumumab (Amado *et al*, 2008) donnés seuls ou en combinaison à la chimiothérapie conventionnelle ont montré que *KRAS* était un facteur prédictif significatif de la réponse aux anti-EGFR. La recherche de mutations *KRAS* est donc un indicateur important pour le choix d'un traitement anti-EGFR, prescrit seul ou en association à la chimiothérapie conventionnelle.

Cependant, des données récentes nous rappellent que les mutations acitvatrices de la protéine KRAS ne sont pas toutes équivalentes. La plupart des mutations de *KRAS* surviennent au sein des codons 12 et 13. En reprenant les prélèvements tumoraux de 215 patients inclus dans un

essai de thérapies ciblées en traitement d'un CBP-NPC avancé, Ihle *et al.* ont recherché un lien entre le type d'activation de la protéine KRAS, la survie sans progression, et l'expression des différents gènes. Les données ont ensuite été confirmées sur 67 lignées cellulaires. Les patients présentant une mutation KRAS - Gly12Cys ou KRAS - Gly12Val avaient une survie sans progression moins bonne que les patients présentant une autre mutation ou deux allèles sauvages. De plus, l'étude des lignées cellulaires montrait que la mutation KRAS - Gly12Asp entraînait une activation des voies de signalisation PI3K – AKT - mTOR et RAF – MEK - ERK, alors que les mutations KRAS - Gly12Cys et KRAS - Gly12Val entraînaient une activation de Ral et une diminution de l'activation d'AKT dépendante des facteurs de croissance. Il sera donc désormais indispensable de prendre en compte le type de mutations de KRAS dans le choix des traitements ciblant les effecteurs de cette protéine (Ihle *et al*, 2012).

3.2 Traitements pharmacologiques ciblés des tumeurs mutées KRAS

Malgré une décennie d'effort, aucune des tentatives d'inhibition pharmacologique directe de la forme activée de la protéine KRAS n'a abouti à un résultat préclinique ou clinique probant (Cox *et al*, 2002 ; Young *et al*, 2009). Les efforts thérapeutiques se sont donc déplacés en amont vers des pan-inhibiteurs HER, et en aval, vers les effecteurs de KRAS, avec des stratégies d'inhibition de HSP90, d'AKT, et d'association thérapeutiques ciblant les effecteurs de RAS.

3.2.1 Pan-inhibition des récepteurs HER

En amont de KRAS, la famille de récepteurs transmembranaires HER comporte 4 membres : HER1/EGFR, HER2/neu, HER3, et HER4. La fixation du ligand EGF sur l'EGFR induit l'homo et l'hétérodimérisation du récepteur, activant les voies de signalisation intra-cellulaire. On sait que les inhibiteurs compétitifs et réversibles de l'EGFR, erlotinib et gefitinib, n'ont pas d'efficacité en cas d'activation oncogénique de KRAS. Cependant, cette approche a été étendue par le développement de pan-inhibiteurs irréversibles de HER1, HER2, et HER4 (Ou *et al*, 2012).

L'un de ces inhibiteurs, le PF-00299804 ou dacomitinib, a été largement étudié en préclinique, puis en clinique jusqu'à un essai de phase 2. *In vitro*, le dacomitinib présente une efficacité supérieure à l'erlotinib et au géfitinib sur les lignées cellulaires mutées pour l'EGFR. Le dacomitinib reste efficace sur des lignées cellulaires rendues résistantes à l'erlotinib et au géfitinib par la transfection de la mutation T790M de l'EGFR, avec des IC50 de l'ordre du nanomolaire. Cependant, le dacomitinib n'était pas efficace sur les 2 lignées de CBP-NPC présentant des mutations de *KRAS*, A549 et H441. Sur la lignée A549, l'IC50 n'a

pas été atteinte. Sur la lignée H441, l'IC50 n'a été atteinte que pour des concentrations d'inhibiteur supérieures à 4 μM, et reflétait plus probablement un effet off-target qu'une inhibition spécifique, conduisant les auteurs à ne pas tester cette lignée cellulaire *in vivo* (Engelman *et al*, 2007).

En clinique, le dacomitinib est bien toléré, et a pu être testé au cours d'un essai de phase 2, randomisé, récemment publié. Chez des patients atteints de CBP-NPC métastatique, résistant à une ou deux lignes de chimiothérapie, et non sélectionnés sur le plan moléculaire, le dacomitinib n'offrait qu'un intérêt limité par rapport à l'erlotinib, avec 1 mois de gain de survie sans progression sur la population globale. Ce bénéfice limité recouvrait des situations différentes selon la présence d'une mutation *KRAS*, avec un bénéfice de 1,8 mois chez les patients *KRAS* sauvage, et une absence de bénéfice chez les patients *KRAS* mutés (Ramalingam *et al,* 2012). Les pan-inhibiteurs HER ne semblent donc pas efficaces dans le traitement des CBP-NPC *KRAS* mutés.

3.2.2 Inhibition de HSP90

Dans la cellule normale, la protéine chaperone heat shock protein 90 (HSP90) est indispensable à la stabilité et à la maturation de nombreuses protéines clientes impliquées dans la transduction du signal intra-cellulaire. Beaucoup de ces protéines clientes jouent un rôle indispensable dans la croissance et la survie cellulaire, comme EGFR, RAF, ou AKT. Dans la cellule tumorale, la machinerie associée à HSP90 est utilisée pour protéger un grand nombre d'oncoprotéines d'une dégradation ciblée, facilitant ainsi l'addiction aux oncogènes et la survie cellulaire (Trepel *et al*, 2010).

L'inhibition de HSP90 conduit à la dégradation de ses protéines clientes par le protéasome. Cette inhibition pharmacologique permet une action coordonnée et simultanée sur plusieurs

voies de signalisation, permettant d'inhiber la transmission du signal malgré sa redondance. Cette inhibition pharmacologique permet également d'échapper à certains mécanismes de résistance utilisés par la cellule tumorale. Les inhibiteurs de HSP90 sont actuellement en cours d'essais cliniques dans plusieurs types de tumeurs solides (Xu *et al*, 2007).

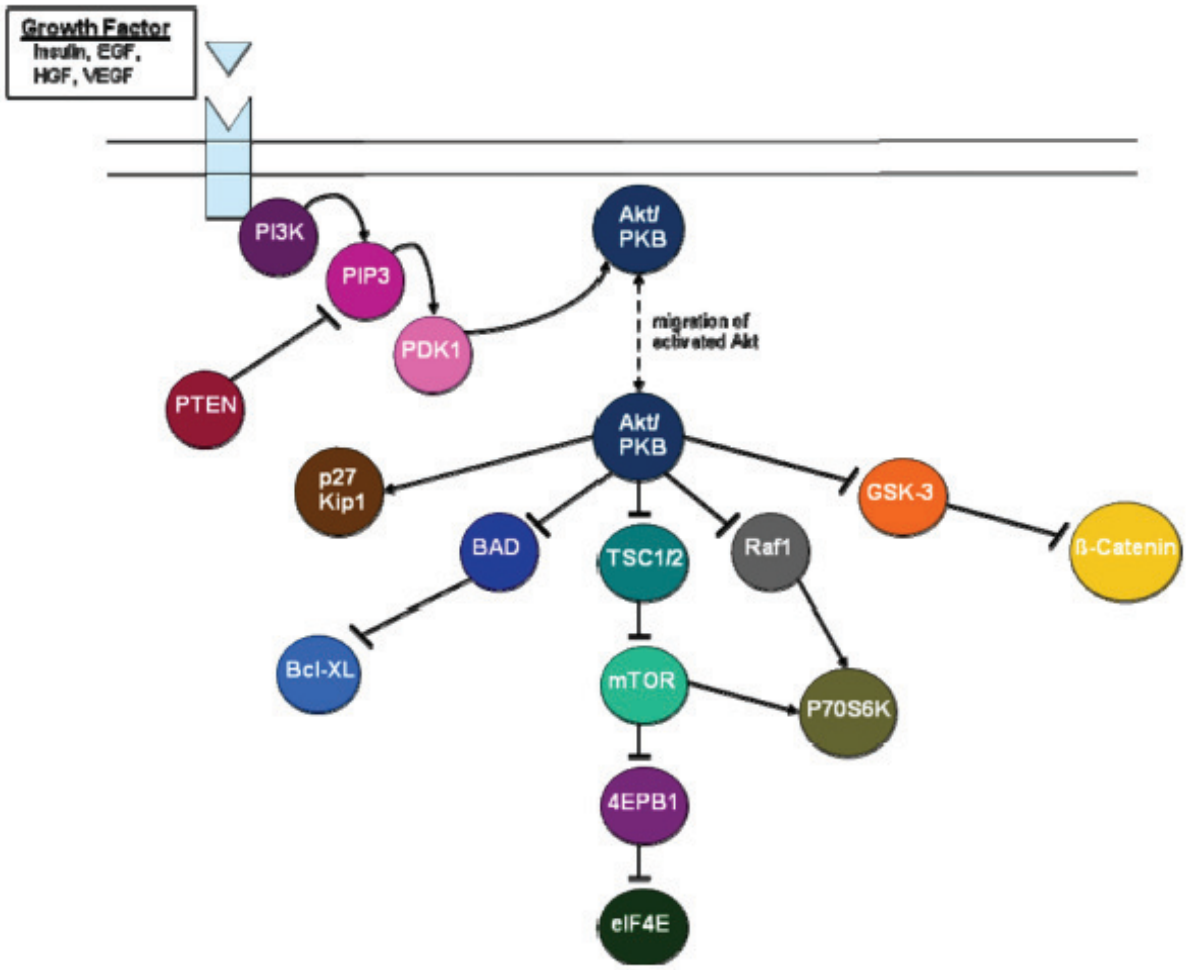
Récemment, Acquaviva *et al*. ont étudié l'efficacité de l'inhibiteur de HSP90 ganetespib sur des lignées de CBP-NPC présentant différents statuts mutationnels de *KRAS*. *In vitro*, le ganetespib entraînait une inhibition de la phosphorylation de RAF, MEK, ERK, et AKT et une activation de l'apoptose sur toutes les lignées cellulaires testées. Il est intéressant de noter que malgré un effet cytotoxique prononcé, l'inhibition de HSP90 n'entraînait une inhibition de la phosphorylation de 4EBP1 que sur la lignée H441, suggérant que l'inhibition de HSP90 n'entraîne pas toujours une inhibition des effecteurs de mTOR (Acquaviva *et al*, 2012).

In vivo, l'association du ganetespib et de l'inhibiteur de PI3K/mTOR BEZ235 était un traitement efficace sur un modèle de xénogreffes SC de lignée A549. Au niveau moléculaire, l'inhibiteur de HSP90 supprime la boucle de rétrocontrôle qui suit l'inhibition de MEK et de PI3K-mTOR, même si cela n'explique qu'incomplètement l'activité de cette association thérapeutique. De plus, le ganetespib sensibilise les lignées cellulaires de CBP-NCP mutées *KRAS* aux agents cytotoxiques classiques, qu'il s'agisse d'inhibiteurs de la topoisomérase ou d'agents alkylants (Acquaviva *et al*, 2012). Des essais cliniques de phase 1 sont en cours, mais le manque de spécificité de l'inhibition de HSP90 reste un obstacle majeur à son utilisation en association avec d'autres thérapies ciblées.

3.2.3 Inhibition de AKT

AKT est un nœud central dans les voies de signalisation situées en aval de RAS. AKT possède de nombreux substrats, avec des implications aussi larges que la prolifération cellulaire, la résistance à l'apoptose, le métabolisme du glucose et des acides gras. AKT peut être activée directement par l'activation de KRAS ou de PI3K ou indirectement par la perte de pTEN. L'activation d'AKT est un facteur de résistance aux inhibiteurs de l'activité tyrosine kinase des récepteurs transmembranaires, tels que HER2, les traitements anti-hormonaux, et les cytotoxiques classiques. L'inhibition pharmacologique d'AKT est donc une piste intéressante en cancérologie (Figure 19 ; Pal *et al*, 2010).

Figure 19. Cascade de transmission du signal dépendant d'AKT (Pal *et al*, 2010)



Malgré une abondante littérature préclinique, seul un petit nombre d'inhibiteurs d'AKT est arrivé au stade clinique, le plus avancé étant la périfosine, actuellement testée dans deux essais de phase 3 incluant des patients atteints de carcinome colorectal métastatique ou de myélome multiple (Pal *et al*, 2010). La principale limite de ces inhibiteurs est liée à la cible elle-même. En effet, l'activation d'AKT n'est pas spécifique de la cellule tumorale, et de nombreuses molécules inhibitrices d'AKT ont été stoppées dans leur développement pour des problèmes de toxicité (Hixon *et al*, 2010).

De plus, l'application de ces molécules aux tumeurs mutées *KRAS* reste limitée, car l'activation de la protéine KRAS aboutit à une activation simultanée des voies de signalisation PI3K-AKT-mTOR et RAF-MEK-ERK, avec des possibilités d'hyperactivation de la voie RAF-MEK-ERK en cas d'inhibition limitée à AKT.

A titre d'exemple, une étude récente a testé l'efficacité de l'AZD5363, un inhibiteur spécifique d'AKT, sur des lignées cellulaires présentant différentes mutations de *KRAS*, *PI3K*, et *pTEN*, et a retrouvé une corrélation significative entre la présence d'une mutation de *PI3K* ou de *pTEN* et la sensibilité à l'AZD5363 d'une part, et la présence d'une mutation de *KRAS* et la résistance à la drogue d'autre part (Davies *et al*, 2012).

Ainsi, la présence de mutations *KRAS* rend la croissance tumorale indépendante de l'axe PI3K-AKT-mTOR, car la traduction protéique dépendant de la coiffe est activée de façon redondante par la voie RAF-MEK-ERK. Certains types tumoraux, comme les cancers du sein et de la prostate, pourraient donc bénéficier d'un traitement par inhibiteur d'AKT, alors que les tumeurs présentant des mutations *KRAS* fréquentes, comme les cancers du colon, du poumon, ou de l'endomètre, pourraient bénéficier d'associations thérapeutiques comprenant un inhibiteur d'AKT et un inhibiteur de l'axe RAF-MEK-ERK.

Mais d'autres mécanismes peuvent également limiter l'efficacité des inhibiteurs d'AKT donnés en monothérapie. *In vitro*, il s'agit des lignées cellulaires présentant des niveaux élevés de phosphorylation de p70S6K à l'état basal, que cette hyperphosphorylation dépende de l'activation des protéines KRAS ou PI3K (She *et al*, 2010). Ces limites favorisent clairement les associations thérapeutiques ciblant les deux voies de signalisation d'aval de KRAS, c'est à dire PI3K-AKT-mTOR et RAF-MEK-ERK.

3.2.4 Association d'inhibiteurs de tyrosine kinase

Les associations d'inhibiteurs des tyrosines kinases (TKI) ont pour but de dépasser les résistances aux monothérapies développées plus haut. Legrier *et al.* ont montré que l'association d'un inhibiteur de MEK et d'un inhibiteur de mTOR était synergique *in vivo* sur des modèles de xénogreffes sous-cutanées de lignées A549 (*KRAS* G12S) mais pas H157 (*KRAS* G12R et *PTEN* G251C) (Legrier *et al*, 2007). Dans une autre étude, Engelman *et al* ont créé un modèle murin d'adénocarcinome pulmonaire initié et maintenu par l'expression de p110-α H1047R (*PIK3CA*). Le traitement de ces souris par le NVP-BEZ235, un double inhibiteur de PI3K et de mTOR, conduisait à une réponse tumorale marquée (Engelman *et al*, 2008). Dans un autre modèle de souris transgéniques, les mêmes auteurs ont montré que les tumeurs liées à une mutation de *KRAS* (G12D) ne répondaient pas au même NVP-BEZ235 utilisé seul. Cependant, quand le NVP-BEZ235 était associée à l'inhibiteur de MEK ARRY-142886, une synergie importante était constatée.

Nous avons constaté que l'ajout du RAF265 à l'everolimus ne diminuait ni la viabilité ni la prolifération cellulaire sur la lignée A549 (*KRAS* G12S). Ces données ne confirment donc pas les études précédentes de Legrier et Engelman. Cette discordance peut avoir plusieurs explications. Tout d'abord, nous avons testé des composés différents. ARRY-142886 inhibe la voie de signalisation RAF-MEK-ERK en aval de MEK, alors que le RAF265 ne diminue pas

la phosphorylation de MEK sur la lignée A549. Au contraire, de faibles concentrations de RAF265 ont augmenté la phosphorylation de MEK dans les lignées A549 et HCT116. Le NVP-BEZ235 peut également inhiber l'axe PI3K-AKT-mTOR à plusieurs niveaux, ce que ne fait pas l'everolimus.

Ensuite, les voies RAS-RAF-MAPK et PI3K-AKT-mTOR se croisent en de nombreux points, expliquant que les inhibitions de RAF ou mTOR d'un coté, et de MEK ou PI3K/mTOR de l'autre, induisent des effets différents. Enfin, l'effet plus important de l'everolimus et du RAF265 *in vivo* par rapport à *in vitro* peut refléter l'effet sensibilisant de l'hypoxie sur l'activité de l'everolimus, et l'inhibition de VEGFR2 pour le RAF265.

Ainsi, si l'association du RAF265 et de l'everolimus n'a pas montré d'effet majeur sur les tumeurs *KRAS* muté – *PI3K* sauvage, l'ajout d'un autre inhibiteur de la voie RAF-MAPK à un inhibiteur de mTOR reste une approche prometteuse.

3.3 Association d'inhibiteurs des voies PI3K-AKT-mTOR et RAF-MEK-ERK

3.3.1 Résultats précliniques

Nos résultats suggèrent que l'association de l'everolimus et du RAF265 est efficace dans le contexte d'une double mutation des voies de signalisation PI3K-AKT-mTOR et RAF-MEK-ERK. Cette efficacité peut avoir deux explications. D'une part, l'inhibition croisée de la phosphorylation de 4E-BP1 induit un déplacement vers la forme hypophosphorylée de 4E-BP1, qui est associée à un niveau réduit de traduction dépendante de la coiffe, permettant à 4E-BP1 de séquestrer eIF4E (Von Manteuffel *et al*, 1996). D'autre part, l'inhibition de la boucle de rétrocontrôle négatif par le RAF265 diminue l'hyperphosphorylation d'AKT induite par l'everolimus, et peut interférer avec les signaux de survie associés à AKT (Cheng *et al*. 2008). De plus, l'association du sorafenib, double inhibiteur de RAF et de VEGFR, avec la rapamycine, inhibiteur de mTOR, induirait une inhibition syngergique de la croissance de lignées cellulaires de mélanome, et l'augmentation de leur apoptose in vitro (Molhoek *et al*, 2005). Des études complémentaires devront s'attacher à clarifier les rôles respectifs d'AKT et de 4E-BP1 dans l'effet synergique observé.

D'autres études ont déjà cherché à établir l'efficacité de l'association d'un inhibiteur de MEK à un inhibiteur de PI3K. Dans une première étude, l'inhibition concomitante de MEK et de PI3K a été testée in vivo sur des lignées cellulaires présentant un déficit de pTEN. Dans les cellules tumorales présentant une délétion de PTEN et une activation de la voie RAF-MEK-ERK, l'inhibition de la voie de signalisation PI3K-AKT-mTOR seule ne suffisait pas à induire une apoptose significative, mais l'inhibition des deux voies de signalisation présentait un effet

synergique, probalement par l'inhibition cumulée de la phosphorylation de BAD aux sites Serine 112 et Serine 136, abrogeant la liaison de BAD à 14-3-3 (She *et al*, 2010).

Dans une deuxième étude, Yu *et al* ont testé les effets antiproliférants de deux inhibiteurs de PI3K, WAY-266176 et WAY-266175, dans différentes lignées cellulaires. Cette étude retrouvait un lien entre une hyperactivation de la voie PI3K-AKT-mTOR et une sensibilité à ces inhibiteurs (Yu *et al*, 2008). Dans la lignée HCT116, les mutations concomittantes de *PIK3CA* et de *KRAS* sont associées à une résistance à ces inhibiteurs, qui était partiellement levée par le remplacement homologue de *KRAS* muté par *KRAS* sauvage, mais pas après la délétion de PTEN. La déplétion de MEK1 par SiRNA dans des lignées résistantes augmentait l'efficacité des inhibiteurs de PI3K testés. De plus, l'association entre d'une part les inhibiteurs de PI3K WAY-266176 ou WAY-266175, et d'autre part les inhibiteurs de MEK1 CI-1040 ou UO126, était associée à une augmentation importante de l'efficacité des inhibiteurs de PI3K sur des lignées précédemment résistantes.

Dans une étude plus récente, plusieurs combinaisons thérapeutiques ciblées ont été testées sur un modèle de souris transgéniques présentant des mélanomes liés à l'activation oncogénique de RAS et au déficit de Ink4a/Arf. Comme chez l'homme, ces mélanomes étaient résistants à la chimiothérapie et aux thérapies ciblées administrées seules. Cependant, l'association de l'AZD6244, un inhibiteur de MEK, et du BEZ235, un double inhibiteur de PI3K et de mTOR, était la seule association efficace, à la fois en terme de régression tumorale et d'amélioration de la survie. Cette association a ensuite été testée sur trois modèles transgéniques de cancer du sein, claudin-low (T11 OST), basal-like (mutations de C3-TAg), et luminal B (mutation de MMTV-Neu), et l'association AZD / BEZ gardait son efficacité sur ces trois modèles (Roberts *et al*, 2012).

Nos travaux retrouvent des conclusions comparables avec une inhibition d'amont de la voie RAF-MEK-ERK et d'aval de la voie PI3K-AKT-mTOR, avec 3 avantages potentiels. Tout d'abord, les inhibiteurs de RAF ont une structure chimique qui permet l'inhibition croisée de VEGFR2, et ajoute des propriétés anti-angiogéniques à l'inhibition du signal intra cellulaire. Ensuite, l'inhibition de mTOR est située en aval de PI3K, et permet d'interrompre également d'autres voies de signalisation intracellulaires qui convergent vers mTOR. Enfin, l'everolimus est disponible en clinique, presente une bonne tolérance et une excellente spécificité; le RAF265 est actuellement testé dans des essais de phase 1, seul chez des patients atteints de mélanome localement avancé ou métastatique (ClinicalTrials.gov NCT00304525), ou en association avec un inhibiteur de MEK chez des patients atteints d'une tumeur solide mutée KRAS ou BRAFV600E (ClinicalTrials.gov NCT01352273).

Ainsi, nos résultats identifient la dérégulation de la voie RAS-RAF-MEK-ERK comme un déterminant majeur de la résistance tumorale aux inhibiteurs de mTOR, et soulignent l'intérêt d'une double inhibition de RAF et de mTOR dans ce contexte.

3.3.2 Résultats cliniques

Deux études récentes ont rapporté l'efficacité des associations de thérapies ciblées en fonction des statuts mutationnels PI3K-AKT-mTOR et RAF-MEK-ERK des tumeurs. Reprenant les 236 patients inclus dans le programme d'essai précoce de San Antonio et traités par inhibiteurs des voies PI3K-AKT-mTOR et/ou RAF-MEK-ERK, Shimizu *et al.* ont étudié la tolérance, l'efficacité et la corrélation entre mutations génétiques tumorales et réponse clinique de ces associations thérapeutiques. Cent-soixante patients ont reçu un traitement par inhibiteur en monothérapie (voie PI3K-AKT-mTOR n=124 ; voie RAF-MEK-ERK n=36), et 76 ont reçu une association. Les taux de toxicité de grade supérieur à 3 et de toxicité limitant

l'escalade de dose étaient plus élevés chez les patients traités par association que chez ceux traités par monothérapie (54% vs 18%, p<.001 ; 18% vs 9%, p=.06 ; respectivement).

L'analyse génétique des tumeurs a permis de retrouver 9 patients présentant une coactivation des deux voies de signalisation. Il s'agissait de tumeurs coliques dans 7 cas : 5 mutations *KRAS* / délétion pTEN ; 1 mutation *KRAS* / amplification AKT ; 1 amplification MAP3K10 / amplification AKT. Il s'agissait de mélanomes dans 2 cas : 2 mutations de BRAF / délétion pTEN. Cinq de ces patients ont été traités par une association ciblant les deux voies, avec des réponses tumorales dans tous les cas, comprise entre 2% et 64% (Shimizu *et al*, 2012).

Parallèmement, Garrido-Laguna *et al.* ont réalisé le même type d'étude sur les patients atteints de carcinome colorectal métastatique et inclus dans le programme de phase 1 du MD Anderson Cancer Center de Houston. Les biopsies de 238 patients présentant un carcinome colorectal ont été soumises à un séquençage ADN à la recherche de mutations de *KRAS*, *PIK3CA* et *BRAF*. Au total, 51% des patients présentaient une mutation *KRAS*, et 15% des patients présentaient une mutation *PIK3CA*. Les mutations de *KRAS* et de *PIK3CA* étaient significativement corrélées. Les patients traités par inhibiteur de l'EGFR avaient une survie sans progression plus courte en cas de mutation *KRAS* ou *PIK3CA* qu'en l'absence de ces mutations.

Sur les 80 patients traités par inhibiteur de l'axe PI3K-AKT-mTOR, 43 patients avaient des mutations *KRAS* (taux de réponse 21%) et 9 n'avaient pas de mutations de *KRAS* (taux de réponse 27%, p=.59), tandis que 15 patients avaient des mutations de *PIK3CA* (taux de réponse 7%) et 63 n'avaient pas de mutations de *PIK3CA* (taux de réponse 27%, p=.27). De façon intéressante, 10 patients avaient des doubles mutations de *KRAS* et de *PIK3CA*, et aucun de ces patients n'a répondu aux inhibiteurs de *PIK3CA* donnés en monothérapie (Garrido-Laguna *et al*, 2012). Ces données semblent donc confirmer que les tumeurs doubles-

mutées *KRAS*- *PIK3CA* ne sont pas sensibles aux inhibiteurs de PIK3CA donnés seuls, mais pourraient être sensibles à une double inhibition des voies PI3K-AKT-mTOR et RAF-MEK-ERK, plaidant pour une caractérisation moléculaire précise des patients avant l'introduction d'un traitement inhibiteur administré seul ou en association.

3.4 Doubles mutations des voies PI3K-AKT-mTOR et RAF-MEK-ERK

3.4.1 Dans les tumeurs solides en général

Des mutations oncogéniques de *RAS*, *PI3KCA* et *BRAF* ont été identifiées dans de nombreux cancers. Il est intéressant de noter que si les mutations de l'*EGFR*, *KRAS*, *RAF*, et *HER2* sont mutuellement exclusives, les mutations de *PI3KCA* peuvent être associées à la mutation d'un de ces 4 gènes (Eberhard *et al*, 2005 ; Yamamoto *et al*, 2008).

Afin de déterminer la fréquence de ces mutations et de leurs associations, Janku *et al.* ont séquencé l'ADN de biopsies tumorales de 504 patients adressés aux services des thérapies ciblés du MD Anderson Cancer Center depuis octobre 2008. Les mutations de *KRAS*, *NRAS*, *PIK3CA*, et *BRAF* ont été recherchées. Au total, ces mutations ont été retrouvées chez 19% des patients testés pour *KRAS*, 8% pour *NRAS*, 11% pour *PIK3CA*, et 9% pour *BRAF*. Les mutations de *KRAS* étaient particulièrement fréquentes dans les cancers du pancréas (56%), colorectaux (51%) et utérins (15%) ; celles de *NRAS* dans les mélanomes (30%) et les cancers du col (18%), celles de *PIK3CA* dans les cancers du col (36%), de l'utérus (25%), du sein (21%), et colorectaux (17%), celles de *BRAF* dans les mélanomes (44%) et les cancers colorectaux (6%) (Janku *et al*, 2011).

De façon intéressante, toutes histologies confondues, des mutations de *KRAS* étaient trouvées chez 38% des patients *PIK3CA* mutés, contre 16% des patients *PIK3CA* sauvage (p=.001). A l'inverse, des mutations de *PIK3CA* étaient retrouvées chez 28% des patients *KRAS* mutés, contre 10% des patients *KRAS* sauvages (p=.001). Cette corrélation forte suggère une physiopathologie commune qui n'a pas encore été élucidée. Au total, seulement 19 patients de

cette série (3,7%) présentaient une double mutation *KRAS* - *PIK3CA* (Janku *et al*, 2011). Cependant, cette étude n'a inclu que des patients présentant des cancers avancés, et aucune double mutation n'a été retrouvée sur les 22 CBP-NPC inclus.

3.4.2 Dans les CBP-NPC en particulier

La fréquence des doubles mutations *KRAS* - *PIK3CA* est moins claire dans le CBP-NPC. En reprenant les pièces chirurgicales des patients inclus dans les essais canadiens JBR-10 et CALG B-9633, Cuffe *et al.* ont retrouvé des mutations de *KRAS* dans 27% des 390 cas interprétables (Cuffe *et al*, 2012). En étudiant les tumeurs de 139 patients japonais atteints de CBP-NPC, Okudela *et al*. ont retrouvé des mutations de *PIK3CA* dans 3,6% des cas, et des amplifications du gène dans 18,3% des cas interprétables. Il est intéressant de noter que dans cette étude, l'amplification et la mutation du gène de *PIK3CA* étaient mutuellement exclusives, suggérant l'effet oncogénique de chacune de ces anomalies (Okudela *et al*, 2007). De même, la mutation ou l'amplification de *PI3K* concernait 20% des patients atteints de CBP-NPC étudiés par Yamamoto (Yamamoto *et al*, 2008).

Une équipe chinoise a recherché la présence de mutations somatiques et le niveau d'expression d'ARNm sur une population de patients atteints de CBP-NPC de stade avancé. Sur 30 patients non sélectionnés, des mutations du gène de l'EGFR on été retrouvées dans 12 cas, avec un lien significatif entre l'incidence des mutations des exons 19 et 21 et la surexpression de l'ARNm de l'EGFR ; des mutations de *KRAS* ont été trouvées dans 3 cas et des mutations de *PIK3CA* ont été retrouvées dans 3 cas également. Aucune double mutation *KRAS* - *PIK3CA* n'a été mise en évidence dans cette étude (Lu *et al*, 2012).

Pour étudier les mécanismes de résistance aux inhibiteurs de l'EGFR, une étude italienne a recherché rétrospectivement la présence de mutations des gènes *EGFR* (exons 18-21), *KRAS* (exons 2, 3), *PIK3CA* (exons 9, 20) et *MET* (exons 14, 15) par séquençage sur une population de 166 patients traités par inhibiteurs de l'EGFR. Comme attendu, les auteurs retrouvaient un lien entre la présence d'une mutation du gène *EGFR* de résistance aux inhibiteurs et la survie globale. Ils ont également retrouvé des mutations de *KRAS* dans 6,8% des 162 cas informatifs, et confirmé que les mutations de *KRAS* et *EGFR* étaient mutuellement exclusives. Surtout, les auteurs ont retrouvé des mutations de *PIK3CA* dans 4,1% des 145 cas informatifs, rapporté 2 cas de doubles mutations *EGFR* - *PIK3CA*, mais n'ont pas retrouvé de doubles mutations *KRAS* - *PIK3CA* (Ludovini *et al*, 2012).

Dans une étude prospective incluant 589 patients adressés pour génotypage tumoral avant prise en charge d'un CBP-NPC avancé et pré-traité, Sequist *et al*. retrouvaient 24% de mutations *KRAS*, 13% de mutations *EGFR*, 4% de mutations *PIK3CA*, et 5% de translocations impliquant *ALK* sur 552 analyses informatives. Les mutations de *PIK3CA* semblaient plus fréquentes chez les patients atteints de carcinome épidermoïde. Cette étude prospective et systématique a permis d'identifier des doubles mutations *KRAS* - *PIK3CA* dans 5 cas (0.9%), et des doubles mutations *KRAS* - *TP53* dans 5 cas également (0,9%). Cependant, cette étude présentait de nombreuses limites, dont l'absence de précisions sur le type de traitements administrés avant le génotypage tumoral, la réponse initiale et l'origine de la biopsie tissulaire (Sequist *et al*, 2011). Actuellement, la fréquence exacte des doubles mutations *KRAS* - *PIK3CA* chez les patients porteurs de CBP-NPC est donc estimée aux alentours de 1% des cas, avec de nombreuses imprécisions, qui limitent d'autant l'interprétation des données (Tableau 5). Cependant, plusieurs cas favorables à l'émergence de cette double mutation peuvent être anticipés.

Tableau 5. Comparaison de la fréquence des mutations dans les carcinomes colorectaux et bronchiques.

	Carcinomes colorectaux	Carcinomes bronchiques
Mutation *KRAS*	51%	27%
Mutation *PIK3CA*	17%	3-4%
Double mutation	? (4% dans les tumeurs solides)	1%

3.5 Situations potentiellement favorables aux doubles mutations des voies PI3K-AKT-mTOR et RAF-MEK-ERK.

3.5.1 Variation selon les populations

Il convient tout d'abord de garder à l'esprit qu'il existe une grande variation dans la fréquence des mutations oncogéniques des CBP-NPC selon la population considérée. Ainsi, dès 2005, une plus grande fréquence des mutations de l'EGFR a été rapportée dans les populations asiatiques, chez les femmes et chez les non-fumeurs (Yang *et al*, 2005).

Plus récemment, des chercheurs sardes ont étudié la fréquence des mutations de *KRAS*, *BRAF*, et *PIK3CA* dans une population de patients atteints de carcinome colorectal, et provenant de toute la Sardaigne. Sur 478 patients ayant bénéficié d'un séquençage ADN complet de façon prospective entre 2009 et 2011, une mutation de *KRAS* était retrouvée dans 21% des cas dans la population du sud contre 43% dans la population du nord ($p<0.001$). De même, une mutation de *PIK3CA* était retrouvée dans 24% des cas dans la population du sud contre 10% des cas dans la population du nord ($p<0.001$). De façon intéressante, cette discordance n'était pas retrouvée pour les doubles mutations *KRAS* - *PIK3CA*, qui concernaient 4,4% des cas dans la population du nord contre 3,5% des cas dans la population du sud (p=ns) (Palomba *et al*, 2012).

Ainsi, si l'origine géographique influe sur la proportion des mutations EGFR pour le CBP-NPC, *KRAS* et *PIK3CA* pour le colon, cette donnée géographique ne semble pas avoir d'influence sur la proportion des doubles mutations *KRAS* - *PIK3CA* chez les patients atteints de carcinome colorectal.

3.5.2 Hétérogénéité tumorale

Le chirurgien et l'anatomopathologiste savent depuis longtemps qu'une tumeur solide ne se présente pas comme une masse homogène, mais comme un agrégat de zones plus ou moins infiltrantes, fibreuses, nécrotiques, kystiques et vascularisées. Cette hétérogénéité tumorale a été récemment traduite en termes scientifiques.

Partant du principe que les traitements personnalisés du cancer se basaient sur une unique biopsie du site le plus accessible, qu'il s'agisse de la tumeur principale, d'une adénopathie périphérique, ou d'une métastase, Gerlinger *et al.* ont soumis plusieurs prélèvements d'une tumeur rénale et de ses métastases à l'étude de la ploïdie, l'analyse des aberrations chromosomiques, le séquençage des exons, le profilage des ARNm, la détermination des conséquences fonctionnelles de ces mutations, et l'analyse immunohistochimique de la tumeur.

Les reconstructions phylogénétiques ont révélé une croissance tumorale en branches, dont près de 70% des mutations n'étaient pas détectables dans toutes les régions tumorales. Une hétérogénéité tumorale a été retrouvée sur l'activation de mTOR, la phosphorylation de S6 et de 4EBP. De même, de nombreux gènes suppresseurs de tumeurs (SETD2, PTEN, KDM5C) n'étaient pas exprimés de la même façon selon la zone tumorale considérée. Enfin, des signatures génétiques de bon et de mauvais pronostic étaient retrouvées dans des régions différentes de la même tumeur. Ces données suggèrent une grande hétérogénéité moléculaire au sein d'une même tumeur, constituant un défi majeur aux thérapies ciblées (Gerlinger *et al*, 2012).

Depuis la publication de cette étude, un groupe de chercheurs allemands a analysé l'hétérogénéité des mutations *KRAS* au sein des CBP-NPC. Partant du principe que la mutation de *KRAS* était un événement précoce dans la carcinogenèse des CBP-NPC, les auteurs se sont demandé si cette mutation était présente de façon homogène dans toute la tumeur. De 3 à 8 zones ont été prélevées dans chacune des 40 tumeurs analysées (20 adénocarcinomes, 10 carcinomes épidermoïdes, 10 carcinomes à grandes cellules). Les adénopathies ont également été analysées dans 19 cas. Une mutation de *KRAS* a été retrouvée dans 13 des 30 adénocarcinomes et carcinomes à grandes cellules. Aucune mutation n'a été retrouvée dans les 10 carcinomes épidermoïdes. Le séquençage a retrouvé 4 cas de positivité hétérogène pour les mutations *KRAS*, mais des analyses complémentaires ont montré que ce signal hétérogène était la conséquence de prélèvements contaminés par des cellules non tumorales. Aucune hétérogénéité intra-tumorale des mutations de *KRAS* n'a donc été détectée dans les CBP-NPC, confirmant que cette mutation survient précocement dans la carcinogenèse pulmonaire (Alsdorf *et al*, 2012).

Cependant, les mutations de *PIK3CA* semblent survenir plus tardivement dans la carcinogenèse pulmonaire, et une hétérogénéité intra-tumorale des mutations *PIK3CA* et des doubles mutations *KRAS* - *PIK3CA* devra être recherchée avant d'initier des essais thérapeutiques sur des combinaisons d'inhibiteurs des axes PIK3-AKT-mTOR et RAF-MEK-ERK.

3.5.3 Discordance mutationnelle entre primitif et métastases

La troisième circonstance à prendre en compte au moment de déterminer la fréquence des doubles mutations *KRAS* - *PIK3CA* correspond aux potentielles discordances mutationnelles entre la tumeur primitive et les métastases lymphatiques ou hématogènes.

Dans le CBP-NPC, l'extension lymphatique survient dans plus de 50% des cas, influençant grandement la prise en charge de ces patients et leur pronostic. En 2011, une équipe chinoise a comparé les statuts *EGFR* et *KRAS* de 80 tumeurs primitives pulmonaires et des ganglions locaux (N1) associés. Sur 160 prélèvements, une tumeur primitive et 7 métastases lymphatiques présentaient des mutations *KRAS*, et 21 tumeurs primitives et 26 métastases lymphatiques présentaient des mutations *EGFR*. Les statuts *KRAS* et *EGFR* étaient discordants chez 6 (7.5%) et 7 (8.75%) patients, respectivement (Sun *et al*, 2011). A contrario, en 2012, dans l'étude citée plus haut, Alsdorf *et al*. ne retrouvaient aucune discordance de statut *KRAS* entre la tumeur primitive étudiée et les adénopathies prélevées (Alsdorf *et al*, 2012). Ainsi, ces résultats contradictoires appellent des études plus larges afin de déterminer la proportion de discordance entre la tumeur principale et ses extensions lymphatiques à la fois sur les mutations *KRAS*, *PIK3CA*, et sur les doubles mutations *KRAS* - *PIK3CA*.

En l'absence d'étude de grande ampleur dans le CBP-NPC, il est intéressant de se tourner vers le carcinome colorectal. En 2010, une équipe allemande a étudié les mutations *KRAS*, *BRAF*, et *PIK3CA* de 100 tumeurs primitives, ainsi que des 55 adénopathies et 20 métastases hématogènes associées. Des mutations de *KRAS* et *PIK3CA* étaient retouvées dans 41% et 21% des tumeurs primitives, respectivement. Une hétérogénéité intra-tumorale entre le centre

et le front invasif de la tumeur était retrouvée dans 8% et 5% des cas, respectivement. Une discordance mutationnelle entre primitif et adénopathie était retrouvée dans 31% et 13% des cas, respectivement. Une discordance mutationnelle entre primitif et métastase hématogène était retrouvée dans 10% et 5% des cas, respectivement. De façon intéressante, des doubles mutations *KRAS* - *PIK3CA* étaient retrouvées dans 9% des tumeurs primitives, mais n'ont pas été étudiées dans les adénopathies ou les métastases hématogènes (Baldus *et al*, 2010).

Dans le CBP, les efforts se sont principalement portés sur les discordances mutationnelles entre tumeurs primitives et métastases hématogènes. La probabilité que la tumeur primitive et ses métastases présentent des mutations différentes a été suggérée dans le cas de l'*EGFR* (Italiano *et al*, 2006, Mordant *et al*, 2010, Annexe 4) et de *KRAS* (Badalian *et al*, 2008), mais les données sur PIK3CA manquent actuellement. Des discordances primitif/métastases sur les bras chromosomiques portant les gènes d'intérêt ont bien été retrouvées dans les carcinomes épidermoïdes pulmonaires (Boelens *et al*, 2009), mais ces données préliminaires doivent être confirmées et complétées.

3.5.4 Maladie résiduelle

Les cellules souches tumorales sont des cellules indifférenciées qui possèdent la capacité de s'auto-renouveler et peuvent représenter un réservoir générant des cellules tumorales (Mordant *et al,* 2011, Annexe 5). Dans le CBP-NPC, les CSCs ont été caractérisées par des marqueurs tels que CD133 et ABCG2, mais les modifications survenant dans les voies de signalisation intra-cellulaires n'ont pas été totalement explorées.

D'un point de vue fondamental, on sait que la Protein kinase Ciota (PKCiota) est un oncogène nécessaire au maintien du phénotype transformé des cellules de CBP-NPC. Pour étudier le rôle de PKCiota dans le développement tumoral, des chercheurs américains ont utilisé un modèle murin transgénique dans lequel l'oncogène *KRAS* est activé par un système Cre/Lox au niveau du poumon, avec ou sans perte du gène murin de PKCiota. La perte du gène de PKCiota était associée à une inhibition importante de l'hyperplasie initiée par KRAS, et donc de la formation de tumeur *in vivo*. Ce lien était lié à l'impossibilité pour les cellules souches bronchiolo-alvéolaires de subir l'amplification et la transformation induites par KRAS en l'absence de PKCiota. De même, l'ajout d'un inhibiteur de PKCiota chez les animaux présentant une activation de KRAS sans délétion de PKCiota inhibait également l'expansion et la transformation cellulaire *in vivo*. Ces données suggèrent donc que PKCiota est indispensable à l'expansion et à la transformation de cellules souches pulmonaires induites par KRAS (Regala *et al*, 2009).

Toujours indirectement, une équipe allemande a comparé l'expression de plusieurs biomarqueurs entre des patients long-répondeurs, répondeurs intermédiaires, ou non répondeurs au géfitinib. L'étude des biopsies avant traitement des patients long-répondeurs montre comme attendu une fréquence plus élevée des mutations *EGFR* et moins élevée des mutations *KRAS*. De façon intéressante, l'expression de l'E-cadherine/vimentine était la même dans les 3 groupes, alors que le CD133 était exprimé chez seulement 40% des long-répondeurs et le BCRP1 était exprimé principalement chez les répondeurs, qu'ils soient long-répondeurs ou répondeurs intermédiaires. Les long-répondeurs présentaient également une évolution particulière, comprenant un nombre moins élevé de sites métastatiques, et des métastases principalement pulmonaires et pleurales. Les antigènes spécifiques des CSCs permettent donc d'identifier un sous-groupe de patients long-répondeurs aux inhibiteurs de l'EGFR (Gottschling *et al*, 2012).

Plus récemment, Singh *et al*. ont exploré directement le lien entre l'activation des voies de signalisations intra-cellulaires dépendant des oncogènes et les capacités d'auto renouvellement des cellules souches. Des *sides populations* (SP) ont été identifiées et isolées à partir des lignées cellulaires de CBP-NPC H1650, H1975, et A549, et des tumeurs primitives humaines conservées sur des souris en sous-cutané. Les cellules SP possédaient des propriétés de cellules souches, telles que l'auto-renouvellement et la croissance en sphère, elles étaient également capables de générer des tumeurs primitives et métastatiques après implantation orthotopique dans le poumon de souris SCID. *In vitro*, les cellules SP présentaient une hyper-expression des marqueurs de cellules souches Oct4, Sox, et Nanog, mais elles présentaient également des signes de transition epithélio-mésenchyateuse. De plus, l'inhibition génétique ou pharmacologique de l'EGFR, de Src ou de AKT inhibait les capacités d'auto-renouvellement et d'expansion des cellules SP, et aboutissait à la sous-expression de Sox2. De même, l'inhibition de Sox2 par des siRNA bloquait le phénotype SP et les capacités d'auto-renouvellement de ces cellules, alors que Oct4 et Nanog semblait jouer un rôle moins important dans le phénotype SP (Singh *et al*, 2012).

Collectivement, ces données suggèrent donc que l'axe EGFR-Src-AKT joue un rôle primordial dans le maintien du phénotype SP par l'intermédiaire de Sox2, mais les rôles spécifiques de KRAS et de PIK3CA dans les CSCs de CBP-NPC restent à établir.

Dans d'autres tumeurs solides telles que le gliome, la voie de signalisation EGFR-PTEN-PI3K-AKT joue un rôle clé pour réguler le phénotype SP des CSCs (Bleau *et al*, 2009). De même, dans le cancer du sein ERα positif, l'isolation de 500 CSCs définies par le phénotype CD44(+)/CD24(-)/CD45(-) a permis une analyse extensive de leur génome et de leur transcriptome. Cette étude a montré qu'au delà des gènes spécifiques des CSCs, ces cellules présentaient une activation des gènes de la voie PI3K, dont l'EGFR, HB-EGF, PDGFRA/B,

PDGF, MET, PIK3CA, PIK3R1 et PIK3R2. Ces données suggèrent que la voie de signalisation de PI3K, qui est responsable de la résistance à l'hormonothérapie des cancers mammaires ERα positifs, est également activée dans les CSCs correspondantes (Hardt *et al*, 2012).

Des études complémentaires seront donc nécessaires afin d'évaluer le rôle et la fréquence des activations et mutations de *PIK3CA* dans les contextes particuliers de certaines populations, au sein d'une hétérogénéité tumorale, en cas d'extension lymphatiques, et dans la sous-population des CSCs. Ces données permettront ensuite de mieux tester l'efficacité de l'association everolimus-RAF265 dans les métastases et cellules souches tumorales de CBP-NPC muté *KRAS*.

Les progrès dans la caractérisation moléculaire des CBP-NPC dévoilent deux situations différentes. Dans le premier cas, le CBP peut être perçu comme une maladie monogénique, survenant le plus souvent en l'absence d'exposition au tabac, et répondant de façon importante et prolongée à un inhibiteur de kinase donné en monothérapie. C'est le cas des mutations de l'EGFR survenant chez la femme non-fumeuse, et bénéficiant pleinement du traitement par erlotinib ou géfitinib. En préclinique, les modèles murins transgéniques rendent fidèlement compte des étapes de cette cancérogenèse, et prédisent correctement l'efficacité des traitements testés. Dans le second cas, plus fréquent, le CBP est une maladie multifactorielle, plurigénique, survenant après une inflammation bronchique prolongée chez un patient exposé au tabac. Ces patients présentent de multiples mutations géniques, et répondent mal aux thérapies ciblées. En préclinique, les modèles murins de xénogreffe sous-cutanés et d'animaux transgéniques ne reproduisent pas cette diversité, nous amenant à mettre au point un modèle orthotopique conduisant à des métastases et permettant de travailler sur la maladie résiduelle (CSCs, CTCs, DTCs).

3.6 Modèles animaux

3.6.1 Modèles actuels

Les xénogreffes SC de lignées cellulaires dérivées de cancers humains sur des animaux immunodéprimés ont constitué la pierre angulaire de l'évaluation préclinique de nouvelles drogues durant des décennies (Dolgin, 2010). Ce modèle présente de nombreux avantages, dont la faisabilité technique (absence d'anesthésie, injection aisée) et l'accessibilité de la tumeur (mesure directe autorisant un suivi longitudinal). Cependant, les modèles SC sont limités par la discordance entre l'origine de la xénogreffe et le micro-environnement de l'hôte, incluant la matrice extra-cellulaire et les signaux paracrine, contredisant la théorie de Paget sur la "graine et le terroir" (“seed and soil”) (Mueller et al, 2004). Cette discordance est à l'origine de variations dans la réponse de la tumeur aux agents cytotoxiques ou à l'irradiation (Teicher, 2006) et modifie certainement la réponse aux nouvelles thérapies anti-cancéreuses. Cette discordance pourrait être particulièrement importante dans le CBP-NPC, où l'hypoxie et la néoangiogénèse jouent un rôle majeur dans la progression tumorale (Hu et al, 2005), et surviennent différemment dans les xénogreffes SC et intra-thoraciques (Graves et al, 2010). Enfin, seule une minorité de modèles SC dissémine et atteint un stade métastatique. L'absence de progression du stade localisé au stade métastatique par l'intermédiaire d'une extension vasculaire et lymphatique constitue donc une limite majeure des modèles SC, alors que les métastases sont la première cause de mortalité par cancer.

Ces différences ont des implications pour la progression tumorale, avec une absence de métastases et la présence d'un nombre limité de CTCs dans notre étude, montrant les limites de l'évaluation préclinique de nouveaux traitements, avec 2 risques potentiels. Le premier

risque est d'introduire en clinique une molécule ou une association thérapeutique peu ou pas efficace. Ainsi, l'association de chimiothérapie et d'inhibiteurs de l'EGFR était très efficace sur des modèles SC (Sirotnak *et al*, 2000 ; Higgins *et al*, 2004), mais inefficace sur un modèle orthotopique (Onn *et al*, 2004), et n'a jamais prouvé son efficacité dans les essais cliniques incluants des patients atteints de CBP-NPC non sélectionnés sur leur statut *EGFR* (Giaccone *et al*, 2004 ; Herbst *et al*, 2004, Herbst *et al*, 2005). Le second risque est de stopper le développement de drogues prometteuses. Ainsi, l'antagoniste de HIF-1alpha PX-478 n'était pas efficace sur des modèles SC, mais inhibait bien la progression et la dissémination tumorale sur des modèles orthotopiques de CBP-NPC, entraînant son évaluation actuelle dans un essai de phase 1(Jacoby *et al*, 2010)

Ces données suggèrent que les modèles SC ne sont pas totalement adaptés à l'étude préclinique du CBP-NPC, et que leurs résultats ne devraient être analysés qu'avec prudence. Des conclusions identiques ont été tirées de l'évaluation préclinique des traitements du CBP-PC (Kuo *et al*, 1993).

Pour prendre en compte l'influence de la spécificité d'organe et du micro-environnement tumoral, des modèles orthotopiques ont été développés progressivement au cours des 20 dernières années, dans le but d'obtenir une seule tumeur intra-parenchymateuse qui reproduise la situation clinique et permette un suivi longitudinal. Les administrations intra-veineuses (Reddy *et al*, 1987), intra-bronchiques (Mc Lemore *et al*, 1987 ; March *et al*, 2001 ; Kang *et al*, 2006) et intra-pleurales (McLemore *et al*, 1988 ; Nagamachi *et al*, 1998) de lignées cellulaires de CBP-NPC ont permis d'obtenir des tumeurs pleurales, localement avancées, ou multiples et synchrones. Ces modèles sont donc intéressants pour étudier ces situations précises, mais leurs résultats ne peuvent être étendus au CBP-NPC intra-parenchymateux

localisé, qui constitue à la fois une situation clinique fréquente et l'origine de la dissémination tumorale.

Des modèles de souris transgéniques (Genetically engineered mouse models - GEMMs) ont également été développés pour étudier le CBP-NPC chez l'animal. Ces animaux sont habituellement mutés pour p53, ce qui favorise l'instabilité génétique et la formation de tumeurs. Ces animaux développent des tumeurs pulmonaires liées à l'introduction d'une mutation oncogénique d'*EGFR* (Li *et al*, 2007), *KRAS* (Meuwissen *et al*, 2001), *PIK3CA* (Engelman *et al*, 2008), *BRAF* (Pritchard *et al*, 2007), ou à la présence d'un oncogène de fusion *EML4-ALK* (Chen *et al*, 2010). Des CBP-NPC peuvent être présents sur des souches murines porteuses de l'oncogène *KRAS*, activé soit après recombinaison homologue spontanée dans tout l'organisme (Singh *et al*, 2010), soit après recombinaison induite dans le poumon par un système Cre-lox après administration intra-pulmonaire d'un adénovirus codant pour la recombinase Cre (Meuwissen *et al*, 2001). Les deux modèles permettent le développement de CBP-NPC mutés *KRAS* sur une période assez courte, mais sont limités par leur éloignement de la situation clinique, autant que les mutations programmées d'un ou deux oncogènes ou gènes suppresseurs de tumeur sont éloignés de la progression multi-étapes d'un cancer bronchique survenant sur une inflammation chronique. De plus, les modèles de GEMMs conduisent à des centaines de CBP-NPC primitifs simultanés, qui sont difficiles à suivre de façon précise et non invasive au cours des investigations précliniques de nouvelles drogues. Malgré ces inconvénients, les GEMMs représentent un modèle inestimable de définition des oncogènes, permettent de prendre en compte la réponse immunitaire, et ont donc été utilisés pour les essais de nouvelles molécules (Singh *et al*, 2010).

L'implantation chirurgicale par thoracotomie de fragments de CBPNPC dans le poumon de souris imunodéprimées a été publiée pour la première fois en 1992 (Wang *et al*, 1992). Les fragments tumoraux provenaient de tumeurs primaires fraîches ou de tumeurs sous-cutanées développées après injection de cellules en suspension. Les fragments tumoraux étaient insérés dans le parenchyme pulmonaire, sur la plèvre viscérale, ou sur la plèvre pariétale, selon l'extension tumorale souhaitée. La mortalité peropératoire était inférieure à 10%, et les taux de prise de xénogreffes étaient situés entre 60 et 100%, mais aucune métastase systémique n'était observée. Le même modèle a ensuite été développé chez le rat (Wang *et al*, 1997) et confirmé en utilisant des cultures primaires de CPB-NPC chez la souris SCID (Fujino *et al*, 2003). L'introduction de la GFP dans la tumeur permettait un suivi tumoral par imagerie in vivo, et la visualisation directe d'une extension métastatique aux ganglions médiastinaux, poumon controlatéral, et os (Hoffman, 1999 ; Yamauchi *et al*, 2007).

Cependant, ces modèles sont techniquement difficiles à réaliser, nécessitent des laboratoires et une main d'oeuvre spécialisés, et n'ont donc pas été généralisés à ce jour malgré des résultats préliminaires encourageants (Kraus-Berthier L *et al*, 2000).

3.6.2 Modèle d'injection orthotopique transpleurale

L'injection orthotopique de cellules de CBP-NPC chez la souris *nude* a été développée pour surmonter la difficulté technique de la thoracotomie (Boehle *et al*, 2001 ; Onn *et al*, 2003). Cette technique est associée à une faible mortalité perprocédure et à un taux de prise important, avec deux limites. La première est l'impossibilité de prédire en perprocédure si la tumeur va être localisée dans le parenchyme pulmonaire ou si un ensemencement pleural va conduire à une tumeur localement avancée. Afin d'obtenir une tumeur unique limitée au

parenchyme pulmonaire, nous avons utilisé un nombre élevé de cellules dans un volume limité de milieu, et avons ajouté de la matrice de sarcome de souris afin d'améliorer l'ancrage de la solution dans le parenchyme pulmonaire. Nous avons injecté cette solution de façon transpleurale, sous contrôle visuel du poumon à travers les espaces intercostaux, combinant ainsi précision et faisabilité technique (absence d'intubation orotrachéale ou de ventilation mécanique).

La seconde limite est la nécessité d'un suivi longitudinal par microscanner en l'absence d'imagerie fluorescente ou bioluminescente. Le microscanner présente une résolution spatiale limitée, et n'est pas disponible partout. Nous avons donc utilisé une lignée cellulaire transfectée par la luciférase, ce qui nous a permis d'obtenir une imagerie bioluminescente *in vivo*. Cette technique a déjà été décrite dans un modèle orthotopique percutané associé à un ensemencement pleural (Matsumoto *et al*, 2009), et a pu être appliquée à notre modèle d'injection orthotopique transpleurale conduisant à un modèle de CBP-NPC intra parenchymateux localisé.

Ce nouveau modèle xénogènique, orthotopique et bioluminescent a permis l'identification de CTCs. Cette nouvelle application du système Cellsearch® aux modèles précliniques de CBP-NPC pourrait présenter un intérêt majeur, à la fois pour étudier le rôle des CTCs dans la progression tumorale, et pour évaluer la réponse à de nouvelles stratégies thérapeutiques.

Cependant, notre étude présente des limites liées à l'utilisation d'animaux immunodéficients et au terrain génétique hétérogène des xénogreffes. Ainsi, en comparaison avec les animaux transgéniques, notre modèle souffre d'une caractérisation moléculaire incomplète et de l'absence d'immunité anti-tumorale. La prochaine étape dans l'amélioration de ce modèle

animal serait d'utiliser des fragments tumoraux issus de tumeurs pulmonaires d'animaux *KRAS* mutés, et de les implanter en position orthotopique chez la souris immunocompétente. L'ultime étape serait de tester les traitements validés du CBP-NPC, afin de valider rétrospectivement le caractère prédictif du modèle préclinique. L'intérêt de cette nouvelle approche devra ensuite être comparé aux autres modèles précliniques disponibles en termes de décision d'introduction des nouvelles molécules dans l'étape clinique. Nous pensons que l'imagerie fonctionnelle et les paramètres biologiques pré et per-thérapeutiques tels que les CTCs peuvent contribuer à l'amélioration de la valeur clinique de ces approches. En particulier, nous pensons travailler sur l'implantation orthotopique de tumeurs pulmonaires provenant d'animaux transgéniques mutés *KRAS*, afin d'évaluer l'immunité anti-tumorale et de surveiller l'efficacité de nouveaux traitements chirurgicaux et ou radiothérapeutiques en association avec des thérapies ciblées au stade localement avancé.

3.7 Perspectives

Afin de rapprocher les deux étapes de ce travail, il serait intéressant d'étudier l'évolution du statut mutationnel PI3K et de l'activation de la voie RAF-MEK-ERK entre une tumeur primitive mutée *KRAS* et ses métastases d'une part, et la tumeur primitive et la maladie résiduelle (CTCs, CSCs, DTCs) d'autre part. Enfin, l'association de l'everolimus et du RAF265 pourrait alors être testée sur le modèle orthotopique bioluminescent par injection transpleurale, afin de suivre l'effet de l'association sur la croissance (imagerie bioluminescente), l'invasion (imagerie bioluminescente et CTCs), et la dissémination métastatique (imagerie bioluminescente).

4 Conclusion

L'association du RAF265, inhibiteur de RAF, et de l'everolimus, inhibiteur de mTOR, est une stratégie efficace pour augmenter l'effet cytotoxique et antiprolifératif sur des lignées présentant une dérégulation concomittante des voies RAS-RAF-MEK-ERK et PI3K-AKT-mTOR. *In vivo*, l'association est additive sur les lignées HCT116 et H460, potentiellement (i) par une inhibition croisée de la phosphorylation des effecteurs de mTOR 4EBP1 et S6, et (ii) par l'interruption de la boucle de rétrocontrôle conduisant à l'hyperphosphorylation d'AKT.

La double inhibition des voies PI3K-AKT-mTOR et RAS-RAF-MEK-ERK est donc une stratégie prometteuse pour dépasser la double activation oncogénique de KRAS et PIK3CA. Ces résultats plaident en faveur d'associations de thérapies ciblées en traitement de certains cancers sélectionnés. Des études complémentaires sont nécessaires pour confirmer le potentiel de l'association de l'everolimus et du RAF265, dans le cadre d'études précliniques de tolérance et d'efficacité, puis de projets translationnels.

Le micro-environnement tumoral joue un rôle majeur dans la promotion de la croissance tumorale (Whiteside, 2008). Dans les modèles de xénogreffes, la localisation tumorale influe sur la sensibilité à la chimiothérapie (Teicher, 2006), et joue un rôle majeur dans la sensibilité aux thérapies ciblées. Pour ces raisons, nous avons développé un modèle intrapulmonaire murin de CPB-NPC humain, associé à un taux de mortalité peropératoire bas, un taux de prise tumorale élevé, une croissance loco-régionale, et le développement de métastases. Ce modèle a permis l'identification de CTCs selon une méthode en cours de validation en clinique.

Il sera maintenant intéressant d'étudier l'évolution du statut mutationnel *PIK3CA* et de l'activation de la voie RAF-MEK-ERK entre la tumeur primitive mutée *KRAS* et les métastases d'une part, et la tumeur primitive mutée *KRAS* et la maladie résiduelle (CTCs, CSCs, DTCs) d'autre part. Enfin, l'association de l'everolimus et du RAF265 pourrait alors être testée sur le modèle orthotopique bioluminescent par injection transpleurale, afin de suivre l'effet de l'association sur la croissance, l'invasion, et la dissémination métastatique.

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5. Annexes

5.1 Annexe 1: Bases moléculaires de l'oncogenèse bronchique

Loriot Y, Mordant P, Fouret P, Deutsch E, Soria JC. Bases moléculaires de l'oncogenèse des voies aérodigestives supérieures et bronchiques. Bull Cancer. 2009;96 Suppl 1:S13-24.

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Bases moléculaires de l'oncogenèse des voies aérodigestives supérieures et bronchiques

Molecular aspects of head and neck, and lung cancer oncogenesis

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Résumé. Le cancer bronchique et celui des voies aérodigestives supérieures (VADS) relèvent d'un processus multi-étapes faisant intervenir l'activation d'oncogènes et l'inactivation de gènes suppresseurs de tumeur. Ces deux processus ont des caractéristiques et des acteurs moléculaires communs, tandis que sur le plan clinique ces deux entités sont étroitement liées à l'intoxication tabagique. Les anomalies moléculaires liées à ces processus multifocaux et multi-étapes sont souvent présentes dans des tissus normaux ou dysplasiques. Une meilleure compréhension des mécanismes moléculaires qui sous-tendent ces processus permet une meilleure sélection des cibles potentielles modulables en curatif ou en préventif par des thérapies moléculaires ciblées. ▲

Mots clés : ORL, poumon, VADS, délétions, EBV, EGFR, RAS, VEGF

Abstract. Lung and head and neck cancers result from a multistep process involving activation of oncogenes and inactivation of tumor-suppressor genes. These two processes share common features and molecular players, while their corresponding clinical entities are both triggered by the tobacco carcinogens. In many cases, the molecular abnormalities associated with these multi-step and multifocal processes can be found in pre-malignant lesions and normal tissue. The growing knowledge of the molecular basis of lung and head and neck carcinogenesis allows to better selecting molecular alterations that can be modulated by molecular targeted agents either in a curative or in a chemopreventive approach. ▲

Key words: head and neck cancer, lung cancer, chromosomal deletions, EBV, EGFR, RAS, VEGF

Introduction

Le cancer bronchique est le cancer le plus fréquent dans le monde et représente la première cause de mortalité par cancer chez l'homme dans les pays occidentaux [1]. En France, les cancers bronchiques primitifs ont une incidence de 70 cas pour 100 000 habitants et représentent environ 30 000 nouveaux cas par an [2]. L'âge médian de survenue du cancer des bronches est de 66 ans, avec une nette prédominance masculine (sex-ratio hommes/femmes proche de 10) [1]. Il est classique de distinguer, sur des critères anatomopathologiques mais également cliniques et évolutifs, les cancers bronchiques à petites cellules des cancers bronchiques non à petites cellules (carcinomes épidermoïdes, adénocarcinomes et carcinomes à grandes cellules). Les cancers bronchiques non à petites cellules constituent environ 80 % de l'ensemble des cancers des bronches. Les cancers des voies aérodigestives supérieures (VADS) [cavité buccale, pharynx, et larynx] ont une incidence de 23 cas pour 100 000 habitants et représentent environ 17 000 nouveaux cas par an, avec une médiane d'âge de 60 ans et un sex-ratio hommes/femmes de 3 à 7 selon les localisations tumorales [3]. Ces cancers représentent donc un problème majeur de santé publique de par leur fréquence et la morbimortalité qu'ils entraînent.

Nous avons choisi de regrouper les cancers des bronches avec les cancers des VADS et de les nommer « cancers des voies aériennes ». Ce regroupement possède plusieurs justifications : continuité anatomique, fonction commune (voies aériennes), même agent carcinogène causatif (tabac), similitudes histologiques au niveau des épithéliums de revêtement et surtout des lésions cancéreuses qui s'y développent, anomalies génétiques communes et constatations cliniques. En

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effet, les patients atteints d'un cancer des VADS ont un risque accru de développer un cancer des bronches (ratio cas observés/cas attendus égal à 3,4). De même, les patients atteints d'un cancer des bronches ont un risque élevé de développer un cancer des VADS (ratio cas observés/cas attendus égal à 2,5) [4]. D'une façon plus générale, tous les patients atteints d'un cancer des voies aériennes ont un risque élevé de développer un second cancer, le plus souvent en rapport avec le tabac (bronches, VADS, œsophage, vessie) [5, 6]. Le but de cette revue est d'identifier les mécanismes principaux d'initiation et de progression des tumeurs des voies aériennes et de souligner le rôle des altérations moléculaires retrouvées dans les muqueuses normales, dans les récidives et dans le développement des seconds cancers.

Susceptibilité génétique

Malgré l'identification du tabac comme principal carcinogène des voies aériennes, des données épidémiologiques suggèrent l'existence d'une prédisposition génétique dans l'apparition des cancers bronchiques et des VADS. Environ 90 % des cancers pulmonaires surviennent chez des fumeurs, alors que seulement 20 % des fumeurs développeront un cancer bronchique [7]. Par ailleurs, le rôle de l'intoxication alcoolique comme facteur de risque indépendant dans la survenue des cancers des VADS est établi, avec un effet multiplicatif pour l'association alcool-tabac [8, 9]. L'étude des gènes impliqués dans le métabolisme des carcinogènes du tabac a permis de mettre en évidence des polymorphismes génétiques enzymatiques, jouant un rôle important dans la survenue des cancers du poumon et des VADS. Ainsi, des mutations génétiques conduisant à l'augmentation de l'activité du cytochrome P450 constitueraient un facteur de risque pour l'apparition de cancers des bronches et du larynx [10]. D'autres altérations, tels les génotypes nuls des gènes codant pour les enzymes de détoxification glutathion-S-transférase (GST) T1 et GST M1 ainsi que le génotype AG ou GG de la GST P1, semblent être des facteurs de risque pour la survenue des cancers des VADS et des bronches [11]. Une étude a suggéré l'implication d'un locus situé en 6q23-25 dans le développement des cancers héréditaires du poumon, cette région contenant plusieurs gènes d'intérêt tels que *ICF2R*, *PARK2*, *SASH1* et *TCF21* [12].

Récemment, plusieurs études ont démontré l'existence de polymorphismes génétiques, en particulier au niveau des gènes codant les récepteurs nicotiniques de l'acétylcholine située sur le locus 15q25.1 [13, 14].

Anomalies chromosomiques

Le profil d'aberrations chromosomiques observé dans les cancers bronchiques est du type « monosomique ». Il se caractérise par la présence de nombreux remaniements chromosomiques aboutissant surtout à des délétions et très rarement à des duplications ou à des amplifications. La formule chromosomique tend à s'abaisser jusqu'à 35 chromosomes. Surviennent alors souvent des endoreduplications, qui font penser à une hypotétraploïdie [15]. De multiples déséquilibres chromosomiques ont été identifiés *in vitro* dans les cancers du poumon et dans des lignées tumorales d'épithéliums bronchiques. Beaucoup d'anomalies chromosomiques sont communes aux cancers bronchiques non à petites cellules et aux cancers bronchiques à petites cellules [16]. Elles concernent la perte en 3p (locus de FHIT et d'autres gènes candidats) et en 9p (locus de CDKN2A/CDKN2B encodant p16 et $p14^{ARF}$). Ces pertes génétiques sont associées à l'exposition aux carcinogènes du tabac et sont reconnues comme des événements précoces du processus carcinogénique bronchique. Elles restent détectables plusieurs années après l'arrêt de l'intoxication tabagique [17]. La perte en 3p intervient dans 96 % des cancers bronchiques et 78 % des lésions préinvasives [18]. La perte en 17p13 (locus de p53) est moins fréquente et suggère que les altérations de p53 sont plus tardives ou bien qu'elles relèvent d'autres mécanismes tels que des mutations. La fréquence et le nombre des anomalies chromosomiques augmentent de façon parallèle à la progression tumorale, depuis la lésion prémaligne jusqu'au cancer invasif [19]. Outre ces pertes en 3p, 9p et 17p présentes dans 50 à 90 % des cancers des bronches, des pertes en 5q, 8p, 11p et 13q ont été démontrées dans 20 à 60 % des cas [19-22]. Certaines anomalies chromosomiques peuvent être rattachées à certains types histologiques. Ainsi, les gains en 7p sont surtout observés dans les cancers bronchiques non à petites cellules, tandis que les gains en 18q ne s'observent que dans les cancers bronchiques à petites cellules. Les adénocarcinomes bronchiques ont des gains en 1q, 7p et 11q plus souvent que les carcinomes épidermoïdes des bronches [16]. La très grande majorité

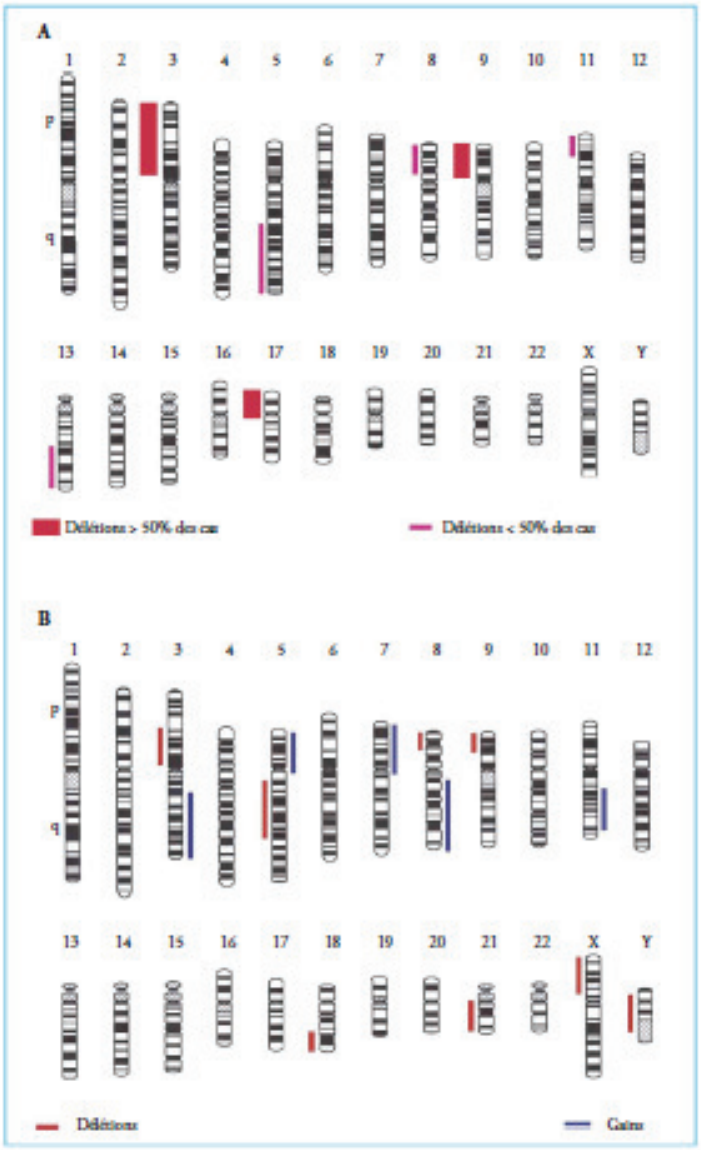

Figure 1. Illustration schématique des principales anomalies chromosomiques dans les cancers des bronches (A) et des VADS (B).

des patients atteints d'un cancer du poumon présente des anomalies chromosomiques non seulement dans leurs cellules tumorales, mais encore dans les cellules des tissus voisins pourtant histologiquement normaux [18]. Les mutations du tissu environnant correspondraient à des événements précoces de la carcinogenèse.

Dans les cancers des VADS, des gains et des pertes chromosomiques ont été identifiés respectivement en 3q21-qter, 5p, 7p, 8q, 11q13-23 ; et 3p13-p24, 5q12-q23, 8p22-p23, 9p21-p24, 18q22-23 et 21q [23]. Des pertes en Xp chez les femmes et des pertes du chromosome Y ont également été décrites [23]. Le profil chromosomique observé dans les cancers des VADS est le plus souvent du type « monosomique » [15]. La *Figure 1* illustre les principales anomalies chromosomiques observées dans les cancers des bronches (A) et des VADS (B). Cette figure est volontairement incomplète et cherche avant tout à donner un aperçu global des profils chromosomiques observés. Les pertes d'hétérozygotie, évocatrices de l'inactivation de gènes suppresseurs de tumeur, concernent les chromosomes 3p, 4q, 5q, 6p, 9p, 11q, 13q, 14q, 18q et 17p [24]. Les pertes en 3p14 et 9p21 ont été observées, avec une fréquence élevée dans les lésions précancéreuses de la cavité buccale [25]. Ces observations laissent à penser que l'altération de certains gènes suppresseurs dans ces régions est probablement un événement précoce de la carcinogenèse des VADS. Il a par ailleurs été démontré que le degré de polysomie était associé à la progression histologique des lésions précancéreuses des VADS [26]. La perte d'hétérozygotie a été reconnue comme un facteur pronostique du risque cancéreux des lésions prémalignes de la tête et du cou [27]. Dans l'étude de Rosin *et al.*, pratiquement toutes les lésions prémalignes ayant progressé vers un cancer invasif présentaient une perte d'hétérozygotie au niveau de 3p et/ou de 9p, alors qu'aucune des lésions avec conservation des deux allèles en 3p et 9p n'avait évolué vers un cancer invasif. Au total, environ 60 % des lésions prémalignes possédant une perte d'hétérozygotie en 3p et/ou en 9p et au niveau d'une troisième région (comme 4q, 8p, 11q ou 17p) ont eu une évolution invasive.

Oncogènes et gènes suppresseurs de tumeur

Oncogènes

La carcinogenèse des voies aériennes correspond à un processus multi-étapes faisant intervenir l'activation d'oncogènes et l'inactivation de gènes suppresseurs de tumeur. Plus de 100 oncogènes ont été identifiés à ce jour. Parmi eux, nombreux sont ceux impliqués dans la carcinogenèse des voies aériennes *(tableau 1)*. Dans le cas particulier des cancers bronchiques, il faut souligner le rôle de ras, c-myc, c-erb-B1 (EGFR) et c-erb-B2 (HER-2/neu). Les mutations activatrices de c-erb-B1 (EGFR) sont fréquentes (24 % des cancers bronchiques non à petites cellules) et touchent systématiquement la région tyrosine-kinase du récepteur [28]. Elles sont caractéristiques des carcinomes bronchiques, mais leur étiologie n'est pas connue. Les mutations les plus fréquentes sont celles de l'exon 19, suivies de celles de l'exon 21, puis celles de l'exon 20 [29]. La fréquence rapportée de ces mutations est de 46 % pour l'exon 19, 40 % pour l'exon 21 et 9 %

Tableau 1. Exemples d'anomalies moléculaires impliquées dans l'oncogenèse des tumeurs des voies aériennes.

Anomalies moléculaires	Rôle	CBNPC	CBPC	Cancer des VADS
EGFR	Prolifération	+	–	+
HER-2	Prolifération	+	–	–
RAS	Prolifération	+	–	+
PI3K/AKt	Prolifération/apoptose	+	+	+
c-myc	Prolifération	+	–	+
IGF	Prolifération/apoptose	+	+	+
Bcl-2	Apoptose	+	+	+
p53	Apoptose	+	+	+
Cycline D1/E	Cycle cellulaire	+	–	+
Rb	Cycle cellulaire	+	+	–
p16	Cycle cellulaire	+	+	+
p21	Cycle cellulaire	+	–	+
Télomérase	Immortalisation	+	+	+
VEGFR	Angiogenèse	+	+	+

pour l'exon 20 [30]. Les mutations sont corrélées à la sensibilité aux inhibiteurs de tyrosine-kinase (certaines sont des mutations secondaires, T790M impliquées dans la résistance aux inhibiteurs de EGFR). Ces mutations sont plus fréquentes chez les femmes, les non-fumeurs, les adénocarcinomes et les sujets asiatiques [31]. L'existence de telles mutations a été décrite au sein de l'épithélium bronchique « normal » adjacent à la tumeur [32]. L'amplification de EGFR (polysomie ou amplification vraie) a également été décrite, et elle est associée à la mutation [33]. C-erb-B1 (EGFR) est surexprimé dans les cancers bronchiques non à petites cellules dans 34 à 62 % des cas [34]. Cette surexpression est dans les deux cas liée à une augmentation de la transcription et de la traduction, et seul un faible pourcentage de tumeurs présente une amplification du gène [33]. Une méta-analyse récente sur données publiées ne retrouve pas d'association de la surexpression d'EGFR et de la survie des patients [35], mais cette surexpression pourrait prédire la réponse et la survie des patients sous inhibiteurs oraux d'EGFR [36]. La fréquence d'amplification du gène de HER2 évaluée par FISH varie de 2 à 10 %. Combinée à la fréquence de la polysomie, l'amplification de HER2 s'élève à 23 % [37]. La surexpression de C-erb-B2 (HER-2) est également un facteur pronostique péjoratif, associée notamment à une plus grande chimiorésistance [38]. Des mutations localisées dans le domaine kinase de HER2 ont été décrites dans 4 % des CBNPC, en particulier dans les adénocarcinomes (9,8 %), celles-ci survenant essentiellement chez les fumeurs ou anciens fumeurs [39, 40] Des mutations de la *PI3K* ont été décrites dans 4 % des CPNPC, conduisant à une activation d'AKT [41]. Une augmentation d'expression de phospho-AKT est observée dans plus de 60 % des cancers bronchiques à petites cellules et non à petites cellules [42, 43]. AKT est également activée dans les lésions bronchiques prénéoplasiques suggérant son rôle dans la carcinogenèse des cancers des VADS [44]. La diminution d'expression de PTEN, régulateur négatif d'AKT, est fréquente (74 %) dans les cancers bronchiques non à petites cellules [45].

Ras est surexprimée dans près de 50 % des CBNPC [46]. Les mutations activatrices de Ras sont retrouvées dans 10 à 15 % des cancers bronchiques non à petites cellules, mais très rarement dans les cancers bronchiques à petites cellules, et localisées le plus souvent au niveau du codon 12 de K-ras, plus rarement au niveau des codons 13 et 61 et très rarement au niveau de N- et H-ras [47]. Les mutations de Ras sont plus fréquentes dans les adénocarcinomes et les carcinomes bronchiques à grandes cellules. Quatre-vingt-dix pour cent des mutations de *Ras* sont des mutations de K-ras. Les techniques de biologie moléculaire les plus sensibles retrouvent une mutation de *K-ras* dans environ 50 % des adénocarcinomes et exclusivement chez des patients fumeurs. Les mutations de *BRAF* sont rares dans les cancers du poumon (3 %) [48-50].

La détection de *c-myc*, au niveau des marges chirurgicales bronchiques histologiquement normales ou altérées, est particulièrement élevée chez les patients atteints d'un cancer des bronches [51]. Ces données suggèrent que l'altération de c-myc précède les modifications histologiques. L'amplification de c-myc est, en revanche, un phénomène tardif, le plus souvent trouvé chez les patients préalablement traités par chimiothérapie [52]. Bien que la surexpression de c-myc ne soit liée à une amplification du gène que dans 10 % des cas, une surexpression de la protéine a été retrouvée dans environ 45 % des cancers des bronches [53]. Le mécanisme précis expliquant la surexpression protéique de c-myc dans les cancers bronchiques n'est pas connu. Il pourrait s'agir d'une altération de la dégradation de l'ARNm de c-myc [52]. Par ailleurs, le proto-oncogène MET qui code pour le récepteur du HGF (*hepatocyte growth factor*) pourrait contribuer à une boucle autocrine de croissance des cancers bronchiques non à petites cellules et à la résistance aux inhibiteurs de l'EGFR [54]. Enfin, 20 à 60 % des cancers bronchiques à petites cellules et un pourcentage plus faible des carcinomes pulmonaires non à petites cellules expriment la bombésine ou GRP (*gastrin-releasing peptide*) [55]. La plupart des lignées cellulaires bronchiques expriment des récepteurs pour la bombésine, suggérant ainsi l'existence d'une boucle autocrine [56]. L'expression plus importante du récepteur au GRP chez les femmes, liée à la présence de ce gène sur le chromosome X et à son absence d'inactivation, pourrait être un des mécanismes expliquant la susceptibilité plus élevée des femmes à l'effet carcinogène du tabac [57]. L'existence d'une boucle autocrine, faisant intervenir l'IGF-1 et -2 (*insulin growth factor*) et le récepteur à l'IGF-1, a également été suggérée dans les cancers des bronches [58]. À ce titre, les sujets ayant un taux élevé d'IGF-1 sérique présentent un risque accru de cancer des bronches [59]. Par ailleurs, la sur-

expression des cyclines E et D1 dans les cancers bronchiques non à petites cellules pourrait avoir un effet oncogénique. La résistance à l'apoptose constitue l'une de ces étapes. La surexpression de la protéine Bcl-2 est retrouvée dans 75 à 95 % pour les cancers du poumon à petites cellules, 10 à 35 % pour les cancers du poumon non à petites cellules.

Le récepteur à l'EGF (*epithelial growth factor*) est surexprimé dans environ 85 % des tumeurs des VADS [60]. Le degré d'expression du récepteur à l'EGF est 29 fois plus élevé dans l'épithélium histologiquement normal de patients atteints de cancer des VADS et 69 fois plus élevé dans la tumeur elle-même, que dans l'épithélium normal de sujets non fumeurs [61]. Cette surexpression est également constatée dans les lésions dysplasiques et semble associée à l'aggravation histologique des lésions précancéreuses [60]. L'amplification du gène *EGFR* en FISH est corrélée à un mauvais pronostic des tumeurs ORL [62] L'amplification du gène de la cycline D1 (situé en 11q13) et sa surexpression sont également des événements importants de la carcinogenèse des VADS, souvent associés à l'inactivation de p16.

Gènes suppresseurs de tumeur

La perte des gènes suppresseurs de tumeur est l'une des étapes les plus précoces de la carcinogenèse des voies aériennes, et son existence était fortement suggérée par le profil caryotypique « monosomique » de la plupart de ces tumeurs [15]. Ainsi, l'inactivation de Rb est très fréquente dans les cancers bronchiques, en particulier dans le carcinome à petites cellules. Une perte d'hétérozygotie au niveau du locus de Rb est observée dans 90 % des carcinomes bronchiques à petites cellules et dans environ 30 % des carcinomes bronchiques non à petites cellules [63, 64]. Une perte d'hétérozygotie en 13q est également retrouvée dans environ 50 % des cas de cancers des VADS. Cette perte est rarement associée avec une inactivation de Rb, ce qui suggère l'existence d'un autre gène suppresseur de tumeur dans la région du gène *Rb* [65].

Environ 70 % des cancers des bronches présentent une mutation de *p53*. Les travaux de Denissenko *et al.* ont démontré que le benzopyrène – un des nombreux carcinogènes contenus dans la fumée du tabac – est directement responsable de la survenue de mutations du type transversion G : T [66]. Les mutations de *p53* sont trouvées dans 70 à 100 % des cas de cancers bronchiques à petites cellules et dans 45 à 75 % des formes non à petites cellules [67, 68]. Une corrélation élevée a été retrouvée entre la fréquence de ces mutations au niveau tumoral et la durée de l'intoxication tabagique. Les altérations de p53 sont également fréquentes dans les cancers des VADS. Elles interviennent sous la forme de pertes alléliques, de mutations ponctuelles et de délétions. L'inactivation par des protéines virales (protéine E6 des papillomavirus oncogènes) a été suggérée dans les localisations oropharyngées. La fréquence des surexpressions et des mutations de *p53* dans les cancers des VADS est estimée entre 20 et 80 %. L'existence d'altérations de p53 est démontrée dans des lésions précancéreuses des VADS. La fréquence des altérations de p53 augmente de façon parallèle à l'aggravation histologique de ces lésions [69]. Il a également été suggéré que des marges chirurgicales, considérées comme morphologiquement saines, étaient souvent envahies par des cellules porteuses de mutations de *p53* [70]. Enfin, la surexpression de p53 retrouvée dans l'épithélium morphologiquement sain des VADS, à distance de la tumeur, pourrait être un facteur prédictif de la survenue de seconds cancers.

La perte d'hétérozygotie en 9p21-p24, fréquente et précoce dans les lésions des VADS, a conduit à l'étude de cette région chromosomique et à la découverte du locus CDKN2A/CDKN2B. Ce locus code pour *p16* et $p14^{ARF}$, gènes suppresseurs de tumeur fréquemment impliqués dans la carcinogenèse de ces tumeurs. Les mutations ponctuelles de *p16* sont rares dans la plupart des tumeurs des VADS, les principaux mécanismes d'inactivation de p16 étant la délétion homozygote ou la méthylation du gène. La perte de *p16* intervient tôt dans la carcinogenèse des VADS.

Les pertes d'hétérozygotie en 3p sont très fréquentes dans les cancers des bronches (> 70 %). Le rôle de FHIT (*fragile histidine triad*), situé en 3p14.2, reste débattu comme possible gène suppresseur de tumeur impliqué dans la carcinogenèse bronchique et probablement des VADS. Le gène *RASSF1* (*ras effector homolog 1*) est situé en 3p21.3 ; son rôle comme gène suppresseur de tumeur dans la carcinogenèse bronchique semble se confirmer, avec une inactivation fréquente du second allèle par méthylation du promoteur du gène chez près de 100 % des tumeurs bronchiques à petites cellules et 60 % des cancers bronchiques non à petites cellules [71].

Une forte proportion de pertes d'hétérozygotie en 5q, à proximité du gène *APC* (*adenomatosis polyposis coli*) ainsi que des pertes d'hétérozygotie au locus d'APC ont été retrouvées dans 80 % des épithéliums dysplasiques, ainsi que dans 67 % des carcinomes *in situ* et dans 50 % des cancers invasifs des VADS [72].

Human papillomavirus (HPV) et *Epstein-Barr virus* (EBV)

Le rôle du HPV dans la survenue d'un sous-groupe de cancers des VADS semble reconnu, avec notamment l'élucidation des mécanismes moléculaires oncogéniques liés aux protéines virales E6 et E7. En effet, un nombre croissant de données suggère un rôle possible des infections à HPV dans la survenue de certains cancers des VADS. Les lésions buccales associées au HPV comprennent des papillomes, des condylomes, des leucoplasies verruqueuses et des carcinomes. La prévalence du HPV dans les cancers de la cavité buccale varie entre 31 et 74 % selon les séries [73]. L'étude de l'infection par HPV et l'étude des mutations/inactivations de *Rb* et *p53* suggèrent très fortement le rôle de ce virus dans l'apparition de certains cancers des VADS [74, 75]. L'HPV-16 est le plus fréquent parmi les cancers ORL positifs pour HPV. Une proportion importante de cancers des VADS, notamment ceux de localisation oropharyngée (en particulier de l'amygdale), de forme histologique faiblement différenciée, ou survenant chez des sujets à faible consommation alcoolique et sans intoxication tabagique, serait en fait associée à une infection par le HPV [76]. Enfin, le pronostic des tumeurs des VADS associées au HPV, serait meilleur que celui des cancers HPV-négatifs [76]. Il n'y a pas d'explication au meilleur pronostic des cancers HPV-positifs des VADS. Une meilleure réponse à l'irradiation, le rôle de l'immunosurveillance induite par les antigènes viraux, une carcinogenèse de champ réduite chez ces patients non fumeurs pourraient être responsables de ces résultats [76]. Il est possible de considérer HPV comme un accélérateur de la carcinogenèse multi-étapes des VADS. Plusieurs études épidémiologiques récentes suggèrent qu'une sérologie positive pour HPV E16 serait un facteur prédictif de la survenue de cancers des VADS [77]. La présence d'une infection orale à HPV est également associée à un risque accru de cancer des cavités orales [78].

Le rôle de l'EBV dans la survenue des carcinomes du nasopharynx est suspecté depuis plus de 35 ans. Des études plus récentes ont mis en évidence la présence du génome de l'EBV dans les cellules tumorales et la sécrétion par les cellules tumorales infectées de facteurs de croissance lymphoïde. Les études sérologiques montrent que la survenue d'un carcinome du nasopharynx (surtout dans son type indifférencié) est associée à des concentrations élevées d'anticorps, dirigés contre les protéines impliquées dans le cycle productif du virus (antigènes précoces : EA avant la réplication de l'ADN et antigènes de structure plus tardifs, tel l'antigène de la capside : VCA) [79].

Télomérase

Les télomères sont des structures ribonucléoprotéiques localisées à l'extrémité des chromosomes eucaryotes dont le rôle est de prévenir leur dégradation et la survenue de réarrangements chromosomiques. Lors de chaque division cellulaire des cellules somatiques normales, les télomères se raccourcissent conduisant *in fine* à un arrêt de prolifération ou au déclenchement de la sénescence réplicative. Un complexe ribonucléoprotéique appelé télomérase permet le maintien de ces séquences télomériques. Celui-ci est composé d'une matrice d'ARN nécessaire à la synthèse des télomères (hTERC) et d'une sous-unité catalytique *reverse transcriptase* (hTERT). La réexpression de la télomérase joue un rôle important dans la biologie de la cellule cancéreuse lui permettant de proliférer indéfiniment. Une activité de la télomérase est détectée dans 90 % des cancers du poumon à petites cellules [80] et dans 60 à 95 % des cancers du poumon non à petites cellules, avec toutefois une fréquence moindre dans les cas des carcinomes bronchioloalvéolaires (40 %) [81]. Plusieurs études ont suggéré que la détection d'une activité forte de la télomérase, où un niveau élevé de l'ARNm de hTERT serait corrélé à une survie moindre dans les tumeurs du poumon non à petites cellules de stade I [82] et à un risque de récidive plus important [81]. L'activation de la télomérase semble jouer un rôle important dans le développement précoce des tumeurs du poumon. En effet, un taux élevé de l'ARNm de hTERT a été retrouvé dans 77 % des hyperplasies alvéolaires atypiques de haut grade contre seulement 27 % en cas d'hyperplasies alvéolaires atypiques de bas grade [83]. L'expression de la télomérase est également un événement fréquent dans les cancers ORL avec une détection de l'ARNm de hTERT dans 75 à

100 % des cas [84, 85], celle-ci survenant également de façon précoce au cours de la tumorigenèse [84].

Angiogenèse

Enfin, le développement de la néoangiogenèse tumorale est également une étape essentielle à la cancérogenèse bronchique et ORL [86]. Celle-ci passe par l'expression de la voie du VEGF (*vascular endothelial growth factor*) et du PDGF (*platelet-derived growth factor*). L'expression du VEGF est retrouvée dans 70 % des tumeurs bronchiques non à petites cellules [87]. Des taux élevés de VEGF intratumoraux ou circulants ont été associés à un mauvais pronostic chez les patients atteints de cancer bronchique non à petites cellules [88, 89]. Une densité élevée de microvaisseaux est également un facteur de mauvais pronostic et prédictif de survenue de métastases [90, 91]. L'expression tumorale du VEGF (détectée par immunohistochimie) pourrait être également un facteur de mauvais pronostique dans les cancers ORL [92]. Le VEGF joue un rôle dans la croissance tumorale en stimulant l'angiogenèse et favorise également la diffusion pleurale des cellules tumorales dans des tumeurs bronchiques [93]. L'inhibition du VEGF ou de son récepteur entraîne une diminution de la vascularisation et de la croissance tumorale de xénogreffes de tumeurs bronchiques humaines [94]. D'autres études sur des modèles de xénogreffes suggèrent que l'inhibition de la voie du VEGF induit l'apoptose des cellules tumorales et augmente l'efficacité d'agents cytotoxiques [95]. D'autres facteurs anti-angiogéniques jouent un rôle dans l'angiogenèse des tumeurs bronchiques. Une expression forte du *basic fibroblast growth factor* constitue un facteur indépendant de mauvais pronostic, de même que l'expression intratumorale de l'*hypoxia-inducible factor 2-α* [90, 96].

Carcinogenèse : un processus multi-étapes

Il est admis que la carcinogenèse correspond à un processus multi-étapes au cours duquel il existe une accumulation progressive d'altérations génétiques, moléculaires et phénotypiques qui permettent l'émergence finale du cancer. Les événements les plus précoces de ce processus correspondent à des altérations du génome des cellules, mais les anomalies primaires restent à identifier. Les altérations génétiques incluent des délétions de partie de chromosomes et de chromosomes entiers (aneuploïdie), des translocations, des amplifications et des réarrangements géniques, des mutations ponctuelles et des duplications. Ces modifications génétiques ne se traduisent pas initialement par des modifications morphologiques cellulaires ou des remaniements de l'architecture tissulaire. L'accumulation, l'interaction et la coopération de toutes ces altérations génétiques concourent aux modifications phénotypiques des tissus (prolifération incontrôlée, pouvoir d'invasion, pouvoir métastatique, etc.). Il a été suggéré que 10 à 20 événements génétiques sont nécessaires pour permettre la progression carcinogénique au sein des tissus bronchiques ou des VADS [97]. Les *Figures 2 et 3* illustrent le processus carcinogénique multi-étapes, respectivement au niveau des VADS et de l'épithélium bronchique. Ce n'est pas tant l'ordre des altérations qui est important pour permettre la progression tumorale, mais leur accumulation successive. Les *Figures 2 et 3* sont inspirées de Califano

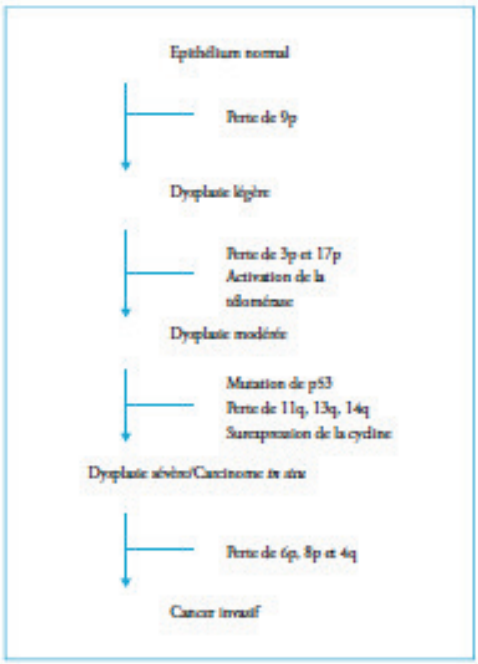

Figure 2. Modèle de carcinogenèse multi-étapes des cancers des VADS.

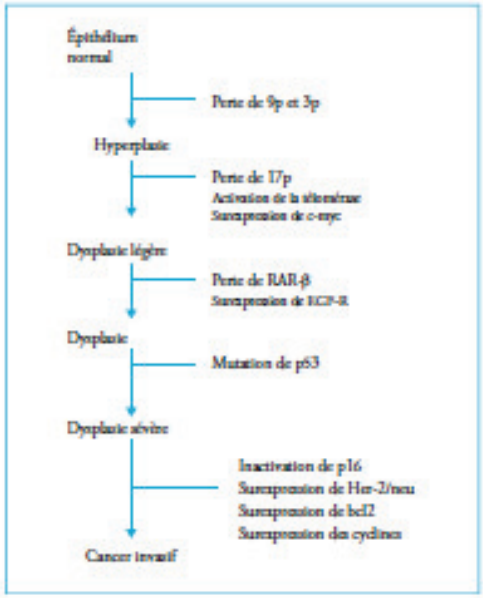

Figure 3. Modèle de carcinogenèse multi-étapes des néoplasies bronchiques non à petites cellules.

et al. et constituent une compilation des principales données publiées dans la littérature [24].

Les techniques de biologie moléculaire, permettant l'analyse à haut-débit du génome ou du transcriptome, aboutissent à l'établissement de signatures moléculaires des voies de cancérogenèses caractéristiques des différents sous-types histologiques des cancers des voies aériennes. Ainsi, plusieurs signatures génomiques et protéomiques pronostiques ont été établies dans le cancer du poumon non à petites cellules [98, 99] De même, l'établissement de signatures reflétant l'expression des ARNmi des tumeurs du poumon se développent rapidement compte tenu de la mise en évidence récente de l'implication des ARNmi dans l'oncogenèse et la progression tumorale des tumeurs du poumon [100].

Conclusion

La carcinogenèse des tumeurs des voies aériennes supérieures et du poumon présentent de nombreuses similarités à la fois dans le type d'anomalies moléculaires impliquées (oncogènes et gènes suppresseurs de tumeurs) et dans la séquence de ces événements (processus multi-étapes).

L'identification précise des différentes altérations moléculaires permet ainsi le développement de thérapies moléculaires ciblant ces anomalies de manière spécifique. De plus, la présence de ces altérations dans les lésions précancéreuses laisse également entrevoir de nouvelles stratégies en termes de détection précoce et de chimioprévention. ▼

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5.2 Annexe 2: Mutation de RAS et efficacité de la chimiothérapie

Loriot Y*, Mordant P*, Deutsch E, Olaussen KA, Soria JC. Are RAS mutations predictive markers of resistance to standard chemotherapy? Nat Rev Clin Oncol 2009;6(9):528-34.

Are *RAS* mutations predictive markers of resistance to standard chemotherapy?

Yohann Loriot, Pierre Mordant, Eric Deutsch, Ken André Olaussen and Jean-Charles Soria

Abstract | *KRAS* mutations maybe predictive of resistance to anti-EGFR monoclonal-based therapy in patients with colorectal cancer (CRC). Screening for *KRAS* mutations in patients with CRC and non-small-cell lung cancer (NSCLC) may provide additional information on optimizing treatment options with targeted therapies. Only limited studies, however, have assessed the predictive value of *KRAS* mutations in response to conventional chemotherapy. We reviewed all relevant papers investigating the association of *KRAS* mutations and conventional chemotherapy-related outcome in NSCLC, CRC, and other solid tumors, both in the adjuvant and advanced settings. Our Review strongly suggests that *KRAS* mutations have no value in response prediction to conventional chemotherapy in NSCLC, CRC and other solid tumors. Therefore, *KRAS* mutations should not be used as molecular predictors of response to conventional chemotherapy.

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Introduction

Three human *RAS* genes have been identified: *HRAS* (homologous to the oncogene of the Harvey rat sarcoma virus), *KRAS* (homologous to the oncogene of the Kirsten rat sarcoma virus) and *NRAS* (first isolated from a human neuroblastoma). *RAS* is one of the most important molecules in the EGFR downstream signaling pathway.[1] *RAS* can activate the serine/threonine kinase RAF, mitogen-activated kinases ERK1 and ERK2, phosphatidylinositol 3-kinase, and a number of proteins that translocate to the nucleus to promote cell proliferation. *RAS* genes, especially *KRAS*, have been implicated in the pathogenesis and prognosis of several cancers, including adenocarcinomas of the lung, colon, and pancreas (Box 1). Point mutations that lead to the loss of GTPase activity are associated with the transforming activation of the protein, resulting in a sustained proliferation signal.

Overall, *KRAS* mutations are found in approximately 30% of human cancers, mainly in pancreatic, colorectal, endometrial, biliary tract, lung, and cervical cancer.[2] Oncogenic mutations in *RAS* genes have been detected in 15–30% of lung adenocarcinomas.[3] These alterations are more common in patients with tobacco-associated lung cancer compared with nonsmokers. Patients with *KRAS*-mutant tumors are more likely to be former or current smokers with locally advanced disease.[3] A meta-analysis study has revealed that *RAS* mutations may be a factor for poor survival in patients with non-small-cell lung cancer (NSCLC).[4] *KRAS* and EGFR mutations are thought to be functionally redundant and therefore mutually exclusive.[5]

RAS mutations have been also implicated in the development of colorectal cancer (CRC). Several clinical studies have shown that *KRAS* mutations are a significant predictor of resistance to anti-EGFR therapy in patients with CRC receiving cetuximab or panitumumab, alone or in combination with chemotherapy.[6–9] Screening for *KRAS* mutations among patients with NSCLC and CRC might provide additional information for the selection of optimal candidates for EGFR-based therapy.

Most patients with lung or CRC are treated with standard chemotherapy regimens. Only a limited number of studies have assessed the predictive value of *KRAS* mutations in response to chemotherapy alone, and have resulted in controversial findings. Therefore, we performed a review of the literature to clarify whether *KRAS* mutations could predict clinical outcome to standard chemotherapy regimens in NSCLC, CRC, and other solid tumors.

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Competing interests
The authors declare no competing interests.

Non-small-cell lung cancer

Preclinical data

Cisplatin is the cornerstone chemotherapy regimen in the treatment of NSCLC. Intrinsic chemoresistance in NSCLC has been suggested to be associated with *KRAS* mutations.[10] In this regard most studies have investigated the association between *RAS* mutations and cisplatin efficacy. Resistance to cisplatin can be mediated through the expression of relevant transporters (for example, copper transporters, organic cation transporters, and P-glycoprotein encoded by *MDR1* [multidrug resistance 1]), expression of small molecular weight trace metal and free-radical scavenging proteins (metallothionein), and alterations of DNA repair processes (nucleotide excision repair and mismatch repair).[11] Limited preclinical data, however, are available to support the involvement of *RAS* mutations in resistance to conventional chemotherapy.

The human *MDR1* promoter was shown to be a target for the oncogene c-Ha-RAS-1 and the tumor suppressor

p53 in 3T3 murine fibroblasts. The stimulatory effect of c-Ha-RAS-1 was not *MDR1* promoter specific, in contrast to mutant p53 stimulation and wild-type p53 repression.[12] Wishart *et al.*[13] confirmed that transfection of mink lung epithelial cells with *HRAS* may induce atypical multidrug resistance. This finding, however, has not been observed with *KRAS* expression in NSCLC cell lines. The expression of telomerase and the inactivation of p53 and pRb (retinoblastoma protein) are the minimal set of changes required for the emergence of pretumorigenic drug-resistant cells, which suggests that the ability to acquire multidrug resistance may not be dependent on *RAS* activation.[14] In comparison with nontransformed cells, murine fibroblasts transformed with the viral-*HRAS* oncogene contain elevated metallothionein-IIa mRNA and protein levels.[15,16] This *RAS*-associated change in metallothionein-II expression is accompanied by a notable increase in the half maximal inhibitory concentration (IC_{50}) for cisplatin.[16,17]

A complex network of DNA repair enzymes are involved in nucleotide excision repair of damaged DNA, including the protein Xeroderma Pigmentosum Complementation group D (XPD) and Excision Repair Cross Complementation 1 (ERCC1). Intracellular XPD protein levels in Chinese hamster ovary (CHO) cells have been found to be under the exclusive control of *RAS*-dependent cascade activation in response to insulin.[18] Furthermore, insulin was found to enhance ERCC1 mRNA levels by activation of the RAS/Erk-dependent pathway without the involvement of the phosphatidylinositol 3-kinase/p70 S6 kinase pathway, both in CHO cells and fully differentiated 3T3-L1 adipocytes.[18]

These findings, however, have never been reported in NSCLC cell lines. Mabry *et al.*[19] have shown that viral-*HRAS* expression in the NCI-H82 small-cell-lung-cancer cell line was associated with profound biochemical and morphological changes resulting in cells presenting a non-small-cell phenotype. Despite these phenotypic alterations, viral-*HRAS* expression was not associated with any changes in cisplatin or etoposide sensitivity.[19] Furthermore, Tsai *et al.*[18] found no correlation between chemoresistance to six commonly used chemotherapeutic agents (cisplatin, doxorubicin, carmustine, melphalan, mitomycin, and etoposide) and the presence of *RAS* gene mutations in a panel of NSCLC cell lines. Therefore, at present, no solid preclinical data support the influence of *RAS* expression on chemoresistance in NSCLC cell lines.

Clinical data

Various studies have assessed the prognostic value of *KRAS* mutations in NSCLC. Despite conflicting results and the lack of prospective data, a meta-analysis has confirmed that *RAS* mutations seem to be a factor associated with poor survival.[4] Only a limited number of studies, however, have assessed the predictive value of *KRAS* mutations in patients treated by chemotherapy alone. These studies are summarized in Table 1. The first study to assess the influence of *KRAS* status in patients treated with chemotherapy alone was published by Rosell in 1995.[20] Sixty patients with stage IIIA NSCLC were randomly assigned to receive either immediate surgery alone or three courses of chemotherapy (that is, mitomycin, ifosfamide and cisplatin) followed by surgery. In total, 30 patients were administered preoperative chemotherapy followed by surgery and 30 patients underwent immediate surgery. *KRAS* mutational status was not available for every patient. The frequency of *KRAS* point mutations was found to be higher in the group that received surgery alone (10 of 24 patients) in comparison with the group that received both chemotherapy and surgery (3 of 20 patients). Furthermore, two of the three patients with *KRAS* mutations who received both chemotherapy and surgery had no objective response to treatment, and one had a partial response. In this preoperative chemotherapy group, 10 of the 27 patients with unspecified or wild-type *KRAS* tumor had no objective response to treatment (37%), and 17 had a partial or complete response (63%). Statistical significance, and hence an effective clinical conclusion, was not achieved, owing to the small number of patients with *KRAS* mutations in the study.

Rodenhuis *et al.*[2] reported the results of a prospective clinical trial including 83 patients with inoperable stage III or stage IV advanced lung adenocarcinomas. All patients

Key points

- Activating *RAS* mutations occur in 30% of human cancers and are the most common gain-of-function mutations
- *KRAS* mutations are molecular predictors for the lack of benefit of anti-EGFR monoclonal-based therapy in colorectal cancer
- In lung cancer, colorectal cancer, and other solid tumors, *KRAS* mutations have no value for predicting response to conventional chemotherapy
- *KRAS* mutations should not be used within the decision algorithm of chemotherapy
- All these findings need to be confirmed in prospective trials with appropriate multivariate analysis taking into account other clinical and biological predictors

Box 1 | Characteristics associated with RAS genes and proteins

- In mammalian cells, three *RAS* genes encode four *RAS* isoforms (*HRAS*, *KRAS4A*, *KRAS4B* and *NRAS*) that are highly homologous but functionally distinct.[4]
- RAS proteins are small GTPases that cycle between inactive GDP-bound and active GTP-bound conformations.
- RAS proteins regulate cellular responses to many extracellular stimuli, including soluble growth factors. Growth factors binding to cell-surface receptors create intracellular docking sites for adaptor molecules and proteins involved in signal transduction.[4]
- *RAS* genes are the most common targets for somatic gain-of-function mutations in human cancers.
- Activating *RAS* mutations occur in 30% of human cancers. Specific *RAS* genes are mutated in different malignancies.
- *KRAS* mutations are prevalent in pancreatic, colorectal, endometrial, biliary tract, lung and cervical cancers; *KRAS* and *NRAS* mutations are found in myeloid malignancies; *NRAS* and *HRAS* mutations predominate in melanoma and bladder, respectively.
- In most cases, the somatic missense *RAS* mutations found in cancer cells introduce amino-acid substitutions at positions 12, 13 and 61.[4]

Table 1 | Clinical studies showing a correlation between NSCLC, RAS status and benefit from chemotherapy

Study	Number of patients and disease stage	Chemotherapy regimen	KRAS mutations (%)	Response rate (%)		Overall survival (months) and P value	
				Wild-type	Mutant	Wild-type	Mutant
Rosell et al. (1995)[22]	30 IIIA	cisplatin, mitomycin, ifosfamide (neoadjuvant)	15	NA	NA	NA	NA
Rodenhuis et al. (1997)[2]	62 III–IV	carboplatin, ifosfamide, etoposide (metastatic)	26	26	19 $P=0.49$*	9	8 $P=0.29$*
Schiller et al. (2001)[23]	184 II–IIIA	cisplatin, etoposide (adjuvant)	24	NA	NA	42	25 $P=0.09$* $P=0.07$†
Eberhard et al. (2005)[3]	264 IIIB–IV	carboplatin, paclitaxel (metastatic)	21	26	23 NS*	11	13 NS*
Tsao et al. (2007)[24]	210 IB–II	cisplatin, vinorelbine (adjuvant)	22	NA	NA	NR	74
Zalcman et al. (2008)[25]	178 I–II	cisplatin, gemcitabine or carboplatin, paclitaxel (neoadjuvant or adjuvant)	16	NA	NA	NA	NA

*Univariate analysis. †Multivariate analysis. Abbreviations: NA, not available; NR, not reached; NS, not significant; NSCLC, non-small cell lung cancer.

received chemotherapy (carboplatin, ifosfamide and etoposide). Among the 62 patients whose tumors were assessed, 16 tumors (26%) harbored *KRAS* mutations. The objective response rate was 19% in the *KRAS*-mutation-positive group and 26% in the mutation-negative group, which was not statistically significant (chi-squared test for trend, $P=0.486$). Median survival and median progression-free survival durations were not significantly different between the two groups (log-rank test, $P=0.29$ and $P=0.22$ for survival and progression-free survival, respectively).

In 2001, a prospective randomized trial assessed the potential benefit of postoperative adjuvant therapy in patients with early-stage NSCLC, that is, completely resected stage II and stage IIIA tumors.[22] Patients were randomly assigned to receive either thoracic radiation therapy or four cycles of cisplatin and etoposide plus concurrent thoracic radiation therapy. Among the 184 assessable tumors, 44 (24%) were positive for *KRAS* mutations. The median survival of patients who received chemotherapy was 42 months for those with wild-type *KRAS* tumors and 24.7 months for those with mutant *KRAS* tumors. Univariate and multivariate analysis revealed no significant differences between the groups.

The TRIBUTE trial enrolled 1,079 previously untreated patients with advanced NSCLC (stage IIIB or IV). Patients were randomly assigned to carboplatin and paclitaxel plus erlotinib or placebo.[3] *KRAS* mutational status was assessed in 274 tumors. *KRAS* mutation status was not determined in 10 of the 274 tumors because of polymerase chain reaction (PCR) and/or sequencing reaction failure for exon 2. *KRAS* mutations were detected in 55 of the 264 tumors (21%). Response rates in the chemotherapy alone arm were not significantly different: 23% in the *KRAS*-mutation-positive group ($n=30$), and 26% in the mutation-negative group ($n=103$). The median time to progression and survival rates were not significantly different between the *KRAS*-mutant group (time to progression: 6 months, 95% CI 4.9–7.1 months; survival: 13.5 months, 95% CI 11.1–15.9 months) and *KRAS* wild-type group (time to progression: 5.4 months, 95% CI 4.4–6.1 months; survival 11.3 months, 95% CI lower limit 9.1 months). Although patients bearing *KRAS*-mutant tumors failed to benefit from erlotinib plus chemotherapy, no correlation between *KRAS* mutational status and chemoresistance was established.

The JBR10 trial enrolled a total of 482 patients with completely resected stage IB or stage II NSCLC.[24] Patients were randomly assigned to vinorelbine plus cisplatin or observation. *KRAS* mutational status was available for the tumors of 450 patients. In the chemotherapy arm, which included 210 patients, 46 (22%) had mutations in *KRAS*. Adjuvant chemotherapy prolonged disease-free and overall survival among 333 patients with wild-type *KRAS* in contrast to 117 patients with mutant *KRAS*. Interaction analysis was not statistically significant ($P=0.29$), which suggests that *KRAS* mutational status had no effect on treatment outcome. *KRAS* mutations, therefore, were not prognostic or predictive (Box 2) of a differential benefit from adjuvant chemotherapy.

The IFCT-0002 phase III trial compared two sequences of chemotherapy (preoperative versus perioperative) and two chemotherapy regimens (cisplatin and gemcitabine versus carboplatin and paclitaxel).[25] A total of 528 patients with stage I or stage II NSCLC were randomized, and 16.3% harbored mutations in *KRAS*. Univariate analysis of data from 178 patients revealed that *KRAS* mutational status was the only molecular alteration associated with the absence of tumor response to chemotherapy (hazard ratio [HR] 1.44, 95% CI 1.13–1.83, $P=0.011$). Multivariate analysis, however, demonstrated that *KRAS* mutations were not significantly associated with the absence of response to chemotherapy (HR 1.25, $P=0.082$). Furthermore, *KRAS* mutations were not associated with progression-free survival outcomes.

Overall, these studies demonstrated that *RAS* mutations have no predictive value of response to conventional chemotherapy or survival. Prospective studies that include multivariate analysis are necessary to further confirm this conclusion.

Colorectal cancer

Preclinical data

Several studies have addressed the predictive value of *RAS* mutational status in colorectal cancer and have reported conflicting findings. 5-fluorouracil (5-FU) is an antimetabolic drug that is widely used in colorectal cancer. It inhibits thymidylate synthase and induces cell-cycle arrest and apoptosis by the incorporation of its metabolites into RNA and DNA.[24] Response to 5-FU-based chemotherapy in CRC has been associated with several biomarkers, including overexpression of thymidylate synthase, thymidine phosphorylase, lower dihydropyrimidine dehydrogenase, wild-type p53 and c-myc overexpression.[25] Preclinical data suggest that response to 5-FU might also be predicted by *RAS* mutational status. For instance, targeted deletion of mutant *RAS* in the human colon carcinoma HCT116 cell line was sufficient to protect cells from 5-FU-induced apoptosis through the expression of gelsolin, a protein with antiapoptotic activity.[26] Transient expression of mutant *RAS*V12, but not wild-type *RAS*, enhanced 5-FU-induced apoptosis in rat intestinal epithelial cells.[27] Furthermore, HCT116 cells were more sensitive to camptothecin-induced apoptosis than Hke-3 cells that had a deleted mutant *RAS* allele. These data were supported by another study, which found that the HCT116, Lovo and SW480 colon cancer cell lines, all harboring *RAS* mutations, were more sensitive to 5-FU treatment than HT29 cells that have wild-type *RAS*.[28]

Some reports have shown that oncogenic *HRAS* increased sensitivity to 5-FU in fibroblasts, and reduction of *HRAS* expression in bladder cancer cells conferred resistance to 5-FU-induced apoptosis.[29] These findings suggest that the *RAS* pathway may modulate the responsiveness of cells to multiple chemotherapeutic agents. By contrast, other studies in colorectal cancer suggest a possible predictive value of *RAS* mutations, especially for *KRAS*. Huang *et al.*[30] demonstrated that colon cancer cells bearing wild-type *KRAS* were more sensitive to 5-FU chemotherapy in comparison with *KRAS*-mutant cell lines, which suggests a correlation between *KRAS* genotype and sensitivity to 5-FU. Furthermore, transfection with mutant *KRAS* in thymidylate-synthase-deficient colon cancer cells showed a significant decrease in the ability of cells to undergo apoptosis in response to thymidine deprivation.[31]

Moreover, a relationship between *RAS* mutations and resistance to irinotecan is supported by several studies. Abnormal expression of the *FOS* oncogene, which is downstream of the *RAS* pathway, has been implicated in the resistance to several anticancer compounds. Resistance to irinotecan has been reported in cells with high *FOS* expression, caused by upregulation of topoisomerase I expression. Furthermore, inhibition of c-fos mRNA by anti-fos ribozyme in irinotecan-resistant cells has been shown to reverse resistance to irinotecan and reduce topoisomerase I expression.[32,33]

Box 2 | Biomarker definitions

Biomarker
Characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[52]

Prognostic
Change in a marker or characteristic that is associated with the disease outcome irrespective of treatment.

Predictive
Predicting the usefulness of a given treatment. The benefit of the treatment is greater, for example, for patients with a positive biomarker value, or might even be restricted to these patients.[53] Four groups of patients are needed to define a predictive biomarker (treated and untreated, those expressing the biomarker and those not expressing the biomarker).

Clinical data

Clinical data regarding the predictive or prognostic value of *RAS* mutations in response to chemotherapy in patients with CRC are also inconclusive. These reports are summarized in Table 2. Most studies addressing this question are retrospective and include a limited number of patients. The first study included 37 patients with colon cancer who received 5-FU and leucovorin. *KRAS* mutations at codons 12 or 13 were present in 19 patients and not present in 18 patients.[34] Response to chemotherapy and overall survival rates were similar in patients with wild-type or mutant *KRAS* (28% and 35 months for patients with wild-type *RAS*; 32% and 31 months for patients with mutant *RAS*). Similar results were obtained from a retrospective study including 55 patients with stage III colon cancer treated with adjuvant 5-FU-based chemotherapy.[35]

Another study assessed the predictive value of *KRAS* mutations in 66 patients with stage II colon cancer and 163 patients with stage III colon cancer. Patients were enrolled in a randomized trial (Intergroup Trial 0035), and underwent surgery and received adjuvant levamisole or 5-FU plus levamisole or observation.[36] *KRAS* mutations were found in 41% of cancers and were associated with poor survival in stage II but not stage III tumors. In the group of patients with wild-type *KRAS* tumors, an improvement in the 7-year survival rate was associated with 5-FU plus levamisole therapy (76% versus 44%; HR 0.4; 95% CI 0.2–0.8), whereas no such benefit was observed in the group of patients with *KRAS*-mutant tumors (52% versus 55%; HR 1.1; 95% CI 0.5–2.7). However, differential treatment interaction analysis between wild-type and *KRAS*-mutant tumors were not significant ($P = 0.28$), which suggests that the therapy hazard ratios were not significantly different among the two groups, but rather overlapped.

Another univariate analysis study, in 273 patients with stage III colon cancer who were randomly assigned to receive adjuvant treatment with 5-FU-based chemotherapy

Table 2 | Clinical studies showing a correlation between colorectal cancer, *RAS* status and benefit from chemotherapy

Study	Number of patients and disease stage	Chemotherapy regimen	*KRAS* mutations (%)	Response rate (%) and *P* value		Overall survival (months) and *P* value	
				Wild-type	Mutant	Wild-type	Mutant
Markowitz (1995)[34]	37 IV	5-FU-based	51	32	26	35	31 *P* = 0.96*
Nemunaitis et al. (1997)[33]	35 IV	Irinotecan	40	NA	NA	5	11 *P* = 0.004*
Ahnen et al. (1998)[36]	229 II–III	5-FU-based	41	NA	NA	NA‡ *P* = 0.26	NA
Bleeker et al. (2004)[38]	55 III	5-FU-based	27	NA	NA	NA	NS*
Ginesasanpanthan et al. (2001)[37]	430 III	5-FU-based	33	NA	NA	NA‡§	NA†
Rosty et al. (2002)[35]	56 IV	5-FU-based	30	41	28 *P* = 0.34*	12	15 *P* = 0.51*
Westra et al. (2005)[41]	391 III	5-FU-based	28	NA	NA	NA	NS*
Etienne-Grimaldi et al. (2008)[39]	93 IV	5-FU-based	39	44	32 *P* = 0.27*	NA	NS*
Van Cutsem et al. (2008)[40]	283 IV	FOLFIRI	33	43	40	NA‖	NA
Bokemeyer et al. (2008)[42]	121 IV	FOLFOX	39	NA	NA	NA‖	NA

*Univariate analysis. †Multivariate analysis. ‡Hazard ratio of death (chemotherapy/observation) mutant *RAS* 1.1 versus wild-type *RAS* 0.4. §Hazard ratio of death (chemotherapy/observation) *RAS* Asp-mt 0.33 (*P* = 0.002), *RAS* non-Asp-mt 0.11 (*P* = 0.001), wild-type *RAS* 0.33 (*P* = 0.001), *P* value for interaction not reported. ‖Progression-free survival for wild-type and mutant *RAS* was 8.7 months versus 8.1 months, respectively; hazard ratio = 0.87 (*P* = 0.87). ¶Progression-free survival for wild-type and mutant *RAS* was 7.2 months versus 8.6 months, respectively; hazard ratio = 1.6 (*P* = 0.15). Abbreviations: 5-FU, 5-fluorouracil; NA, not available; NS, not significant.

(5-FU and levamisole or 5-FU, levamisole and leucovorin), demonstrated that *KRAS* status was not associated with disease-free survival.[37] A study conducted in 93 patients with stage IV CRC with inoperable liver metastases, analyzed the effects of *KRAS* mutations in patients who received 5-FU therapy exclusively.[38] *KRAS* mutations were identified in 36 of the 93 metastases; 30 in codon 12 of *KRAS*, and 6 in codon 13. The objective response rate was 44.4% in those with mutant *KRAS* compared with 32.1% in wild-type *KRAS* metastases ($P = 0.27$).[38] Disease-specific survival was not influenced by *KRAS* status, which suggests no predictive and/or prognostic value of *KRAS* mutations in response to combined anti-EGFR monoclonal antibody and 5-FU therapy.[38]

In a small trial of 35 patients with refractory colon cancer who received irinotecan-based chemotherapy (CPT-11), *KRAS* mutations in tumors were associated with poor survival.[33] The median survival of patients with wild-type *KRAS* was 332 days, in comparison with 169 days for patients with *KRAS*-mutant tumors ($P = 0.0036$). There were no differences in sex, age, performance status, prior treatment, current treatment and stage of disease at the time of treatment with CPT-11 among the groups of patients, which suggests a possible predictive value of *RAS* mutations to the lack of benefit from chemotherapy in colon cancer.[33]

A larger, retrospective study in 430 patients with stage III CRC, of whom 208 (48%) had received adjuvant chemotherapy with 5-FU and levamisole or 5-FU and leucovorin, addressed whether there is a specific predictive value for different *KRAS* mutations.[40] A total of 140 mutations were detected; 58% were glycine to aspartate changes in codons 12 and 13. There were no differences in survival benefit based on *KRAS* status. Univariate analysis, however, revealed that patients with wild-type *KRAS* or nonaspartate mutations exhibited a significant survival benefit from chemotherapy, whereas those with aspartate mutations had a moderate benefit.[40] In multivariate analysis, all patients benefitted from adjuvant chemotherapy irrespective of mutational status. Patients with nonaspartate mutations had a greater benefit, confirming the results of the univariate analysis ($P < 0.001$ for wild-type status; $P = 0.019$ for aspartate mutations; $P < 0.001$ for nonaspartate mutations). An interaction test confirming the differences in response rates between mutated or wild-type *KRAS* groups was not reported; therefore, no firm statistical conclusions can be made. Of interest, the confidence intervals overlapped among groups, which suggests a probable negative interaction test.

Two other studies that were reported at the ASCO 2008 meeting addressed the predictive value of *RAS* mutations in CRC. The CRYSTAL study evaluated the effect of *KRAS* mutations on the outcome of patients treated with irinotecan-based chemotherapy (FOLFIRI) with or without cetuximab.[42] Among the 1,998 patients included in this study, 263 patients received FOLFIRI alone. *RAS*

mutations were not predictive of progression-free survival: median survival for patients with wild-type tumors ($n = 176$) and mutant tumors ($n = 87$) was 8.7 months and 8.1 months, respectively (HR 0.97; $P = 0.87$). Furthermore, response rates were not significantly different between the two groups (43.2% [35.8–50.9%] and 40.2% [29.9–51.3%], for the wild-type and mutant groups, respectively). The second study, OPUS, assessed the role of *KRAS* status on progression-free survival and response rate in 337 patients treated with the FOLFOX regimen with or without cetuximab.[41] Among the 337 patients, 121 were administered FOLFOX alone. Median progression-free survival in CRC patients with wild-type *KRAS* ($n = 73$) and with mutant *KRAS* ($n = 47$) was 7.2 months and 8.6 months, respectively. This difference, however, was not statistically significant (HR 1.4; $P = 0.1655$).

Others solid tumors

The predictive value of *RAS* mutations has rarely been evaluated in other solid tumors. A 2008 study reported no predictive value of *NRAS* mutations in patients with metastatic melanoma undergoing isolated limb infusion with melphalan and actinomycin-D.[42] A retrospective study in 76 patients with pancreatic adenocarcinoma who received chemotherapy, radiation therapy or other therapies, or no treatment, also analyzed *KRAS* mutations. Patients with wild-type *KRAS* pancreatic cancer showed improved survival benefit in response to radiation therapy compared with patients harboring mutant *KRAS* tumors. This result, however, was not observed with chemotherapy.[43]

Inconsistent results are reported on the effects of *KRAS* mutations in head and neck cancer. In a study presented at the ASCO 2008 meeting, PCR analysis showed that only 3.5% of 197 head and neck carcinomas presented with *KRAS* mutations in codon 12. There were no significant differences in the characteristics of the patients. The complete response rates to chemoradiotherapy were 71% and 73% ($P = 0.32$), and local relapse rates were 83% and 32% ($P = 0.03$) for patients with mutated and nonmutated tumors, respectively. Although a significant difference regarding local relapse rate was observed, no such difference for overall survival was seen. No significant difference was observed for patients treated with chemotherapy alone according to their *KRAS* status.[43]

In gynecologic cancers, long-term use of tamoxifen in postmenopausal women is associated with a twofold to threefold increase in the risk for developing endometrial cancer. After 24 months of treatment with tamoxifen, *KRAS* mutations were found in 50–60% of endometrial-tumor-free samples of patients.[44] However, the significance of this finding to endometrial carcinogenesis, and its predictive value to standard chemotherapy, remains to be elucidated.[45]

Biliary cancers comprise carcinoma of the gallbladder as well as the intrahepatic, hilar and extrahepatic bile ducts. Cholangiocarcinoma is a rare malignancy associated with poor prognosis and high mortality. *KRAS* mutations have been reported both in dysplasias and carcinomas. Although *KRAS* mutations are rarely detected in gallbladder carcinomas associated with gallstones, they occur often and early in the course of the disease in tumors associated with congenital abnormalcy of the pancreatic bile-duct junction.[46] Surgical resection is the only chance for cure depending on careful selection of patients. There are no conclusive studies regarding the role of adjuvant chemotherapy. The literature regarding treatment results with specific regimens in the adjuvant setting is limited and no general recommendation can be given. In this context, the predictive value of *KRAS* mutations is still to be assessed.[46]

Conclusions

This Review assessed the role of *KRAS* mutations on the effectiveness of chemotherapy in NSCLC, CRC and other solid tumors. *KRAS* mutations have no predictive value of conventional chemotherapy benefit. *KRAS* mutational status may still represent a suitable tool for guiding whether a targeted therapy (such as an EGFR-blocking antibody) or conventional chemotherapy should be used. However, a statistically significant relationship between *KRAS* mutations and median survival was not observed when bevacizumab was combined with chemotherapy in metastatic CRC.[18] These findings need to be confirmed in prospective trials with appropriate multivariate analysis taking into account *KRAS* mutational status together with other clinical and biological predictors.

Review criteria

We searched the MEDLINE database for articles published from the 1 January 1966 to the 1 November 2008. The following search terms were used: "colorectal cancer", "non-small cell lung cancer", "melanoma", "pancreatic cancer", "endometrial and cervical cancer", "biliary tract cancer", "head and neck cancer", "chemotherapy", "anticancer treatment", "therapy" and "RAS mutation". We reviewed all relevant papers investigating the association of RAS mutations and outcome in non-small-cell lung cancer, colorectal cancer and other solid tumors, both in the adjuvant and advanced setting. We reviewed published abstracts from international meetings such as ASCO, American Association for Cancer Research, European Society for Medical Oncology and European Cancer Organization.

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5.3

Annexe 3: Oligonucléotide anti Bcl-2

Loriot Y, Mordant P, Deutsch E. Antisense oligonucleotide targeting Bcl-2 messenger RNA in cancer: bad drug, bad target, neither or both? Ann Oncol 2009; 20(3):596-7.

Annals of Oncology

Antisense oligonucleotide targeting Bcl-2 messenger RNA in cancer: bad drug, bad target, neither or both?

We have read with a great interest the letter entitled "Targeting bcl-2 protein in treatment of melanoma still requires further clarifications" by Pisano et al. [1]. In this letter, the authors suggested that oblimersen, an antisense oligonucleotide (ASO) targeting Bcl-2 messenger RNA, may induce a Bcl-2-independent cellular apoptosis in seven melanoma cell lines. To support this conclusion, the authors observed that oblimersen induced a more important cell growth reduction in Bcl-2-negative cell line than in an appropriate control. At the same time, oblimersen demonstrated the poorest antiproliferative effect in the Bcl-2-expressing cell line. We think that this conclusion requires some comments to avoid a misleading message.

First, the authors have not used an appropriate control to assess the specific effect of the anti-Bcl-2 oligonucleotide. Some studies have reported that oblimersen exhibited immunostimulatory properties [2], and appropriate controls, such as reverse oligonucleotide or antisense mismatch control, are mandatory to blind this nonspecific class effect. Secondly, the concentration used in this publication is a lot higher than the concentrations used in previous publications [3]. The authors used a 250 μM concentration; whereas other studies have shown that oblimersen effectively down-regulates Bcl-2 expression at concentrations below 1 μM. This high concentration increases the likelihood of nonspecific effect. Thirdly, the authors provided no information about Bcl-2 expression in cells treated with oblimersen. Additional data are required to demonstrate that oblimersen effectively down-regulates Bcl-2 expression and finally induces apoptosis in the selected cell lines [4]. Finally, oblimersen may have a greater antitumoral effect in combination with other treatments. Some studies have shown that apoptosis-modulating agents are more efficient in association with chemotherapy or radiotherapy than alone [5].

Hence, we agree with the authors that targeting a single component within the multiple signaling pathways involved in the biology of cancer, and in particular in apoptosis, is unlikely to induce significant antitumor responses. However, preclinical evaluation of molecular targeted therapies should address the following aspects in order to answer the question of efficacy:

1. Does the ASO get into the cells? (cellular uptake)
2. Is the ASO effective at down-regulating the target? (pharmacodynamic criteria)
3. Is the activation of the target a prerequisite for the efficacy of the drugs?

We do agree that the oligonucleotide approach may not be optimal, but the main pitfall is probably an insufficient tumor penetration, rather than hypothetic nonspecific side-effects. Our conviction is that oblimersen may have provided better clinical results if patients had been selected for Bcl-2 over-expression. Pharmacological Bcl-2/Bcl-xl dual inhibitors are more attractive but these drugs will also fail if reasons for the failure of oligonucleotides are not clearly depicted.

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Annexe 4: Discordance mutationnelle entre primitif et métastases

Mordant P, Loriot Y, Castier Y, Deutsch E. Concordance between epidermal growth factor receptor status in primary non-small-cell lung cancer and metastases: still to be established. Eur J Cardiothorac Surg. 2011 Mar;39(3):427-8

the lesion is accomplished with a monopolar device, endocardially. Most of the procedure is carried out on pump and in cardioplegic arrest due to the concomitant mitral valve procedure; diastolic arrest and the opening of the left atrium can help in mobilizing the structures and visualizing the device position and lesions directly. This does not alter the transmurality of the lesions and does not prolong the cross-clamp or the total cardiopulmonary bypass time. In conclusion, our technique is easy, reproducible, and effective in performing a complete set of left-sided lesions for the concomitant treatment of AF in patients undergoing minimally invasive mitral valve surgery.

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Letter to the Editor

Concordance between epidermal growth factor receptor status in primary non-small-cell lung cancer and metastases: still to be established

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Keywords: Non-small-cell lung cancer; EGFR mutation; Targeted therapy

We have read with great interest Watzka's retrospective study of 39 patients with primary non-small-cell lung cancer (NSCLC) and related metastases [1]. In this study, the determination of epidermal growth factor receptor (EGFR) expression by immunohistochemistry (IHC) on formalin-fixed paraffin-embedded (FFPE) tissue revealed good or perfect concordance between primary tumour and all corresponding metastases in 69% of corresponding pairs. The authors' conclusion suggested that the EGFR status of primary tumours correlates with the EGFR status of corresponding metastases in most NSCLCs. We think that the conclusions drawn are partially misleading, in particular because of potential methodological pitfalls.

First, the study population may not be representative of the general NSCLC patients population. Here, the frequency of EGFR overexpression is higher than in recent meta-analysis. From the literature, positivity for EGFR over-expression has been reported to differ between histological types, in a range from 39% in adenocarcinomas to 58% in squamous cell carcinomas [2]. Here, metastatic sites are also unusual, with only 3% of patients with brain metastases at the time of death, compared with 35% in larger series [3].

Second, the methodology used, IHC analysis of EGFR expression on FFPE tissue, is questionable. The results of IHC depend on the fixative used and the time of storage of cut tissue sections as well as the scoring system used. Therefore, this method suffers from lack of standardisation and intra-observer variability, an aspect that has not been addressed by the authors. On the other hand, EGFR mutation analysis would have provided more relevant information including outcome correlation, as opposed to protein expression by IHC, which predicts neither overall survival [2] nor response to EGFR inhibitors [4].

Third, post-mortem analysis has never been correlated to clinical studies regarding relevant NSCLC biomarkers. At least, the interval between death and autopsy should be in the same range as that between surgical removal of a tumour specimen and sectioning in pathology to minimise artefacts in protein expression [5]. This interval is not clarified in the article.

Even though discussed by the authors in the article, this observation is in sharp contrast with previous studies published on the subject. Taking all these above-mentioned considerations into account, we think that the correlation between EGFR expression or mutations in primary NSCLC and related metastases remains to be established. Finally, the prognostic and predictive values of these findings are limited compared with other molecular findings.

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Reply to the Letter to the Editor

Reply to Mordant et al.

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Mordant et al. [1] raise several critical points questioning the representativeness of our patient group and the validity of our methodology, which deserve to be addressed in further detail:

The frequency of epidermal growth factor receptor (EGFR) expression in our study cohort (69%) is higher than in a meta-analysis cited by the authors, in which 49% of tumors were reported to be EGFR positive [2]. The latter analysis included individual studies in which positivity for EGFR overexpression ranged from 27% to 83%. Importantly, these studies used immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), and messenger RNA (mRNA) levels to assess EGFR expression and each differed by the employed cutoff for EGFR overexpression [2]. By contrast, several large prospective randomized clinical trials of EGFR-targeted therapies in non-small-cell lung cancer (NSCLC)-assessed EGFR expression status in formalin-fixed, paraffin-embedded (FFPE) tissues uniformly by the same immunohistochemistry (IHC) test and scoring system and reported a prevalence of EGFR positivity that very much resembles our data [3,4]. We used a commercially available, standardized IHC assay that is widely used in routine assessment of EGFR status in surgically retrieved FFPE samples. Accordingly, intra- and interobserver variability were minimal in our study.

Mordant et al. also note the low prevalence of brain metastases at the time of death in our study cohort. As stated clearly in our article, more than one-third of patients included in our study had not received any form of chemotherapy or radiotherapy, whereas most patients with brain metastases would have received some form of radiotherapy. Moreover, to study the evolution of EGFR expression in different stages of disease, our study also included 15% of patients with locally advanced disease. The observed concordance of EGFR status in paired tumors and metastases in both, pretreated and treatment-naïve patients with different stages of disease, strongly undermines our conclusions that neither the natural history of disease nor its treatment influence EGFR expression status.

While we agree with the authors' reiteration of published literature that EGFR expression of primary tumors does not predict for benefit from EGFR-targeted therapies in NSCLC, their suggestion to prefer EGFR mutation analysis is misleading: While patients with EGFR mutations may derive the greatest benefit from tyrosine kinase inhibitors, a clear overall survival benefit has also been shown for patients with EGFR wild-type tumors [5]. Moreover, EGFR mutations are not predictive for responses to EGFR antibodies [3].

In our study, post-mortem interval ranged from 6 to 12 h. Any alterations in EGFR staining would be expected to affect both primary and metastasis in a similar way. Given the high prevalence of EGFR staining in our cohort, antigen loss unlikely occurred in this interval, while negative staining of adjacent normal tissue precludes false positive staining.

In conclusion, we believe that the results of our study do not contrast, but well support, previously published studies showing that EGFR expression status remains unaltered during the course of disease of NSCLC and its treatment.

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5.5 Annexe 5: Maladie résiduelle en oncologie

Mordant P, Loriot Y, Lahon B, Castier Y, Lesèche G, Soria JC, Massard C, Deutsch E. Minimal residual disease in solid neoplasia: New frontier or red-herring? Cancer Treat Rev 2011 May 23.

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Controversy

Minimal residual disease in solid neoplasia: New frontier or red-herring?

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ABSTRACT

Despite recent advances in prevention, screening, molecular characterization, and treatment, cancer evolution is still associated with late local, regional, or metastatic recurrence, even in early stages. Residual tumor cells can persist locally as cancer stem cells, in the blood flow as circulating tumor cells, and in distant organs as disseminated tumor cells or micrometastasis, defining three faces of minimal residual disease. Definition, preclinical models and clinical implications of these patterns will be detailed, with emphasis on overlaps and therapeutic implications, to determine whether minimal residual disease is only an old concept currently revisited, or a major shift in cancer paradigm.

Introduction

Recent advances in prevention, screening, surgical resection, medical treatment, and radiation therapy have significantly improved cancer outcome. Vaccines that prevent the spread of the human papilloma virus (HPV) promise to dramatically reduce cervical cancer.[1] A growing arsenal of screening technologies has allowed physicians to diagnose and treat cancer earlier in its progression than ever before. Each of the classical pillars of cancer therapy, including surgical resection, chemotherapy, and radiation therapy, has made significant technological strides, including functional imaging, individualized profiling, and targeting systems that improve both prognostic accuracy and individualized treatment for patients.[2]

These evolving strategies result in a significant decrease of cancer related morbidity and mortality in most developed countries.[3] Defined as a tumor confined to the primary site, localized cancer is now associated with excellent initial control rates, approaching 100% in many situations. Nevertheless, even these favorable situations are associated with decreased long-term survival rates. In the US Surveillance Epidemiology and End Results (SEER) program, that covers more than one fourth of the American population and allows long-term follow-up of patients diagnosed with cancer between 1999 and 2005, 5-year relative survival rates of patients diagnosed with localized cancer range from 52.9% in non-small cell lung cancer to 90.4% in colorectal cancer, 98% in breast cancer, and 100% in prostate cancer.[4]

The vast majority of these cancer-related delayed deaths are due to metastatic disease. Malignant primary tumors can often be surgically resected, but the cells that gain the ability to migrate, seed and proliferate in distant organs, are often the most harmful and difficult to target therapeutically.[5] After local and systemic treatment, residual tumor cells can persist locally as cancer stem cells (CSCs), in the blood flow as circulating tumor cells (CTCs), and in distant organs as disseminated tumor cells (DTCs) or micrometastasis, defining three faces of minimal residual disease. Definition, preclinical models and clinical implications of these patterns will be detailed, with emphasize on overlaps and therapeutic implications.

The cancer stem cells hypothesis

CSC definition

Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature tissue

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through differentiation. Key features of normal stem cells are quiescence, asymmetrical division and multipotency. The concept of cancer stem cells arose from the similarities observed between the self-renewal mechanism of normal organs and continuous proliferation in cancer. The cancer stem cells hypothesis suggests that only a small subpopulation of cells in the tumor, called cancer stem cells are able to proliferate and self-renew. These CSCs are thought to come from dormant stem cells that acquired tumorigenic DNA mutation, began dividing inappropriately, and finally directed the neoplastic process.[6] Resulting cancer is organized in a hierarchical fashion, in which CSCs generate a population of non-renewing cells that form the bulk of the tumor.

CSCs characteristics

The hypothesis that cancer growth depend of a small fraction of progenitor cells has been defended for decades before the first experimental documentation by Lapidot et al.[7] Studying acute myelogenous leukemia, these authors demonstrated that only a minority of the leukemic cells was able to proliferate extensively, self-renew and form a new tumor, when injected to an immunodeficient mouse.[7] Since then, CSCs are defined in vivo as a purified cell subpopulation with the ability to form a heterogeneous tumor from a small number of cells injected subcutaneously or orthotopically to an immunodeficient mouse (Fig. 1).

Although CSCs have no specific definition in vitro, some growth properties, such as spheroid formation in culture, seem to be a potential CSC feature.[8–10] Furthermore, CSCs have been largely characterized in vitro, according to the expression of surface proteins; the ability to actively exclude the dye Hoechst 33342 attributed to the expression ATP-binding cassette (ABC) transporter ABCG2; and the high aldehyde dehydrogenase (ALDH) activity.[11]

However, the most convincing demonstration of CSC identity comes from serial transplantation of cellular subpopulations isolated from freshly dissociated human solid tumors, and injected orthotopically into immunodeficient animals.[12] Using in vitro characterization, in vivo assays, and isolation from freshly dissociated solid tumors, stem cell subpopulations have been identified in many cancer localizations, such as breast,[13] brain,[14] colon,[15] prostate,[16] head and neck,[17] pancreas,[18] lung,[19] liver,[20] melanoma,[21] and sarcoma.[22] Markers of cancer stem cells are summarized in Table 1.

CSCs as a residual disease

Due to their low replication rate, high expression of Multi Drug Resistance (MDR) transporters, and increased tumorigenicity, CSCs are thought to be resistant to cytotoxic drug and responsible for tumor regeneration after chemotherapy.

In vitro, using repeated exposition to various concentration of chemotherapy (Fig. 2), drug-surviving cells have been proven to share many CSCs features, including increased expression of membranous (CD133, CD117) or nuclear proteins (Oct-4 and b-catenin); loss of differentiation markers (cytokeratins); ability to grow as tumor spheres, maintain self-renewal capacity, and differentiate; and high tumorogenic potential following inoculation into SCID mice.[23] All this features supported the classification of drug surviving cells as CSCs in models such as breast,[24–26] lung,[23,27] sarcomatous[28] and ovarian cancers.[29] In colon cancer, the drug-selected derivatives of HT29 cell line was associated with induction of CXCR4 and upregulation of ASCL2, which has been shown to play a role in the maintenance of intestinal stem cells.[30] In prostate cancer, human primary tumor cells culture onto human epithelial extra-cellular matrix, allowed the selection of a new prostate cancer cell line, IGR-CaP1, that exhibit the original features of both basal prostate tissue and cancer stemness, but no drug selection was applied.[31]

In vivo, treatment of mice carrying pancreatic[18] or colon[32] carcinoma xenografts increased the proportion of putative CSCs inside the tumor. Such findings have been confirmed in the clinical setting. In breast cancer, chemotherapy-treated patients have a higher proportion of $CD44^{+}/CD24^{-/low}$ cells than untreated patients. This chemotherapy-induced enrichment of the tumor by putative CSCs has also been described in paired specimens from patients before and after neoadjuvant chemotherapy.[24]

Altogether, these data suggest that exposure of a tumor to cytotoxic treatment induced an enrichment in drug-resistant cells, whose features closely mimic CSCs.

The difference between the recent hypothesis of CSCs and former concept of drug resistance may be overestimated. The classic multidrug-resistance (MDR) phenotype was accompanied by cross-resistance to structurally different compounds, and was mediated by ABC transporters. Despite more than 20 years of effort, no pharmacological inhibition of the ABC transporters has proven to be an effective clinical tool to overcome the MDR phenotype.[33] However, drug-resisting and CSCs may be the same cells, but the CSCs hypothesis has gathered additional explorations over time, and benefited from recent progress in cellular biology and molecular pathways analysis.

The link between stemness and radio resistance has also been explored. Intra tumoral hypoxic niches, which play a major role in radio resistance, have been proven to induce the activity of hypoxia-inducible factors (HIFs). These factors induce the expression of oct-4[34] and MYC,[35] both of which are up regulated in stem cells. Furthermore, as underlined by Baumann et al., recent experiments have shown that CSCs isolated using surface markers are more resistant to radiotherapy than non-CSCs.[36] This radio resistance appears to be correlated with more efficient repair of DNA damage in CD133 + cells.[37]

If CSCs are radio resistant, the stemness properties of radio resistant cells should also be proven. Krause et al. have proven that following irradiation, survival of CSCs and expansion of their progeny can be directly visualized in the experimental AT 17/7 tumor, where surviving CSCs are histologically visualized due to the rapid lysis of dead cells.[38] Despite encouraging preliminary results, the selection of resistant cells after exposure to ionizing radiations, and subsequent determination of stem cells marker expression, remains to be established in different tumor types on a systematic basis.[39]

CSCs and cancer dormancy

Cancer dormancy is defined as a protracted stage in tumor progression, in which tumors remain occult and asymptomatic for a prolonged period of time. It can be present as an early stage in tumor or metastasis development, but also as a minimal residual disease after specific treatments.[40]

In untreated primary tumor, dormancy is defined as the time between the carcinogenic transformation event and the onset of inexorable progressive growth. Systematic imaging as well as autopsy studies suggested that primary dormant tumors are frequent in the general population,[40] especially regarding thyroid,[41] breast,[42] and prostate cancer.[43] Several mechanisms have been proposed to explain tumor dormancy and its shift toward inexorable tumor growth, including impaired angiogenesis followed by angiogenic shift,[44] tumor microenvironment balance followed by urokinase receptor (uPAR) dysregulation,[45] potent immunosuppression of tumor growth by the immune system followed by tumor escape.[46] Maintenance of primary dormancy requires a high level of angiogenesis inhibitors thrombospondin[47] and angiomotin.[48] Escape from dormancy requires the activation of

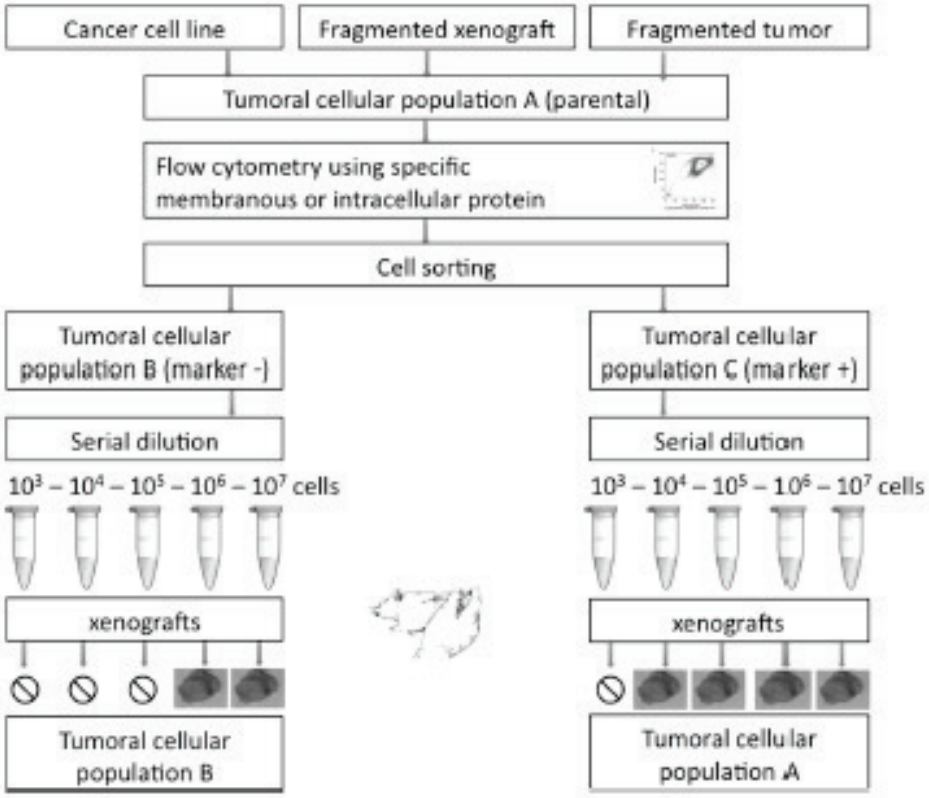

Fig. 1. Schematic representation of the experiment to define stemness in a cellular population expressing a specific marker. The parental cell population (A) is heterogeneous. Flow cytometry and consequent cell sorting is based on the expression of a specific membranous protein (CD133, CD44, etc). The resulting subpopulation are homogeneous regarding the membranous expression of this protein. Serial dilutions are performed, and 10^3–10^7 cells are implanted in mice. The number of cells required to give birth to xenografts is 1–3 logs lower in the CSCs (C) than in the non-CSCs (B) subpopulation. Furthermore, analysis of xenografts resulting from the CSCs subpopulation (C) demonstrates reconstitution of the parental heterogeneity (A).

Table 1
Markers of cancer stem cells in solid tumors.

Localization	Marker
Central nervous system	CD133+
Head and neck	CD44+
Breast	CD44+/CD24−
Lung	CD133+
Pancreas	CD44+/CD24+/ESA+
Liver	CD133+
Colon	CD133+
Melanoma	ABCB5+
Prostate	CD133+

the PI3K/c-MYC pathway and subsequent decrease of thrombospondin secretion.[47] Primary dormant tumoral cells should not be considered as putative CSCs, as increased tumorigenicity has never been reported. The link between normal tissue stem cells and primary dormant tumoral cells remains also to be established.

After specific treatment of a growing tumor, the concept of cell dormancy intersects with those of CSCs and drug-selected cells, arguing for the inclusion of this classical entity in the CSCs hypothesis. In one hand, we have seen that chemotherapy treatment lead to an enrichment of the tumor in putative CSCs in vitro, in vivo, and in the clinical setting. In the other hand, after initial treatment, tumor late recurrences are clearly associated with cell dormancy.[48] Therefore, post treatment cell dormancy and late recurrence have been interpreted as an initial dormancy of CSCs. In a recent study, identification of tumor dormancy was addressed in terms of isolation of therapy-refractory residual tumor cells from tumors that persist in a state of quiescence as label retaining cells. Consequent characterization revealed that label-retaining cells encompass two different populations capable of remaining in a state of quiescence: (i) stem-like cells express a reversibility of quiescence and exhibit therapeutic refractoriness; and (ii) aneuploid cells seem to be either quiescent or proliferation-arrested at steady-state.[50] Whether this dormant CSCs lie in the initial tumor bed, in the bloodstream, or in remote organs remains controversial. Potential cross talks between local, circulating and distant residual tumor cells will be addressed below.

Collectively, these findings reveal that tumor-derived CSCs contribute to drug resistance and post-treatment tumor cell dormancy. However, small size and slow replication remain a major issue when considering non-invasive imaging of these dormant

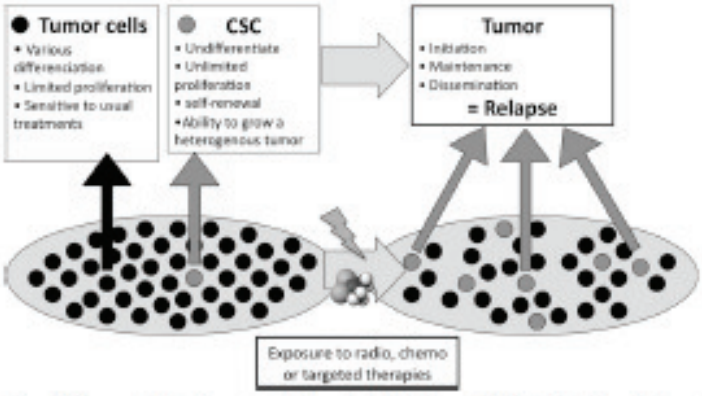

Fig. 2. Selection of drug-surviving cells. Exposure to radio, chemo, or targeted therapies is efficient in non-CSCs, but ultimately results in tumor CSCs enrichment, explaining later tumor relapse.

cells on the site of the primary tumor. In this setting, CTC and DTC detection could represent a valuable alternative to detect occult minimal residual disease.

Circulating tumor cells

Definition

CTCs are released into the bloodstream from tumors of epithelial origin.[51] As hematogenous dissemination of tumor cells is the main mechanism for distant metastasis, the assessment of cancer patients' blood is a highly desirable approach for detecting systemic tumor cell spreading[52] and residual disease. CTCs may be identified directly after automatic enrichment and immunocytochemical detection (CellSearch® System, Veridex®, Warren, NJ, USA[53]), magnetic bead enrichment and laser scanning cytometry (Mainzrac System,[54]), microfluidic harvesting and molecular characterization (CTC-Chip[55]), or membrane filter device (Isolation by Size of Epithelial Tumor cells – ISET[56]). The CellSearch® system is the most available, and is now FDA-approved in breast, colorectal, and prostate cancers.

CTCs may also be identified indirectly, using PCR directed toward circulating mRNA[57] or DNA.[58] Identification of CTC-derived nucleic acid in plasma may account for dissemination of tumor cells. Current approaches include determination of KRAS mutation, P53 mutation, microsatellite alterations, epigenetic changes, mitochondrial DNA, microRNA detection, Cytokeratine or Carcinoembryonic antigen plasma mRNA detection, total circulating plasma DNA levels, and circulating plasma DNA methylation.[59] Circulating plasma DNA was initially though to result either from primary tumor excretion, primary tumor spontaneous lysis, or CTCs spontaneous lysis. However, a strict correlation between circulating total DNA and CTCs has been reported in breast cancer,[59] suggesting that circulating plasma DNA results mostly from CTCs spontaneous lysis, and may serve as a surrogate marker for CTCs, a potential diagnostic indicator in screening programs and a prognostic marker in the clinical follow up. Last but not least, a molecular signature by RT-PCR using epithelial specific genes has been used to detect CTCs in colorectal[60] and breast[48] cancers.

Many studies addressed the prognosis value of CTCs, using either direct CTCs detection or indirect nucleic acid amplifications. Such studies have established CTCs as a prognosis factor in metastatic breast,[52] colorectal,[59] and prostate cancer[54]. In most of the studies, CTCs are selected as cells expressing surface markers EpCAM and cytokeratins, presenting intra cellular DNA, and not expressing hematopoietic marker CD45. As isolated CTCs are rare and only partially sorted, these cells are surrounded by a high number of contaminating leukocytes, making further molecular characterization technically difficult.

Molecular characterization of CTCs

Molecular characterization of CTCs carries multiple basic and therapeutic implications, and has been focused on biomarkers expression. In preliminary reports, CTCs detected with anti cytokeratin antibodies frequently display very heterogeneous tumor specific aberrations, particularly in patients with early stage cancer without overt metastases.[61]

In lung cancer, EGFR mutations have been described in CTCs in vitro[55] and in the clinical setting[58] using microfluidic device, PCR on isolated CTCs, and PCR on circulating plasma DNA. Unfortunately, no direct comparison of EGFR mutational status between primary tumor, CTCs, and circulating plasma DNA has been performed in untreated patients.[58]

In breast cancer, another promising characterization approach has been reported by Sieuwerts et al., using a set of genes with no or minor expression by leukocytes, and allowing further CTC-specific gene expression profiling.[61] A recent publication described an altered hypoxia response and enhanced aggressive phenotype of breast CTCs, in vitro and in vivo.[62] In the clinic, the CellSearch® method and FISH allowed determination of HER2 gene amplification status in CTCs, with discrepancies regarding HER2 status comparison between primary tumor and CTCs.[63,64] A recent study found a rate of concordance of HER2 status between primary tumor and CTCs of 86% at baseline.[70]

In colorectal cancer, a high-throughput method for detection of *KRAS* mutations by a PCR and membrane array has been developed, and suggested a strong correlation between *KRAS* mutation status in the tumor and in the peripheral blood, but cannot strictly conclude that CTCs are the source of the mutation.[71]

In prostate cancer, murine models showed that CTCs are transcriptionally similar to the primary tumor, with notable changes in transcript abundance. As an example, an increase in BCL2alpha gene expression levels has been observed in CTCs relative to primary tumor or metastatic tissue.[72]

CTCs after treatment

The influence of cancer treatment on CTCs levels and molecular characteristics, defining minimal residual disease, has been mostly studied after surgery and treatment with targeted therapies. During surgery, disturbing tumor cells mechanically and causing shedding of tumor cells have long been a concern. The question of whether these tumor cells remain viable and what they subsequently may do is still largely unanswered. And the question has progressively moved to the prognosis role of postoperative CTCs, as a surrogate for residual disease.

In lung cancer, serial analysis of CTCs during treatment with EGFR inhibitors showed that (i) a reduction in the number of captured cells was associated with a radiographic tumor response; and (ii) an increase in the number of cells was associated with tumor progression, with the emergence of additional EGFR mutations in some cases. Interestingly, acquired EGFR mutations have been found in CTCs, but not in the primary tumor.[105] After cytotoxic treatments, the concentration of circulating DNA proved to be an important risk factor for the presence of the illness, and repeated determinations may add a prognostic index in the follow up.[73] In the surgical setting, a preliminary report using the Maintrac System suggested that postoperative CTCs count may be a prognostic factor of early relapse.[74] However, these data need to be confirmed, as in a recent study, no relation between postoperative CTCs level using the CellSearch® system in one hand, and disease relapse or overall survival in the other hand, has been found.[76] After radiation therapy, the value of CK19 mRNA in peripheral blood decreased dramatically compare to that of pre-treatment, but remained positive in 70% of the patients.[57] After chemoradiation, patients with positive CK19 mRNA in peripheral blood had a shorter survival compared to the negative patients.[75]

In breast cancer, a recent study found that HER2 overexpression in CTCs was acquired in 18% and lost in 19% of the patients during a treatment containing trastuzumab. The overall discordance rate between the primary tumor and CTCs was 18%. Patients with HER2 overexpression in CTCs had poorer progression-free survival compared with those without CTCs or with HER2-CTCs.[82] Finally, there has been very limited consideration of estrogen and progesterone receptor determination on CTCs, which might prove useful in predicting the transition from hormone receptor–positive to hormone receptor–negative disease.[56]

In colon cancer, a large-scale study involving patients with stage III and IV disease undergoing curative resection found that postoperative relapse was strongly correlated with CTCs level at pre or postoperative time points, with no differential analysis.[77] More recently, postoperative CTCs levels have been found to be predictive of overall survival after surgical resection of hepatic metastases.[78] In limited disease, a systematic review of the literature confirmed that the presence of CTCs in peripheral blood at least 24 h after resection of CRCs is an independent prognostic marker of recurrence.[79] Further studies are needed to clarify the optimal time point for blood sampling and determine the benefit of chemotherapy in CTC-positive patients with stage-II disease. In patients treated with chemo and targeted therapies, CTCs count before and during treatment independently predicts progression-free and overall survival.[80] However, the impact of targeted therapies on CTC gene expression has not been studied to date.

In castration-resistant prostate cancer, prospective data have shown that changes in CTC number after 4, 8, or 12 weeks of chemotherapy were more strongly predictive of survival than changes in PSA.[83] CTCs can also be assayed for Androgen Receptor gene amplification, PTEN and EGFR expression,[82] fusion proteins and chromosomal rearrangements (e.g., TMPRSS2-ERG) or broad gene expression profiles,[83,86] but once more, the direct impact of targeted therapies on gene expression has not been evaluated.

Therefore, the fact that molecular analysis of CTCs offers the possibility of monitoring changes in epithelial tumor genotypes during the course of treatment, and requires treatment modifications, remain questioned.

Disseminated tumor cells (DTCs) and micrometastasis

From CSCs to CTCs: Epithelial–mesenchymal transition and migrating CSCs

Epithelial to mesenchymal transition (EMT) is the developmental process through which epithelial cells acquire a mesenchymal phenotype. This process is physiologic during development, wound healing, tissue regeneration, and organ fibrosis. EMT is thought to confer tumor cells the capacity to detach from the primary mass by losing cell adhesive properties and acquiring more motile features, thus enabling local invasion, intravasation into blood or lymph vessels, extravasation and the recapitulation of the primary mass at distant sites through the reverse process of mesenchymal to epithelial transition (MET), constituting an essential feature of tumor invasion[103] (Fig. 3).

An important hallmark of EMT is the activation of Wnt signaling, associated with translocation of ß-catenin from adherens junction to the nucleus and subsequent loss of membranous E-cadherin in adherens junction. In colorectal cancer and related liver metastasis, nuclear ß-catenin is accumulated in dedifferentiated tumor cells at the tumor-host interface that have undergone EMT. Interestingly, Wnt signaling is also involved in the self-renewal properties of CSCs, with two groups of target genes. The stemness/proliferation group of genes is activated early and throughout all progression steps, whereas the EMT/dissemination group is expressed later and transiently, mainly at the tumor-host interface.[104]

This dynamic two-phase expression pattern logically leads to a concept based on the existence of two forms of CSCs, stationary CSCs and migrating CSCs (MCSCs). This concept faces major limitations. First, CTCs are currently detected by epithelial markers, pointing a technical limit to MCSCs detection in the bloodstream. Second, MCSCs are defined as tumor cells with both stemness and motility properties, a feature that has not been described to date. Third, as MCSCs are capable of degrading the surrounding matrix, they also enable the detachment and dissemination of non-EMT tumor cells. Whether the CTCs detected using epithelial markers are non-EMT cells, former MCSCs with transient phenotypic modifications, or a mix of both population, remain unknown.

From CTCs to DTCs and micrometastasis: Mesenchymal-to-epithelial transition

Once in the bloodstream, tumor cells will disseminate in homing tissues, such as bone marrow, lymph nodes, lung, liver, or brain. Migrating mesenchymatous cells can then re-activate their epithelial properties through a mesenchymal-to-epithelial transition (MET), the reverse mechanism of EMT, and are then named disseminated tumor cells (DTCs). DTCs and micrometastasis are equivalent (Fig. 3). The bone marrow is a common homing tissue for blood-borne DTCs derived from primary epithelial carcinomas. Studies of bone marrow DTCs present many advantages, including accessibility, liquid aspect, and flow cytometry. This advantage has long assimilated CTCs and DTCs, as with current methods, both can be approached through flow cytometry and present similar cytologic aspect and membrane markers.

However, CTCs and DTCs represent two faces of the same cells but are not equivalent, as their phenotypes should be mesenchymatous in CTCs and epithelial in DTCs. Current methods based on epithelial

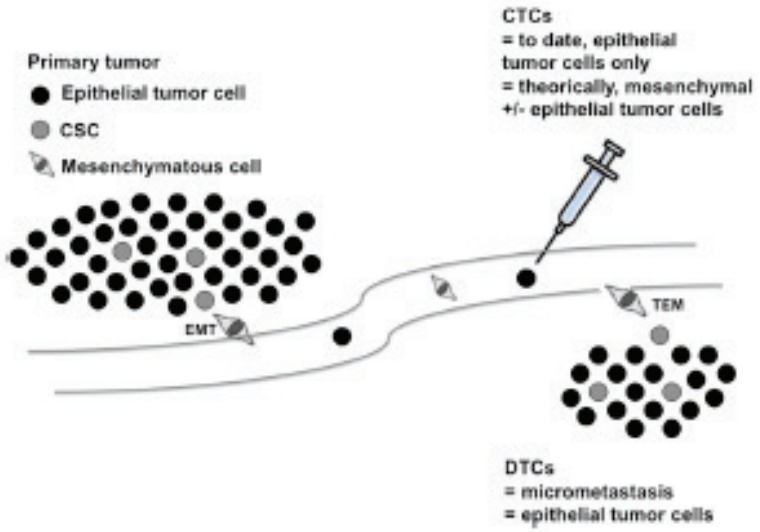

Fig. 3. Hypothesis of tumor dissemination. EMT occurs in the primary tumor. Mesenchymal cells gains the ability to enter blood vessels, follow the bloodstream, and disseminate to remote organs. Here, mesenchymal cells undergo TEM and reproduct primary tumors. The link between CSC and EMT, as the presence of mesenchymal cells in the blood, are still under investigation.

markers detect more easily DTCs than CTCs, as suggested by studies in breast cancer patients.[97] Furthermore, DTCs does not concern only bone marrow, but may also be found in solid organs.

DTCs have been found long after curative treatment of localized tumor, even in patients without overt metastasis, suggesting that DTCs may have a dormant state from seeding to metastasis development. At present, little is known about the factors that might stimulate dormant DTCs to enter uncontrolled proliferation. Hypotheses include changes in DTCs (additional mutations, or epigenetic modifications in genes controlling cell proliferation and apoptosis), modification of tumor microenvironment (release of growth and angiogenic factors), and variation in immune surveillance.[100,101]

After the escape from cell dormancy, DTCs and micrometastasis proliferate to form a complete metastasis that reflects the heterogenous composition of the primary tumor. This implies that DTCs-derived metastasis include both differentiated and more stem-like cellular types. Whether only the EMT-competent MCSCs are capable of forming distant metastases or, when exposed to specific environmental cues, more differentiated tumor cells can also trigger colonization at distant sites, remains unknown.[102]

EMT and CTC in advanced cancer patients

If the hypothesis of preclinical models suggests that EMT is important to metastasis, EMT antigen will be visualized in CTCs from patients with epithelial malignancies.[98]

However, there are only limited data concerning CTCs and EMT in cancer patients. But different reports showed that CTCs express EMT markers or stem cell markers in cancer patients.[92,103] CTCs with mesenchymal and epithelial protein coexpression are detectable in patients with CRPC and women with metastatic breast cancer, in the majority of their detectable CTCs.[104]

These results have several important implications. First, they could explain that EMT may elucidate the under detection of CTCs in many common epithelial malignancies despite a large metastatic burden such as lung cancer. Second, these results with several preclinical findings provide evidence for epithelial plasticity (EMT > MET) in breast and prostate cancer. Third, these results are also important to extend to how CTCs are captured and thus potentially undetected using current technologies. Finally, improving our understanding of CTCs biology may allow for more efficient therapies in the era of personalized medicine.

Therapeutic implications

Understanding the molecular circuitry that contributes to maintenance of stem cells and dormancy may give insight into molecular mechanism of cancer and thus new approaches for cancer treatment. This new model has important implications for the study and treatment of cancer. The analysis of CTC/DTCs could allow the physicians to assess the prognosis and real-time monitoring of the efficacy of systemic therapies (Fig. 4, Table 2).

A major problem in the evaluation of the treatment of advanced cancer is the lack of strong surrogate markers for disease outcome and clinical benefit. But it is also difficult to obtain tumor tissue in metastatic advanced cancer patients in daily practice. Usually, the physicians use the tumor material at time of diagnosis to study different molecular alterations in cancer cells. However, the molecular portrait performed on material at time of diagnosis does not always predict for the molecular portrait of the current disease.

Detecting CTCs or DTCs in cancer patients has a number of potential applications in patients with advanced cancer including the assessment of prognosis, recurrence after definitive local treatment, response to therapy, and obtaining a tumor sample to assess target modulation by drugs without invasive procedure in patients with metastases. Analysis of CTCs/DTCs could provide novel biomarkers, and also a source of putative targets and a marker of resistance to conventional therapies.

Potential biomarkers in clinical practice: CTCs and DTCs

CTCs are tumor cells release in bloodstream, frequently found in blood of patient bearing metastatic cancers. Different CTC isolation

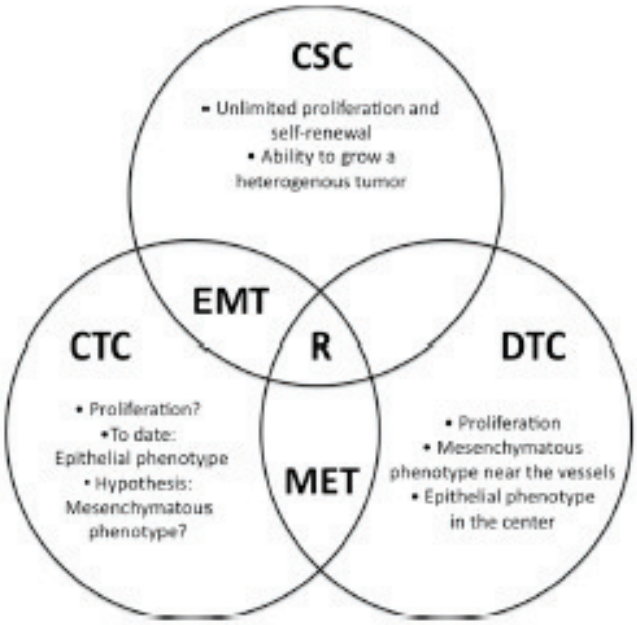

Fig. 4. The multiple faces of minimal residual disease. CSCs, CTCs, and DTCs are all responsible for resistance to treatment (R), but present different proliferation features and cellular phenotypes.

Table 2
The multiple faces of minimal residual disease.

	Identification		Clinical interest			
	Isolation	Markers	Diagnosis	Disease F-U	Molecular F-U	Specific target
CSC	Serial dilution	Stem cell	0	0	0	+
CTC	BC depletion	Epithelial	+	+	+	0
DTC	BC depletion	Epithelial	+	+	0	0

F-U: follow up. BC: blood cells.

methods are available with their advantages and drawbacks. CTC detection can be based on slide-based methods (immunochemistry or FISH), molecular analysis (RT PCR) or flow-based cell sorting methods after immunofluorescent identification and immunomagnetic labeling of cells (anti-Ep-CAM and CK against CTC and antiCD45 against leukocytes).[92]

The CellSearch® System is a semi-automated fluorescent-based performing immunomagnetic cell with anti epithelial marker antibody (anti EpCAM) then labeling with anti-cytokeratins antibodies. This analysis is very simple and feasible due to advances in technology (CellTracks®; Immunicon, PA) that allow the automated separation, visualization and quantitation of cancer cells from blood (7.5 ml samples). The CellSearch® system allows the analysis of CTCs in a standardized, objective manner. CTC enrichment, specific for epithelial cells, is performed automatically with antibody-coated magnetic beads and fluorescent labeling. The specific antibodies for epithelial CTC enrichment and detection are respectively Ep-CAM and Cytokeratin 8/18/19. The leukocyte component is detected with CD45 antibodies. CTCs are defined as nucleated cells lacking CD45 expression and exhibiting Ep-CAM and cytokeratin expression.

Several recent studies have shown that CTC measurement provide prognostic information regarding progression free and overall survival in metastatic breast, colorectal and prostate cancer in large cohort of patients.[96–97] Those studies shown that Favorable/Unfavorable CTC counts (CTC < 3/≥3 or < 5/≥5 in 7.5 ml of blood) is strongly associated to the probability of overall survival for patients at monthly intervals after initiation of therapy. At all time points tested the difference in survival between the Favorable and Unfavorable CTC groups was highly significant.[98] Implications of these findings are that CTC may predict outcomes at any time during treatment and could can be used to monitor treatment – any treatment. Moreover, in all three cancers, patients with persistent CTC counts above threshold had the worst outcome. These data led to Food and Drug Administration (FDA) clearance of CellSearch® system for monitoring metastatic breast, colorectal and prostate cancer. Currently this system is the only system to detect CTC having FDA approval and that enable reliable detection of CTC in blood, suitable for the routine assessment of metastatic cancer patients in the clinical laboratory setting.

Other studies showed that it was possible to isolate CTC using on CellSearch® systems in different type of cancers such as gastric cancers, esophageal cancers, bladder cancers or kidney cancers.[99,100–108]

Putative targets

The cancer stem cell and dormancy hypothesis could have a fundamental and profound implication for cancer therapy. Cancer stem cells as normal stem cells are more resistant to conventional chemotherapy than other more differentiated cancer cells. Therefore, to cure cancer it is also important to use therapies that

target not only proliferating cells but also stem cells. A better knowledge of molecular signaling pathways that controls stem cell proliferation, differentiation and dormancy could lead to the development of new anti cancer strategies (Fig. 4, Table 2).

Besides the quantitative use of CTC count to predict prognosis or response to treatment, collecting CTCs may be used as an elegant technique to sample malignant tissue for molecular analyses. As CTC have been shown to be a powerful prognostic marker in several different tumor types of epithelial origin such as prostate cancer, we considered that CTC could also be a good surrogate material for tumor tissue, potentially useful for testing potential biomarkers predictive of drug efficacy.

Greater knowledge of advanced cancer biology has led to the identification of a number of molecular alterations, some of which are promising potential targets. The discovery of recurrent gene fusions in prostate cancers,[102,103] EGFR mutation and ALK translocation in lung cancer,[104] BRAF mutation in melanoma patients[105] has important clinical and biologic implications. Moreover, various molecular abnormalities in the tyrosine kinase receptors lead to resistance to targeted therapies. Detection of molecular abnormalities in a subpopulation of CTCs will complete information on tumor biology or tumor sensitivity to treatment, and may constitute a surrogate marker of primary or metastatic tumors that ultimately permit a more personalized therapeutic approach.[106,107] CellSearch® technology and other technologies may allow the molecular characterization of CTC using immunofluorescence (IF) for protein expression and FISH for DNA amplification. These techniques have been used to sequence the androgen receptor and to detect TMPRSS2/ETS gene translocations in CTC in prostate cancer.[107]

Resistance to conventional treatment

CTC counts and variation of number of CTC detected was proposed as an independent biomarker associated to overall survival in several types of metastatic cancer. This biomarker measure at the initiation of new therapy as well as during treatment follow-up (as soon as 2–4 weeks) was proposed as predictor of response determined by RECIST imaging modalities. Cristofanilli et al. demonstrate that the detection of CTCs in patients with metastatic breast cancer at 3–4 weeks after the initiation of therapy predicts treatment efficacy as determined by traditional imaging modalities (RECIST) 5–6 weeks later.[96] The association between CTC at 1 month and RECIST response is also true for patient harboring metastatic colorectal cancer.[101] It has been also shown that assessment of CTCs is an earlier, more reproducible indication of disease status than current imaging methods and the authors hypothesized that CTCs may be a superior surrogate end point, as they are highly reproducible and correlate better with overall survival than do changes determined by traditional radiology for patients with metastatic breast cancer, colon cancer and prostate cancer. These data suggest that after the first cycle of therapy, the clinician could determine which patients are showing less than optimal response and who should therefore be potentially placed on an alternative therapy.

An old concept revisited?

Previous hypothesis

The concepts of cell dormancy, MDR, and CSCs result for the same clinical observation that when exposed to radio, chemo, or targeted therapies, tumor have partial response concerning the bulk of the tumor, but residual tumor resist and finally give birth to local and/or systemic relapse, which are usually cross-resistant to common treatments.

These three concepts then differ at the cellular level. In one hand, dormancy is defined mostly before treatment, and includes progressive cellular transformation, microenvironment changes, and immune response modifications, in the interval from the carcinogenic transformation event to the onset of inexorable progressive growth. In the other hand, MDR is defined after treatment, as a particular phenotype associated with specific membrane transporters expression and consequent resistance to multiple therapies. Finally, cancer stemness is defined both before and after treatment, as a particular ability to grow a heterogenous tumor from a homogenous cellular population. Therefore, from the cellular point of view, cancer stemness summarizes pre treatment dormancy and post treatment MDR in a single concept.

Furthermore, some molecular characteristics are common between the three definitions. As an example, membrane efflux pumps are present both in MDR and CSCs, and the ability to retain or exclude a specific dye is the cornerstone of MDR and CSCs side-population definitions. All together, these data suggest that the differences between the recent hypothesis of CSCs and former concept of cell dormancy and MDR may be overestimated. The CSCs hypothesis has gathered additional explorations over time, and benefited from recent progress in cellular biology and molecular pathways analysis, but probably widely overlaps with dormancy and MDR concepts.

New features

Despite large overlaps, the progresses allowed by the CSCs hypothesis are crucial. First, as shown in this review, CSCs are now integrated in a wider concept of minimal residual disease, with transient state of EMT and MET being explored. Second, integration of CSCs, CTCs, and DTCs open the door to the validation of CTCs and DTCs as surrogate markers, overwhelming the technical difficulties to image CSCs in patients. Third, molecular dissection of CSC allows identification of novel targets and development of related therapies for all stages of disease. The CSCs hypothesis may be an old story; it is still to be continued.

Conclusions

The residual disease (cancer stem cell, dormancy, circulating tumor cells) hypothesis is a promising new paradigm that could potentially influence cancer diagnosis and management. Current cancer treatments (surgery, chemotherapy, radiotherapy) have been evaluated on their ability to kill proliferating cells. However, these therapies can fail due to survival of residual disease.

The detection of DTCs and CTCs has been shown to be of clinical relevance in many tumor types, and particularly in breast, colon and prostate cancers. The prognostic value of baseline CTC counts in advanced cancer is just the one early example of a wave of novel blood-borne biomarkers in cancer patients. New platforms might open new opportunities for CTC detection, circulating free DNA, microRNAs studies and allow monitoring of disease in advanced cancer patients. These new biomarkers have additional uses as pharmacodynamic and/or intermediate markers of activity and molecular characterization of CTCs to help optimize treatment selection.

Furthermore, the cancer stem hypothesis is only one of the important aspects in the current understanding of cancer biology. An appropriate local microenvironment, an immunologic tolerance, the emerging role of the genetic background of the host in dissemination and homing of CTCs as well as newly developed vessels are key elements necessary to allow tumor growth, and their targeting is also of the highest importance.

Conflict of interest statement

The authors declare that they do not have any competing financial interests or potential conflict of interest.

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