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GLIOBLASTOME MULTIFORME ET ÉPIGÉNÉTIQUE : DE LA PRÉVENTION AU DÉVELOPPEMENT DE NOUVEAUX TRAITEMENTS

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RÉSUMÉ

Depuis de nombreuses années, la lutte contre le cancer est un enjeu de santé publique majeur. Le glioblastome multiforme est une tumeur primitive du cerveau particulièrement agressive, avec une survie à 5 ans inférieure à 5%. Lors de ce travail de thèse, je me suis intéressée à la lutte contre cette pathologie sous différentes formes, en me concentrant sur l'aspect épigénétique. Dans un premier temps, je me suis penchée sur les causes moléculaires à l'origine de l'apparition d'un glioblastome, afin d'améliorer sa prise en charge. Nous avons mis en évidence la capacité du diuron, un herbicide couramment utilisé jusqu'en 2008, à induire la gliomagenèse lorsque son exposition est couplée à la surexpression d'AKT dans des progéniteurs astrocytaires. Le diuron a aussi un impact sur la cytotoxicité des pDC et diminue leur capacité à détruire des cellules tumorales. Ensuite, nous avons démontré que TET2, une enzyme participant à la déméthylation de l'ADN n'était pas facteur pronostic au moment du diagnostic, mais que son augmentation était liée à un temps plus court entre deux résections, c'est-à-dire au moment de la rechute. La troisième partie de mon travail consistait à m'intéresser à la question du suivi de l'évolution de la réponse au traitement. Pour cela, nous avons démontré que la surexpression d'un exomiR était corrélée à la diminution du granzyme B dans le sang des patients. Enfin, nous avons étudié la mise au point de nouveaux traitements, basés sur les microARN, que ce soit tel quel ou sous forme d'une prodrogue méthylée. Derrière ce travail, l'idée était de s'intéresser aux différentes phases par lesquelles le patient passe, pour essayer de répondre aux nombreux enjeux thérapeutiques et de participer à une meilleure prise en charge du patient à chaque stade.

ABSTRACT

Since many years, fight against cancer is a major public health issue. Glioblastoma multiforme is a particularly aggressive primary brain tumor, with a 5 years survival inferior to 5%. During my thesis, I focused on struggling this pathology under several forms, concentrated on epigenetics. First, I tried to find a way to anticipate glioblastoma emergence, in order to improve its care. We brought to light diuron capacity, an herbicide often used until 2008, to induce gliomagenesis when its exposure is coupled to AKT overexpression in astrocytary progenitors. Diuron also has a negative impact on NK cells cytotoxicity. Secondly, we demonstrated that TET2, an enzyme implicated in DNA demethylation was not a prognosis factor at diagnosis time, but its increase is correlated to a shorter time between two resections. Third part of my work consisted in interesting in monitoring the evolution to patient response to treatment. To this end, we demonstrated that an exomiR overexpression was correlated to granzyme B decrease in patients' blood. Finally, we studied development of new treatments, based on microRNA, unaltered or under a methylated prodrug form. With this work, the idea was to be concerned by several phases which patients undergo, in order to try to answer to numerous therapeutic issue and to participate to a better care of patients to every level: before, during and after disease.

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Abréviations

5caC : 5-carboxycytosines

5fC : 5-formyl-cytosines

5hmC : 5-hydroxyméthyl-cytosines

5mC : 5-méthylcytosines

AKT : RAC-alpha serine/threonine-protein kinase

AMPD : active modification followed by passive dilution

APC : Adenomatous Polyposis Coli Protein

BCDIN3D : Bicoid interacting 3 domain containing RNA methyltransferase

Bcl-w : Bcl-2-like protein 2

Cas9 : CRISPR associated protein 9

ChIP-Seq : Chromatine ImmunoPrecipitation-Sequencing

CTLA4 : cytotoxic T-lymphocyte associated protein 4

CXXC5 : CXXC-Type Zinc Finger Protein 5

DICER1 : endoribonuclease Dicer

DMOG : dimethyloxallyl glycine

DNMT : ADN méthyltransférases

DNMTi : inhibiteurs des méthylases de l'ADN

domaine ADD : ATRX-DNMT3-DNMT3L domain

domaine de liaison aux protéines à doigts de Zinc : Zn-binding CXXC-type

EGFR : epidermal growth factor receptor

EPCAM : epithelial cell adhesion molecule EPCAM

EpiSAVMEN : Epigénétique - Santé - AgroAlimentaire - Végétal - Mer - Environnement - Nutrition

ESC : cellules souches embryonnaires

FTO : Fat Mass And Obesity-Associated Protein

GBM : glioblastome multiforme

GW182 : TNRC6A, trinucleotide repeat-containing gene 6A protein

HDAC : histones déacetylases

HDACi : inhibiteurs des histones déacétylases

HNPCC : Hereditary Non Polyposis Colorectal Cancer

hnRNPA2B1 : hnRNPQ heterogeneous nuclear ribonucleoprotein A2/B1

ICO : Institut de Cancérologie de l'Ouest

IFN : interféron

IGF2 : insulin-like growth factor

INK4A : CDKI2A, Cyclin Dependent Kinase Inhibitor 2A

IR : radiothérapie

IRF7 : Interferon Regulatory Factor 7

ISGF3γ : Interferon-Stimulated Transcription Factor 3, Gamma

KRAS : Kirsten rat sarcoma viral proto-oncogene

KRAS : Kirsten rat sarcoma viral proto-oncogene

LLT1 : CLEC2D, C-type lectin domain family 2 member D

METTL3 : Methyltransferase Like 3

MGMT : O-6-Methylguanine-DNA Methyltransferase

MICA : Major Histocompatibility Complex Class I Chain-Related Protein A

miR : microARNs

miRISC : miRNA-induced silencing complex

MLH1 : DNA Mismatch Repair Protein Mlh1

MMR : MisMatch Repair MMR

MSH2 : DNA Mismatch Repair Protein Msh2

MVB : multivesicular bodies

NGS : Next Generation Sequencing

NK : cellules Natural Killer

nSMase : neutral sphingomyelinase

PARylation : poly-adénosine ribosylation

PBMC : cellules mononucléées périphériques du sang

P-body : processing body

PCNA : Proliferating Cell Nuclear Antigen

PD-1 : programmed cell death protein 1

pDC : cellules dendritiques plasmacytoïdes

PD-L1 : programmed death ligand 1

PKC : protein kinase C

pri-miRNAs : miRNA primaires

PU.1 : hematopoietic transcription factor PU.1

RB1 : retinoblastoma 1

RCAS : replication-competent ASLV

SAHA : suberoylanilide hydroxamic acid

SMAD4 : Mothers against decapentaplegic homolog 4

SYNCRIP : hNRNPQ heterogeneous nuclear ribonucleoprotein Q

TDG : thymine ADN glycosylase TDG

TET : Ten Eleven Translocation TET

TMZ/IR : témozolomide/irradiation

TRBP : trans-activation responsive RNA-binding protein

UHRF1 : Ubiquitin-like containing PHD and RING finger domains 1

WT1 : Wilms tumor 1

YBX1 : Y-box-binding protein

YTH : YT521-B homology

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1. Introduction

1.1) L'ÉPIGÉNÉTIQUE

En 1910, Thomas H. Morgan s'intéresse à la transmission de caractères phénotypiques tels que la couleur des yeux chez la Drosophile, et met en évidence que les chromosomes portent des informations transmissibles d'une génération à l'autre [1]. En 1934, il se pose la question suivante : « Si les caractères de l'individu sont déterminés par les gènes, pourquoi toutes les cellules d'un organisme ne sont-elles pas identiques ? » [2]. Sans le savoir et sans que l'ADN ne soit connu, il met le doigt sur la notion d'épigénétique.

Cette idée est ensuite reprise par Conrad Hal Waddington en 1942, qui invente le mot « épigénétique » pour parler du lien entre le génotype et le phénotype [3]. Il développe alors le concept du paysage épigénétique, où chaque cellule suit son propre parcours pour arriver à des phénotypes différents (Figure 1). L'ADN et donc la génétique correspond au support de l'hérédité, et est modulée par l'épigénétique.

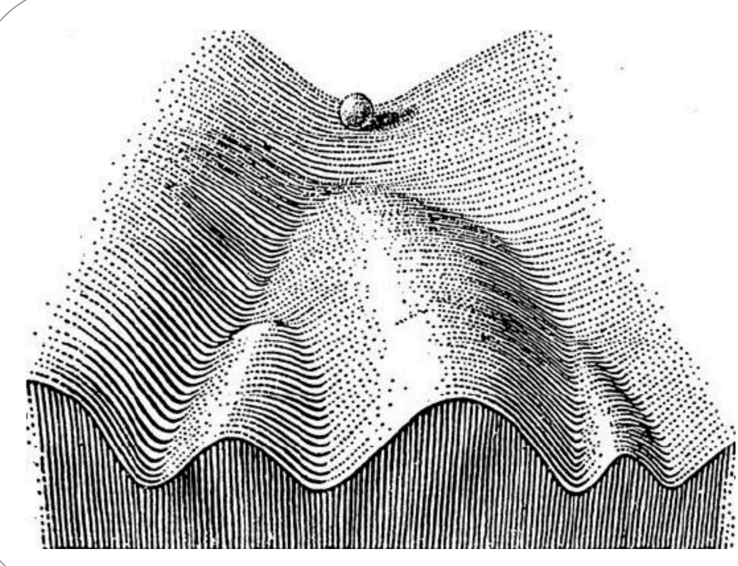

Figure 1 : Le paysage épigénétique de Conrad Waddington

Décrit en 1942, le paysage épigénétique façonne le devenir d'une cellule et fait le lien entre génotype et phénotype. Source : [4]

En 1953, l'ADN est découvert par Watson et Crick [5], et même si l'avancée scientifique est bien évidemment majeure, elle n'explique pas comment l'expression de l'ADN est régulée.

S'en suivent alors de nombreuses publications, découvrant tour à tour les modifications chimiques des histones [6], puis la méthylation de l'ADN [7, 8] et l'impact de ces modifications sur l'expression des gènes. De nos jours et grâce au développement de séquenceurs toujours plus puissants, les scientifiques sont capables de détecter la

méthylation de l'ADN à la base près, et d'étudier les séquences de l'ADN liées à une modification d'histone précise par ChIP-Seq (Chromatine ImmunoPrecipitation-Sequencing).

Aujourd'hui, en s'appuyant sur les travaux de Robin Holliday, l'épigénétique peut se définir comme étant les mécanismes transmissibles et réversibles de régulation de l'expression des gènes, sans modification de la séquence des bases de l'ADN [9]. En 2007, Adrien Bird complète cette définition en parlant de l'épigénétique comme étant l'étude des « adaptations structurales des régions chromosomiques qui permettent d'enregistrer, de marquer ou de perpétuer des états modifiés d'activité des gènes » [10].
Enfin en 2019, lors d'un entretien pour « CNRS, le journal », Edith Heard définit l'épigénétique comme étant « tout changement d'expression des gènes qui n'implique pas de changement dans la séquence ADN, qui est stable mais demeure réversible ».
Au fil des études, il est apparu que certaines marques épigénétiques ou certains changements d'expression de gènes pouvaient être transmis d'une cellule à une autre et d'une génération d'individus à un autre. En effet, les modifications épigénétiques sont transmises après la mitose, mais aussi après la méiose. Cela explique le fait que des modifications épigénétiques puissent être retrouvées plusieurs générations après celle où elles ont été imprimées, en dépit du fait qu'au moment de la fécondation, les marques épigénétiques sont entièrement effacées puis réimprimées [11, 12]. Ce phénomène est très précoce dans le développement, puisqu'il a été démontré qu'une fécondation *in vitro* augmentait le risque d'une mauvaise réimpression des marques épigénétiques et le développement du syndrome de Beckwith-Wiedemann, une maladie se caractérisant notamment par une croissance excessive du fœtus et un risque accru de développer des tumeurs embryonnaires [13].

En extrapolant, nous pourrions dire qu'à l'origine, nous sommes donc tous issus de la combinaison d'un héritage génétique et d'un héritage épigénétique.

D'un point de vue mécanistique, l'épigénétique peut se définir comme se composant des mécanismes qui gouvernent l'activité/l'expression du génome sans affecter la séquence de

l'ADN, à savoir la méthylation de l’ADN, les modifications des histones et la dynamique de condensation/décondensation de l’ADN.

D’autres mécanismes sont impliqués dans la régulation de l’expression des gènes comme ceux impliquant les ARN non codants, mais leur inclusion dans le terme « épigénétique » est encore, à ce jour, source de débat.

1.1.1) Les modifications d’histones et condensation de la chromatine

La première grande famille de modifications épigénétiques est la modification des histones, influant directement sur l’état de compaction de la chromatine. L’ADN d’une cellule entièrement décompacté mesurerait environ 2 mètres, or il est capable de rentrer dans cette cellule dont le diamètre est d’environ 20 µm [14]. Il est donc compacté en différents stades : la double hélice d’ADN s’enroule sous forme de « collier de perles », puis en fibres, en boucles et finalement en chromosomes (

Figure 2).

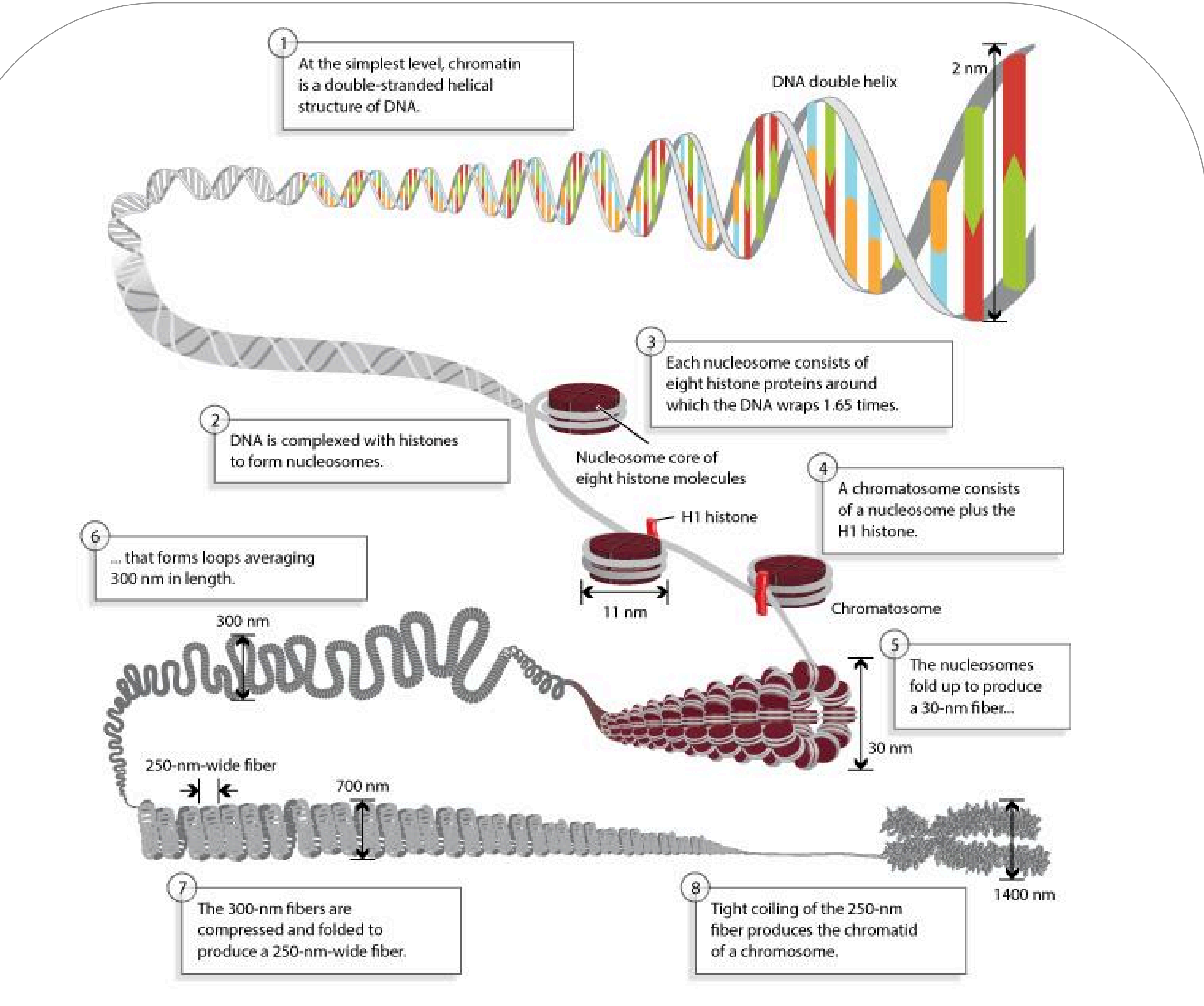

Figure 2 : Les différents niveaux de compaction de l'ADN

De la double hélice au chromosome, l'ADN subit différents niveaux de compaction. Source : [14]

Au-delà du fait qu'il est indispensable que l'ADN soit compacté pour être contenu dans les cellules, la compaction de l'ADN est un point crucial dans la régulation de la transcription. En effet, l'ADN sera plus ou moins accessible aux enzymes de transcription selon son niveau de compaction. Cet état de condensation de la chromatine est régulé par les modifications d'histone et est considéré comme une modification épigénétique.

Les histones sont les protéines autour desquelles l'ADN s'enroule, formant les nucléosomes. Chaque nucléosome est composé d'un cœur de 8 histones (H2A, H2B, H3 et H4) et d'environ 146pb d'ADN s'enroulant autour du cœur d'histones [15]. Suite à l'ajout d'un linker (H1) au niveau de la particule cœur du nucléosome, celui-ci est appelé chromatosome.

Les modifications chimiques des histones sont impliquées dans la modification du niveau de compaction de la chromatine, libérant ou au contraire emprisonnant les sites de fixation des facteurs de transcription et/ou des polymérases. Les principales modifications sont la méthylation et l'acétylation. La même modification peut avoir des rôles opposés selon l'acide aminé et l'histone sur laquelle elle a lieu et sa position sur le génome (Figure 3).

Modification d'histone	Fonction
H3K4me1	Activation
H3K4me3	Activation
H3K36me3	Activation
H3K9me2	Activation
H3K9Ac	Activation
H3K27Ac	Activation
H4K16Ac	Activation
H3K27me3	Répression
H3K9me3	Répression

Figure 3 : Principales modifications d'histones et rôles sur la transcription

L'acétylation est généralement associée à l'activation de la transcription, alors que la méthylation a un rôle ambivalent selon sa position. Source : [15]

Plusieurs familles d'enzymes capables de modifier les histones existent, et il est possible de les classer en deux types : les « writers » et les « erasers ». Les premières seront capables de catalyser la mise en place des modifications sur l'histone, comme les HAT (Histone AcétylTransférase) ou les HMT (Histone MéthylTransférase); les secondes enlèvent ces modifications, comme les HDAC (Histone DéAcétylase) ou les histones déméthylases [16] (Figure 4).

Modification	Writers	Erasers
Acétylation	Histone acétyltransférase (HAT)	Histone déacétylase (HDAC)
Méthylation	Histone méthyltransférase (HMT)	Lysine déméthylase (KDM)

Figure 4 : Enzymes de modification des histones

Pour l'acétylation et la méthylation des histones, des writers et des erasers existent. Source : [17]

Dans la section « Environnement » de cette introduction, nous aborderons le fait que ce mécanisme est dynamique et modulable selon les besoins de la cellule et son environnement, et bien qu'étant crucial dans la régulation de la transcription, il n'est pas unique, il existe aussi la méthylation de l'ADN.

1.1.2) La méthylation de l'ADN

Chez les mammifères, la méthylation de l'ADN correspond à l'ajout d'un groupement méthyl (CH_3) sur le carbone 5 des cytosines, la plupart du temps contenues dans des dinucléotides CpG, avec comme donneur la molécule de SAM (S-adénosine méthionine) par des enzymes appelées DNA Methyl Transferases (DNMT) (

Figure 5). Cette molécule de SAM résulte du métabolisme du folate, présent dans l'alimentation.

Figure 5 : Méthylation de la cytosine par les DNMT

Adapté de [18]

Ces cytosines deviennent alors des 5-méthylcytosines (5mC). La méthylation de l'ADN peut avoir lieu en dehors des dinucléotides CpG, ce phénomène est cependant restreint à certains organismes et plus précisément à certains types cellulaires: les cellules souches pluripotentes, les oocytes, les neurones et les cellules gliales, mais leur rôle n'est pas totalement décrit et plutôt controversé [19].

Chez l'Homme, 70 à 80% des cytosines contenues dans les CpG sont méthylées [20]. Les dinucléotides CpG peuvent être inclus dans des îlots CpG, définis comme des segments de plus de 500 pb où les dinucléotides CpG représentent plus de 55% de la séquence avec un ratio « CpG observés/CpG attendus » supérieur à 0,65 [21]. Ils sont retrouvés dans des zones de contrôle transcriptionnel tels que les promoteurs [22], et en conditions physiologiques, les îlots CpG ne sont pas méthylés, exception faite pour les gènes spécifiques des cellules germinales dans les cellules somatiques [23, 24]. La méthylation des îlots CpG empêcherait donc l'activation de gènes méiotiques dans les cellules somatiques.

1.1.2.1) Acteurs de la méthylation de l'ADN

Les acteurs de la méthylation de l'ADN sont regroupés dans une famille de protéines présentant des homologies de séquence : les ADN méthyltransférases (DNMT) (Figure 6).

Ces DNMT possèdent un domaine de régulation et un domaine catalytique, avec des points communs entre les différentes classes (Figure 6). Le domaine de régulation confère aux DNMT leurs propriétés d'interaction avec l'ADN mais aussi avec d'autre protéine. Par exemple, le domaine de liaison à PCNA (Proliferating Cell Nuclear Antigen) n'est inclus que dans la DNMT1, pour former le complexe DNMT1/PCNA/UHRF1 (Ubiquitin-like, containing PHD and RING finger domains, 1) [25–27]. Certaines DNMT possèdent un domaine de liaison aux protéines à doigts de Zinc (Zn-binding CXXC-type), leur conférant leur propriété de liaison à l'ADN [28, 29]. Le BAH domain (bromo-adjacent homology) correspond, quant à lui, au domaine favorisant l'interaction avec des protéines régulant l'état de la chromatine [30]. Le PWWP domain (proline–tryptophane-tryptophane-proline) est un motif de 90 à 130 acides aminés hautement conservé chez les eucaryotes et qui reconnait la méthylation aussi bien sur l'ADN que sur les histones [31, 32]. Les DNMT3A, 3B et 3L possèdent

également un domaine ADD (ATRX-DNMT3-DNMT3L domain) leur permettant de d'interagir avec les H3K4 non méthylées [33]. Le domaine catalytique des DNMT est hautement conservé aussi bien chez les eucaryotes que chez les procaryotes [32], et 6 motifs sont bien décrits (I, IV, VI, VIII, IX et X). C'est d'ailleurs la présence de ces motifs qui ont permis d'inclure la DNMT2 dans la famille des DNMT suite à son clonage [34]. Mais le rôle de cette protéine fait débat, elle aurait un rôle majeur dans la méthylation des tRNA et mineur dans la méthylation de l'ADN [35–37].

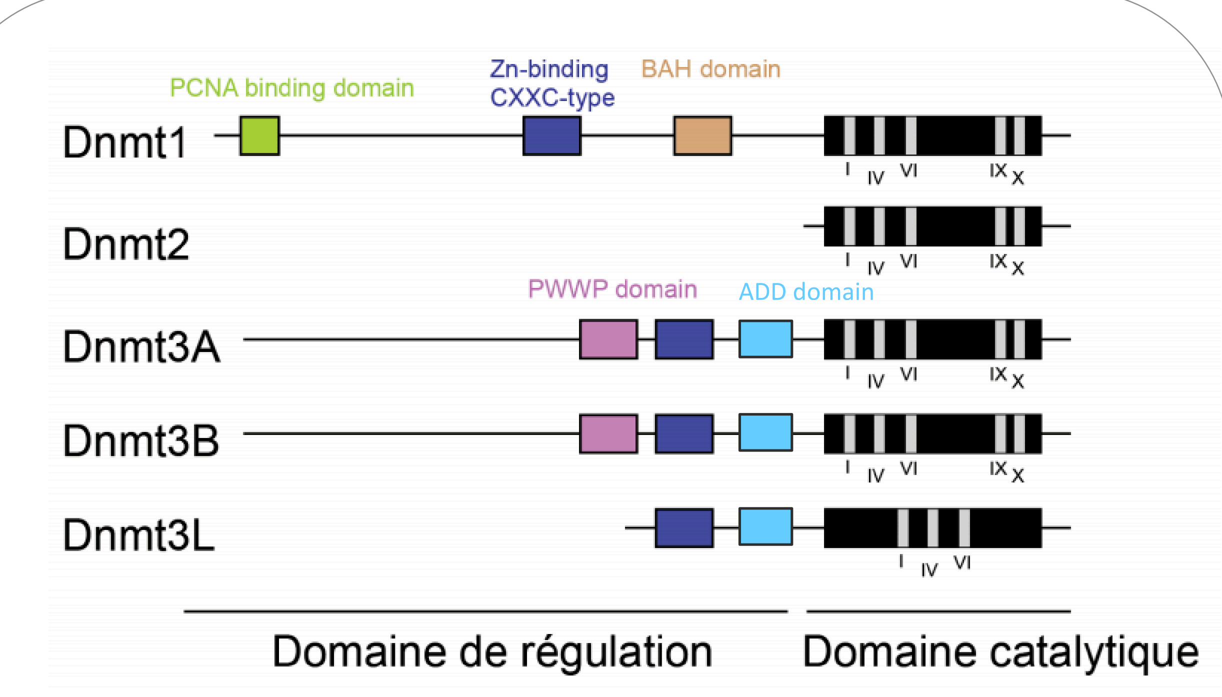

Figure 6 : Structure des différentes enzymes de la famille DNMT

Toutes les DNMT possèdent un domaine de régulation et un domaine catalytique, mais chacune possède ses propres spécificités. Adapté de [38]

1.1.2.2) La méthylation de maintenance

La méthylation de maintenance est essentielle au maintien des marques épigénétiques d'une cellule à une autre après la réplication de l'ADN. Elle a lieu via la DNMT1, qui reconnait majoritairement l'ADN hémi-méthylé [39] et qui agit notamment en complexe avec PCNA qui assure le positionnement de la DNMT1 sur la fourche de réplication [26], et

avec UHRF1 qui se lie à l'ADN hémi-méthylé et recrute la DNMT1 sur la fourche de réplication (Figure 7) [27].

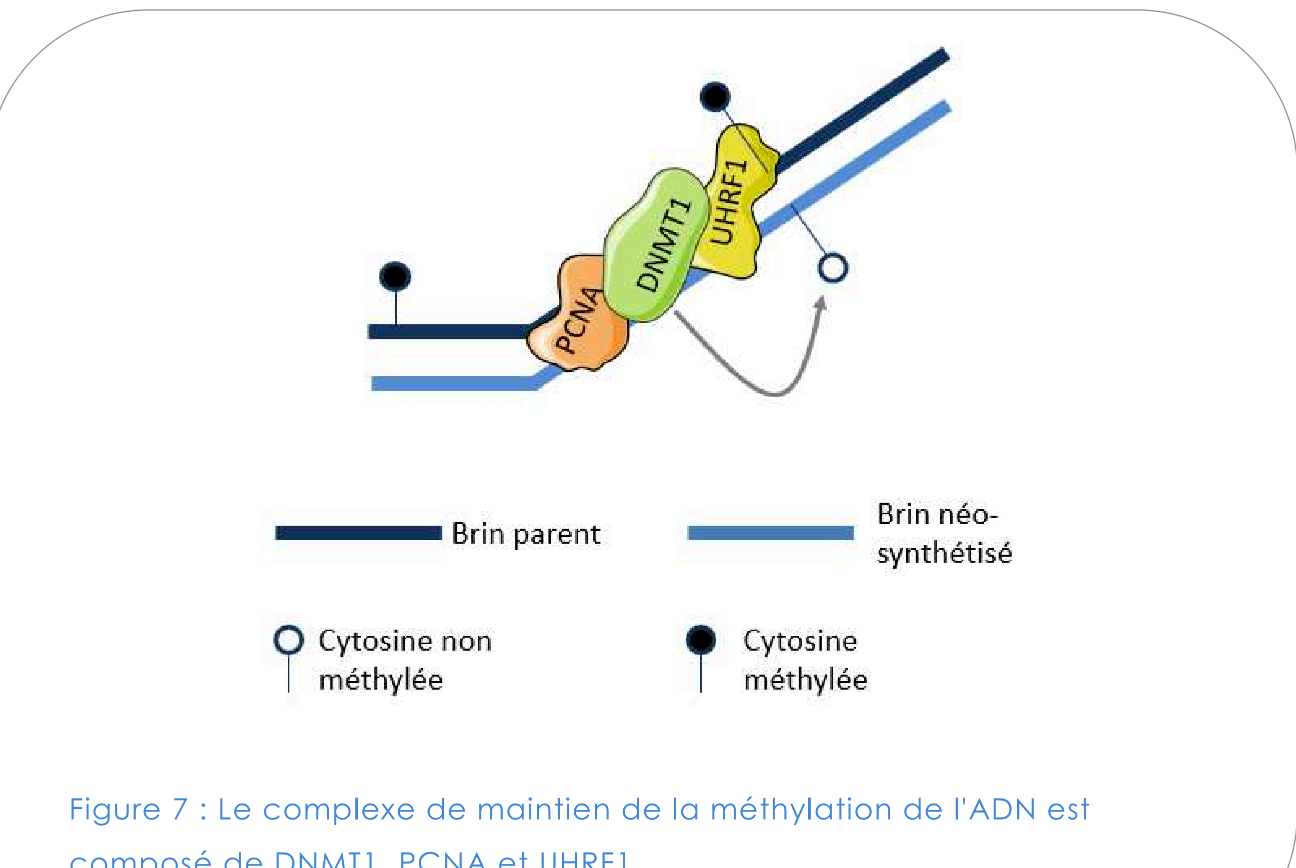

Figure 7 : Le complexe de maintien de la méthylation de l'ADN est composé de DNMT1, PCNA et UHRF1

Adapté de [40]

1.1.2.3) La méthylation *de novo*

Les DNMT3A et 3B catalysent principalement la méthylation *de novo*, or d'après Jeltsch elles ont la même affinité pour l'ADN non méthylé que pour l'ADN hémiméthylé in vitro [39], mais sont aussi capables de méthyler l'ADN ailleurs que sur les CpG [41, 42]. Lin *et al.* ont défini une séquence consensus de méthylation de l'ADN pour la DNMT3A : YNCGY (Avec Y = C ou T, N = A, C, T ou G). Bien que consensuelle, cette séquence n'est pas très restrictive puisqu'elle représente ¼ des séquences possibles de 5 nucléotides contenant un CG [43] (Figure 8).

La DNMT3L ne possède presque uniquement un domaine catalytique puisque son domaine régulateur est fortement réduit, pourtant elle ne présente pas d'activité catalytique propre. Cela est dû au fait que dans son domaine catalytique manquent les motifs IX et X. Son rôle

est de stabiliser le complexe DNMT3A/DNMT3B et augmente leur activité catalytique, bien que chaque DNMT puisse agir indépendamment de ce complexe [44–46]. De plus, la DNMT3L est capable de lier H3K4 non méthylé grâce au domaine ADD et de recruter DNMT3A et DNMT3B pour méthyler les CG au niveau des promoteurs [47]. H3K4me3 au contraire protège les TSS de la méthylation [48]. Enfin, grâce à leur domaine PWWP, les DNMT3A et B sont capables de reconnaitre H3K36me3 dans le corps des gènes [49].

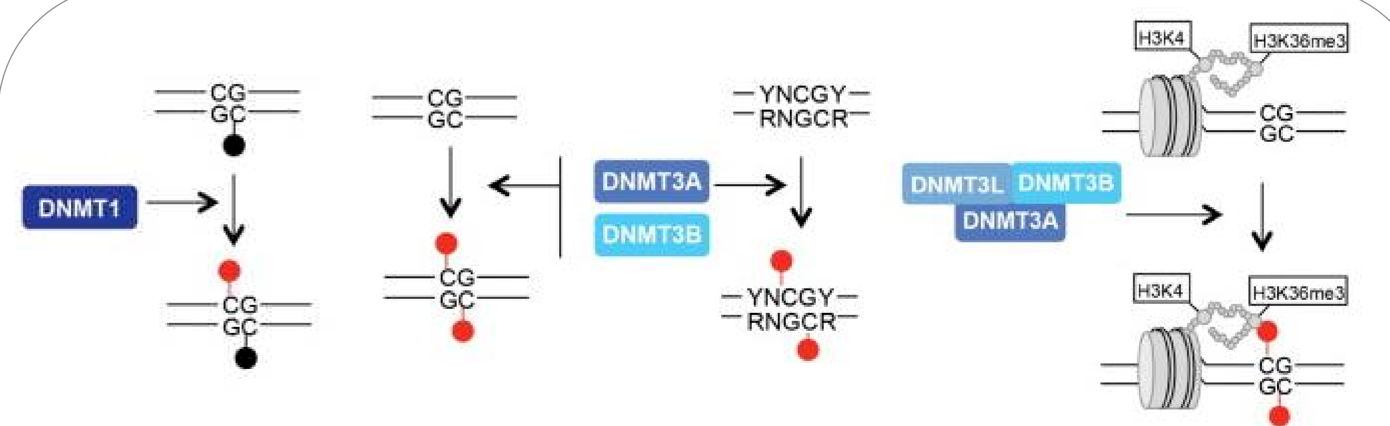

Figure 8 : Mécanismes de méthylation de l'ADN via les différentes DNMT

Suite à la réplication, la DNMT1 maintient la méthylation au cours des divisions cellulaires. Les DNMT3A et 3B sont capables de méthyler un CG de novo, et la DNMT3A ajoute aussi cette modification sur la séquence consensus YNCGY. Le complexe DNMT3A/3B/3L méthyle les CG à proximité des modifications d'histones H3K4 et H3K36me3. Source : [38]

Même si les DNMT sont capables de méthyler l'ADN à proprement parler, elles ne fonctionnent pas de manière monomérique, mais en complexes. Ce sujet sera approfondi dans le paragraphe 1.1.4) Régulation des mécanismes de méthylation et déméthylation de l'ADN.

1.1.2.4) Effets de la méthylation de l'ADN

Le rôle de régulateur transcriptionnel de la 5mC est admis par tout le monde et décrit de nombreuses fois, si bien qu'elle est parfois considérée comme la 5ème base du génome [50]. Toutefois, cette notion de 5ème base est discutée étant donné qu'elle ne joue aucun rôle dans le code génétique lors de la traduction.

La méthylation de l'ADN participe à la régulation de la transcription, puisque lorsqu'elle a lieu sur le promoteur d'un gène elle est globalement associée à sa répression, soit de manière directe en empêchant la fixation des facteurs de transcription [51], soit indirectement en recrutant des protéines de la famille des methyl-CpG binding domain (MDB) [52, 53] (Figure 9).

Quant au locus de la méthylation, elle n'aura pas le même impact si elle a lieu sur le promoteur, sur un enhancer ou sur une séquence régulatrice. En effet, au niveau du promoteur, les MDB empêchent la fixation des facteurs de transcription, sauf quand elle empêche la fixation d'un répresseur transcriptionnel tel que CTCF qui se lie principalement à l'ADN non méthylé. La méthylation de l'ADN au niveau du TSS bloque également la transcription, notamment via le recrutement de MeCP2 [54]. Au niveau des enhancers, même s'ils possèdent globalement peu de CpG, la méthylation est très dynamique et le lien avec la transcription n'a pas été approfondi. Au niveau des insulators, séquence bloquant les interactions entre enhancer et promoteur, la méthylation empêche le recrutement de CTCF [55]. Enfin, lorsque la méthylation de l'ADN a lieu dans le corps du gène, elle n'est pas associée à la répression [56]. Dans le corps du gène, la méthylation de l'ADN aurait un rôle dans la régulation de l'épissage alternatif en recrutant MeCP2 [57].

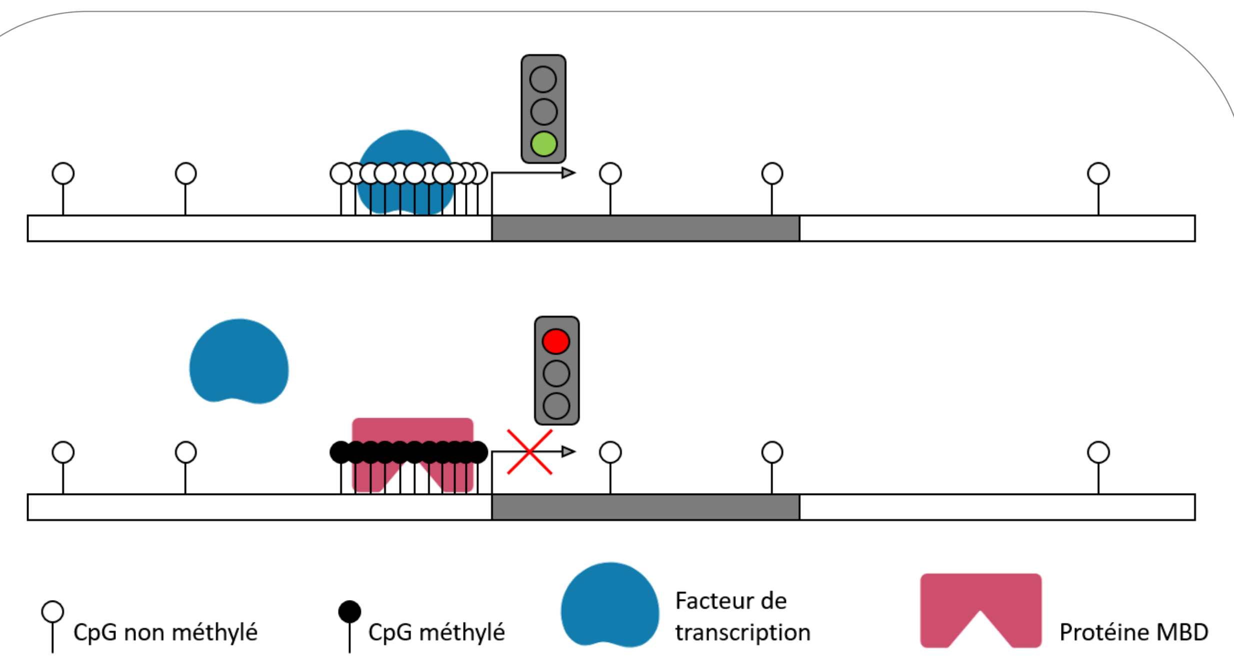

Figure 9 : Effet de la méthylation du promoteur

Dans cet exemple, lorsque le promoteur est déméthylé (en haut), le facteur de transcription peut se fixer et permettre la transcription. Lorsqu'il est méthylé (en bas), les MDB protéines sont recrutées, empêchant la fixation du facteur de transcription. La méthylation peut également à elle seule empêcher la fixation du facteur de transcription.

De plus, ces protéines MDB appartiennent souvent au même complexe que les histones déacétylases (HDAC), capables de compacter la chromatine et rendant sa transcription difficile [58].

Les mécanismes épigénétiques étant réversibles, il existe des mécanismes de déméthylation de l'ADN.

1.1.3) La déméthylation de l'ADN

La déméthylation de l'ADN est due à deux mécanismes : la déméthylation passive, impliquant la perte de fonctionnalité, d'intégrité et/ou de recrutement de la protéine DNMT1, et la déméthylation active, faisant intervenir notamment des protéines de la famille TET.

1.1.3.1) La déméthylation passive

La déméthylation passive est due à la perte de la méthylation au fur et à mesure des divisions cellulaires, puisque l'ADN néosynthétisé suite à la réplication de l'ADN n'est pas méthylé. Elle est due majoritairement à la perte du complexe DNMT1/PCNA/UHRF1, responsable du maintien de la méthylation [25, 59] (Figure 10).

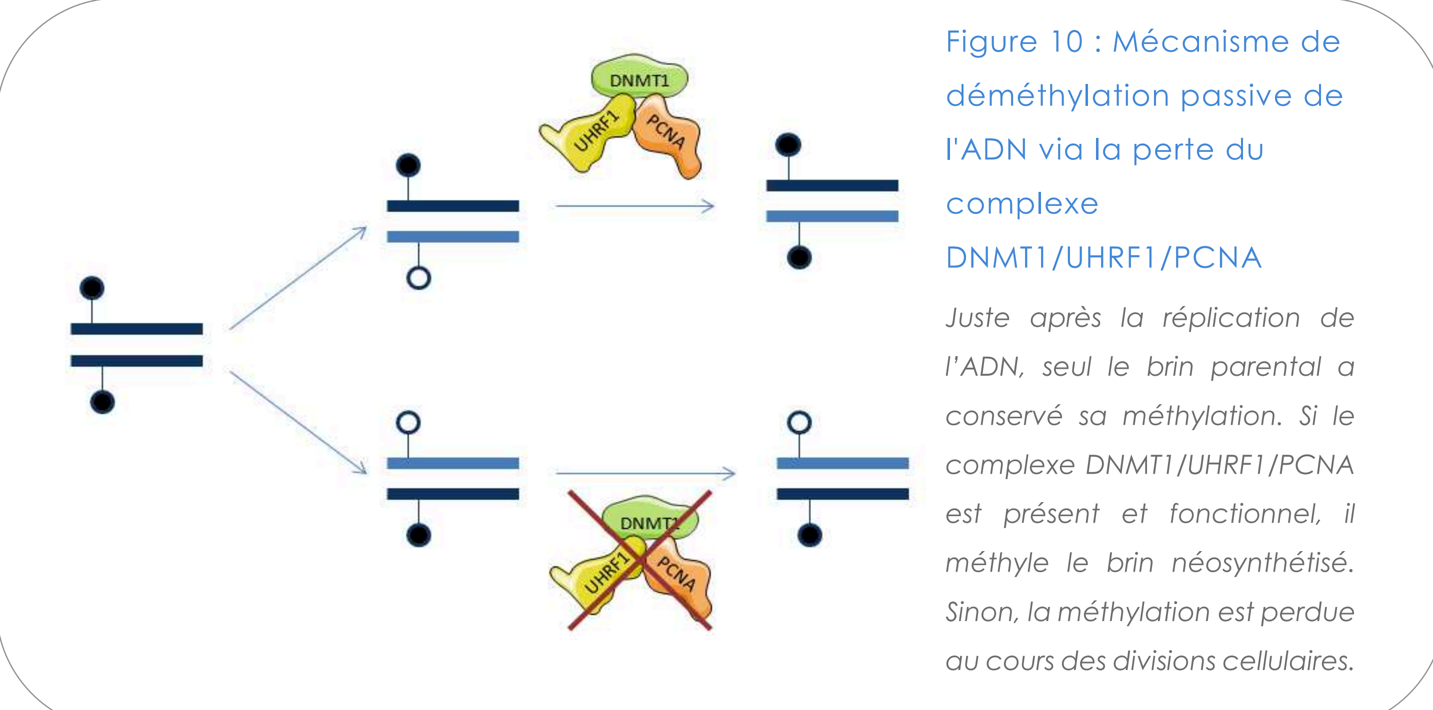

Figure 10 : Mécanisme de déméthylation passive de l'ADN via la perte du complexe DNMT1/UHRF1/PCNA

Juste après la réplication de l'ADN, seul le brin parental a conservé sa méthylation. Si le complexe DNMT1/UHRF1/PCNA est présent et fonctionnel, il méthyle le brin néosynthétisé. Sinon, la méthylation est perdue au cours des divisions cellulaires.

1.1.3.2) La déméthylation active

La déméthylation active, quant à elle, implique les protéines de la famille des Ten Eleven Translocation (TET), qui sont capables de transformer les 5mC en 5-hydroxyméthyl-cytosines (5hmC) puis en 5-formyl-cytosines (5fC) et 5-carboxycytosines (5caC) [60] . Le système TDG/BER (thymine ADN glycosylase/Base Excision Repair) reconnaît le mésappariement 5caC/G et enlève la 5caC, produisant ainsi un site abasique puis y place une cytosine non modifiée [61, 62] (Figure 11).

En parallèle, le système AID/APOBEC est capable de directement désaminer une cytosine méthylée ou hydroxyméthylée, faisant là aussi intervenir le système TDG/BER. Cependant, la désamination par les enzymes AID/APOBEC induit la modification de la 5mC en T, et donc un mésappariement G/T. Si BER ne répare pas ce mésappariement, il existe un risque de mutation.

Enfin, les 5hmC peuvent être directement remplacées par des cytosines via le processus de "active modification followed by passive dilution" (AMPD) [24], c'est-à-dire que la déméthylation passive présentée précédemment a lieu à partir des 5hmC (Figure 11).

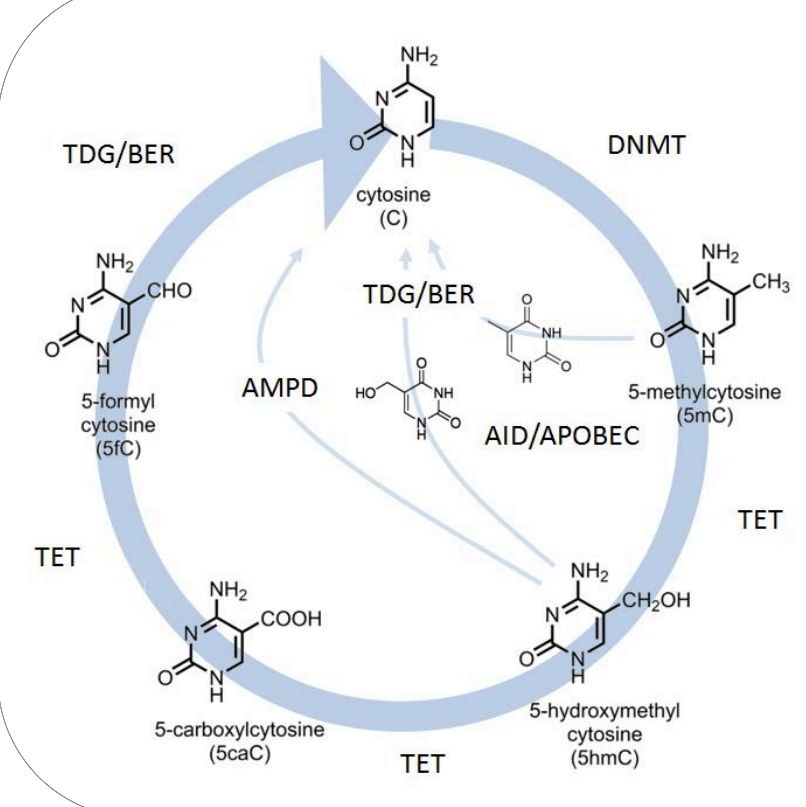

Figure 11 : Mécanismes de méthylation et déméthylation de l'ADN : principaux acteurs

La méthylation de l'ADN est assurée par les enzymes de la famille DNMT, alors que la déméthylation a lieu via les TET et des enzymes de réparation de l'ADN. Dans le cas de la déméthylation, il existe une voie indépendante de TET via AID/APOBEC, et une autre où la déméthylation passive suit directement la modification en 5hmC, on parle alors de « active modification followed by passive dilution ». Adapté de [63]

1.1.3.3) Acteurs de la déméthylation de l'ADN

1.1.3.3.1) Les TET

La famille des TET est composée de 3 membres : TET1, TET2 et TET3, tous impliqués dans la déméthylation active de l'ADN. Ces 3 protéines, tout comme les DNMT, possèdent une région régulatrice ainsi qu'une région catalytique (Figure 12). Cette dernière est très bien conservée et commune aux trois TET, la différence majeure étant dans la longueur des différentes protéines. De manière intéressante, TET2 ne possède pas de domaine CXXC de liaison à l'ADN. Une théorie supporte l'idée qu'à l'origine, un gène voisin de TET2, IDAX qui lui possède un domaine de liaison à l'ADN, faisait partie de la séquence de TET2. Aujourd'hui, IDAX participerait à la régulation de TET2 en se liant à des CpG non méthylés et en interagissant directement avec le domaine catalytique de TET2, mais aussi en activant des caspases responsables de la dégradation de TET2 [64].

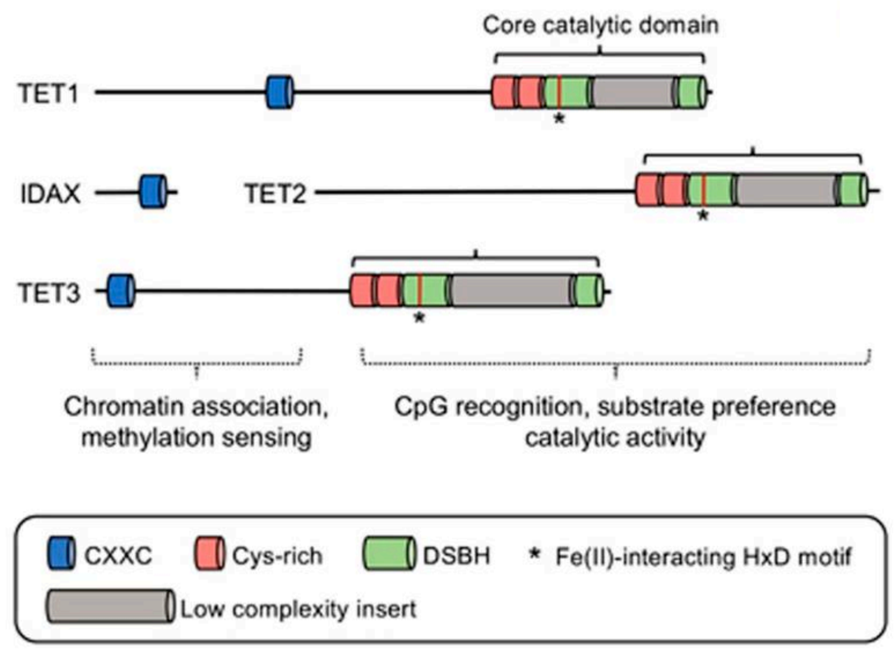

Figure 12 : Structure des différentes enzymes de la famille TET

Toutes les TET possèdent un domaine de régulation et un domaine catalytique, mais chacune possède ses propres spécificités, notamment au niveau du domaine régulateur. Il est à noter que TET2 ne possède pas de domaine de liaison à l'ADN.
CXXC : domaine de liaison aux proteins à doigt de zinc; Cys-rich : domaine riche en cystéine ; DSBH : double stranded β-helix domain. Source : [65]

Cependant, chaque membre est exprimé différemment en fonction du type cellulaire. TET1 et TET2, contrairement à TET3, sont exprimés dans les cellules souches embryonnaires (ESC) [66]. TET3 quant à lui est exprimé dans les zygotes et les ovocytes [67, 68]. Dans les cellules adultes, les trois TET sont exprimés dans la grande majorité des tissus. TET1 est très exprimé dans la partie proximale du tube digestif, TET2 dans le sang et TET3 dans le cerveau (Human Protein Atlas).

1.1.3.3.2) Les cofacteurs

Le domaine DSβH (double stranded β-helix) des protéines TET leur permet de se lier à l'ion Fe2+ et à l'αcétoglutarate (αKG, ou 2-oxoglutarate 2-OG). En utilisant l'oxygène, les TET vont catalyser la décarboxylation d'αKG, qui en présence de Fe2+ va permettre la conversion des 5mC en 5hmC [69, 70] (Figure 13).

La présence de 2-hydroxyglutarate (2-HG), un inhibiteur compétitif de l'αKG, inhibe l'activité catalytique des TET [71]. De ce fait, l'équilibre αKG/2-HG est crucial pour l'activité de TET, tout comme la présence de mutations au sein des protéines IDH1 et/ou IDH2 (isocitrate dehydrogenase 1 et 2) qui convertissent αKG en 2-HG [72] (Figure 13).

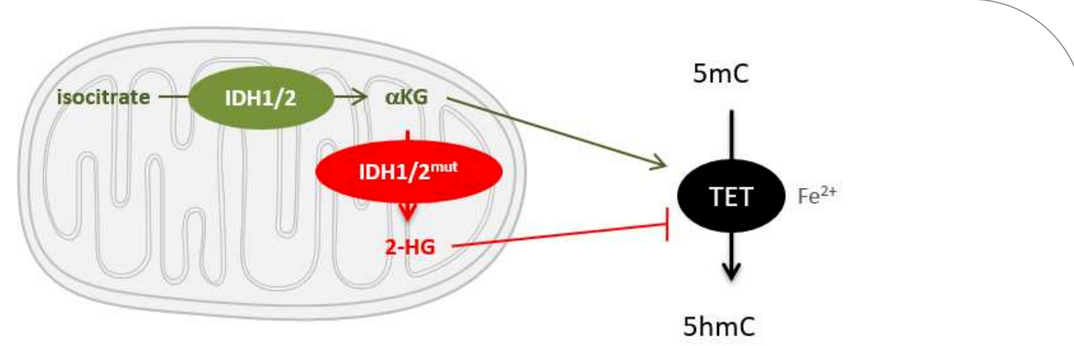

Figure 13 : Les cofacteurs des TET

Dans le cycle de Krebs, l'isocitrate est convertit en acétoglutarate (aKG) par les enzymes IDH. L'aKG, comme le Fe^{2+} sont ensuite utilisés par les TET pour déméthyler l'ADN. Lorsque les enzymes IDH sont mutées, du 2-hydroxyglutarate (2-HG) est produit à la place de l'aKG, empêchant les TET de déméthyler l'ADN. Adapté de [73]

1.1.3.4) Rôles de la 5-hydroxyméthylcytosine

Si le rôle de la 5mC est plutôt bien décrit et compris chez l'Homme, celui de la 5hmC reste partiellement identifié. Il a été démontré que l'abondance de la 5hmC serait dépendante du type cellulaire et du stade de développement [74]. De plus, elle ne serait pas une simple étape de la déméthylation, mais elle aurait un rôle physiologique.

Le fait que MeCP2, qui se fixe à l'ADN méthylé et empêche la transcription du gène, ai la même affinité pour la 5mC que la 5hmC va plutôt dans le sens d'un rôle similaire à celui de la 5mC [75]. Cependant, dans des cellules souches embryonnaires humaines, il a aussi été démontré que le taux de 5hmC était positivement corrélé à l'expression des gènes et ceci est notamment dû à la corrélation avec une conformation de chromatine ouverte via des modifications d'histones telles que H3K4me1 et H3K27ac [76]. De plus, des pics enrichis en 5hmC ont été identifiés comme coïncidant avec des sites de fixation de facteurs de transcription tels qu'OCT4 ou NANOG, deux facteurs de transcription impliqués dans le développement embryonnaire [76]. Enfin, certaines protéines telles que RPL26 (60S

ribosomal protein L26), PRP8 (pre-mRNA-processing-splicing factor 8) ou MSH6 (DNA mismatch repair protein 6) ont une plus grande affinité pour la 5hmC [77].

Enfin, contrairement à la 5mC, la répartition de la 5hmC peut être asymétrique entre les deux brins d'ADN [78].

1.1.4) Régulation des mécanismes de méthylation et déméthylation de l'ADN

Plusieurs mécanismes de régulations de la méthylation existent, impliquant différents types de molécules.

1.1.4.1) Mécanismes de méthylation/déméthylation de l'ADN médiés par les ARN

Dans un premier temps, des mécanismes de RNA-directed DNA methylation ont été décrits. Chez *Arabidopsis thaliana*, environ 30% des cytosines sont méthylées suite au recrutement de la DRM2 (domains rearranged methyltransferase 2, l'équivalent de la DNMT3 chez les mammifères) via des siRNA sur les séquences complémentaires [79, 80]. De la même manière chez l'Homme, il a été démontré qu'un siRNA était capable de recruter les DNMT et donc d'induire la méthylation d'un gène, et que donc que le "Small Interfering RNA-Induced Transcriptional Gene Silencing" existait chez l'Homme [81]. Par la suite, plusieurs articles ont confirmé ce résultat [82–84], toutefois il existe peu d'articles récents sur ce sujet.

1.1.4.2) Mécanismes de méthylation/déméthylation de l'ADN médiés par les facteurs de transcription

Il existe aussi des mécanismes de "transcription factor directed DNA methylation", où les facteurs de transcription recrutent les DNMT sur leur site de fixation à l'ADN [85, 86] et sont donc impliqués dans la régulation de la méthylation de l'ADN. En effet, de nombreux facteurs de transcription ont été décrits comme interagissant avec les DNMT *in vitro* [85, 86] (Figure 14).

Interaction with Dnmt1

AES, AP2α, ASH2L, ATF1, ATF2, ATF3, ATF4, C/EBPα, CREB1, CRSP9, DLX4, DMTF1, ELK, E2F3, E2F4, E2F5, EGR2, EGR4, ER-α, ER-β, ERRγ, ETS1, ETS2, FOS, FOSB, FOSL1, FOSL2, GATA1, GR, HAND1, HAND2, HNF4G, HOXA5, IRF1, ISGF3G, JUN, JUNB, KLF7, LHX2, LRH-1, MADH-3, MAX, MeCP2, MEF2A, MEF2B, NFE2, NFκB-p65, PAX6, PBX1, PPAR α, PPAR β, PPARγ1, PPARγ2, PXR-2, p53, Sp1, STAT1, YY1

No interaction with Dnmt1

BLZF1, BTG2, CART1, CBFB, CDX2, CREBL2, CERM, DDIT3, DR1, E2F6, F2RL1, GCNF, GMEB1, GTF2B, GTF2H2, GTF2I, GTF3C5, HDC1, HEY, HNF4G, HOXB13, HOXC11, ID1, ID2, ISL1, KLF12, LDB1, MADH1, MADH4, MAFK, MEF2C, MEF2D, MSX1, NAB1, NFIL3, NFκB-p50, NFYB, NR1H2, NR1L2, NR2E1, NR5A2, PALM, PAX9, PBX1, RXR-α, Sp4, v-myc

Interactions with DNMT3A

AP2α, DDIT3, Elk, GTF2H2, HOXC11, HEY, ID1, ID2, IRF1, ISGF3γ, JUN, KLF7, MADH1, MAFK, MAX, MSX1, NFE2, NFIL3, NFκB-p50, NFYB, NR1H2, NR1/2, NR1/3, NR5A2, p53, PAX6, PBX1

Interactions with both DNMT3A and DNMT3B

AES, ATF2, ATF4, CREB1, CREBL2, CRSP9, DLX4, DMTF1, DR1, Elk, E2F5, EGR1, EGR2, EGR4, ER-β, ERRγ, ETS1, F2RL1, FOS, FOSB, FOSL1, GATA1, GR, HAND1, HAND2, HDC1, HOXA5, HNF4γ, JUNB, KLF12, LDB1, LHX2, MADH3, MADH4, MECP2, MEF2A, MEF2C, MEF2D, PPARγ1, PPARγ2, v-myc, YY1

Interactions with DNMT3B

CART1, E2F6, ER-α, FOSL2, GMEB1, NR2E1, PXR-2, SP1, SP4, STAT1

ASH2L, ATF1, ATF3, BLZF1, BTG2, C/EBPα, CBFB, CDX2, CERM, E2F3, E2F4, ETS2, GCNF, GTF2I, GTF3C5, HOXB13, ISL1, LRH1, MEF2B, NAB1, NFκB-p65, PALM, PAX9, RXR-α

Figure 14 : Tableau des facteurs de transcription interagissant ou non avec les différentes DNMT

À gauche, les facteurs interagissant ou non avec DNMT1. À droite, les facteurs interagissant avec DNMT3A, 3B, les deux ou aucune. Source : [85, 86]

La revue de Marchal *et al.* détaillent les interactions facteurs de transcription/DNMT en fonction de la zone de l'ADN (Figure 15).

	Promoters / Enhancers	Tumor suppressor genes	Germline genes promoters	Imprinted regions	DNA repeats ERVs	Peri-centromeres
GAIN OF METHYLATION	DNMTs H3 lysine 9 methylation	DNMTs Zfp354b ZNF304 Znf217 cMyc PML-RAR	DNMT3B E2F6 Max	Kap1 Setdb1 DNMTs ZFP57 ZNF274?	Kap1 DNMT3A DNMT1 Zfp809 Zfp819 PLZF Rb others?	DNMT3B Lsh ATRX ZBTB24?
LOSS/ MAINTENANCE OF METHYLATION	H3 lysine 4 methylation REST CTCF TET BER pathways KDM2A Transcription factors CXXC factors	BER pathways TGF-beta RON Transcription factors	H3 lysine 4 methylation			

Figure 15 : Résumé des facteurs nucléaires et des marques épigénétiques impliquées dans la maintenance de la méthylation de l'ADN dans différentes régions du génome

Source : [87]

In vivo, E2F6 a été décrit comme interagissant avec DNMT3B chez la souris [88].

Les mêmes phénomènes de RNA-directed DNA demethylation et de transcription factor DNA demethylation sont décrits pour la déméthylation de l'ADN [89, 90].

1.1.4.3) Régulation post-transcriptionnelle des acteurs de la méthylation/déméthylation de l'ADN

Il est important de souligner que les enzymes épigénétiques sont issues de gènes comme les autres, leur traduction peut donc être régulée par des microARN. Par exemple, le miR-29a-3p a été décrit comme étant capable de réguler la traduction des trois protéines de la famille TET [28–30], mais aussi les 3 principales enzymes de la famille des DNMT [91, 92]. Bien évidemment, de nombreux autres miR ont été décrits comme ciblant des enzymes épigénétiques, tels que le miR-185-5p pour DNMT1 [93] ou le miR-22-3p pour TET2 [94].

1.1.4.4) Régulation post-traductionnelle des acteurs de la méthylation/déméthylation de l'ADN

De plus, comme toute protéine, des modifications post-traductionnelles peuvent modifier l'activité catalytique et/ou le recrutement sur différents sites de l'ADN des enzymes des familles DNMT et TET. Par exemple, certaines modifications post-traductionnelles telles que l'acétylation et la méthylation de DNMT1 vont la déstabiliser et induire son ubiquitinylation, alors que la phosphorylation et la désacétylation vont participer à sa stabilité [59, 95, 96]. Au laboratoire, il a été démontré que la phosphorylation de DNMT1 par AKT (RAC-alpha serine/threonine-protein kinase) et PKC (protein kinase C) sur les sérines 127 et 142 inhibait les interactions DNMT1/PCNA et DNMT1/UHRF1 [59]. Au contraire, l'acétylation de TET2 va augmenter sa demi-vie, alors qu'une poly-adénosine ribosylation (PARylation) non covalente de TET1 va diminuer sa liaison à l'ADN, mais lorsque cette modification est covalente elle augmente l'activité de cette protéine [97].

Si la méthylation de l'ADN est un mécanisme majeur de la régulation de la transcription, il existe d'autres phénomènes qui agissent en aval de la transcription, tels que les microARN.

1.2) Les microARN

Environ 80% du génome est transcrit, mais seulement 2% codent pour des protéines [98]. Au contraire des ARN messagers qui sont traduits en protéines, certains ARN non transcrits mais non traduits : les ARN non codants.

S'il existe de nombreuses sous familles d'ARN non codants (Figure 16), nous nous concentrerons ici uniquement sur les microARN, ces derniers étant ceux étudiés lors de mon travail de thèse.

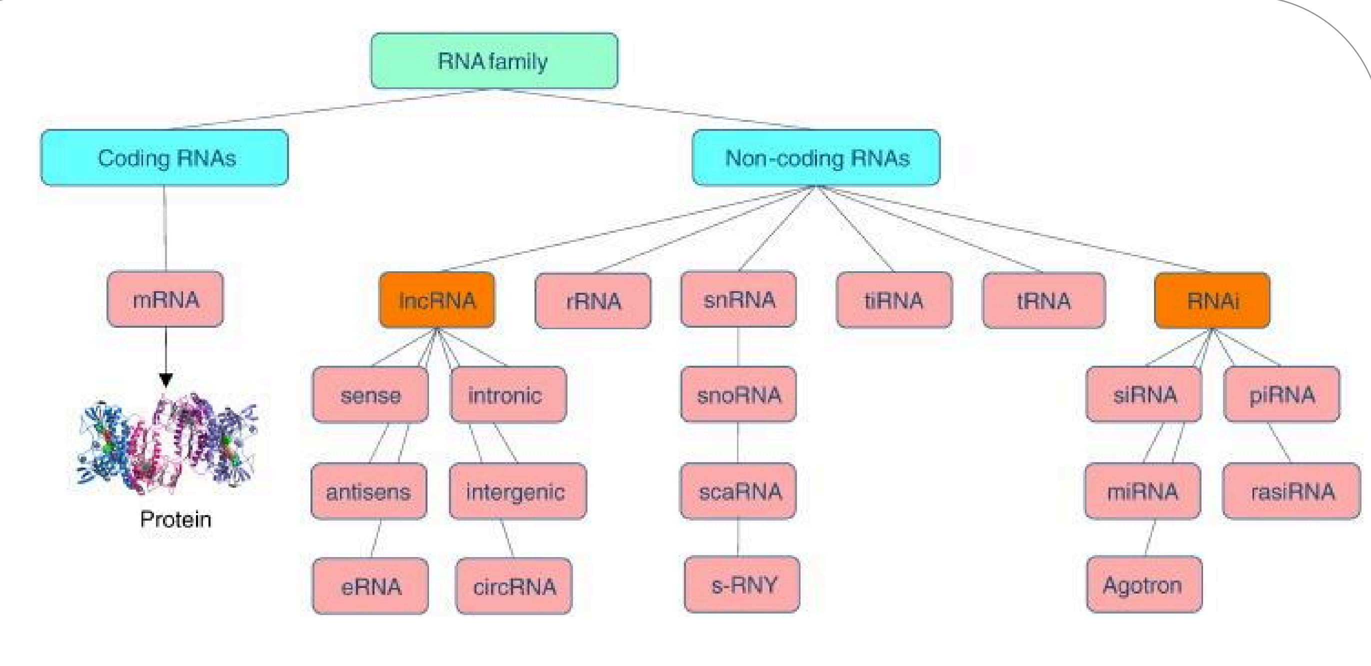

Figure 16 : Les différentes classes d'ARN non codants

Source : [99]

À l'origine, les microARN ont été découverts chez *C. elegans*. Le premier à être décrit fût lin-4 et ne portait pas encore le nom de miR. Il a été identifié comme étant une petite séquence d'ARN complémentaire au 3'UTR de l'ARNm lin-14, formant un duplex ARN/ARN inhibant la traduction de la protéine LIN-14 [100].

De manière très générale, les microARN sont de petits ARN non codants d'environ 20 nucléotides impliqués dans la régulation de l'expression des ARNm. En effet, leurs séquences étant complémentaires à celles d'ARNm, ils sont capables de s'y fixer et ainsi d'empêcher leur traduction en protéines ; soit en induisant leur dégradation, soit en ayant un effet d'encombrement stérique.

1.2.1) Synthèse des miRNA

Dans le génome, les gènes des miRNA sont présents dans plusieurs régions : intronique, exonique ou intergénique. Les miRNA sont transcrits de manière classique, comme un gène, par l'ARN polymérase II dans le noyau [101]. D'ailleurs, chez les Eucaryotes ont été décrits des éléments classiques des gènes, tels que des promoteurs [102], des Transcription Start Sites (TSS) [103], des sites de fixation pour des facteurs de transcription [104] ou encore des supers enhancers [105].

Environ la moitié des miRNA sont transcrits simultanément, autrement dit sous forme de cluster [106]. En effet, l'expression de plusieurs miRNA est liée à un seul promoteur [107]. Par exemple, les séquences des miR-17, -18a, -19a, -20a, -19b-1 et -92a-1 sont sur le même locus du chromosome 13 et transcrites simultanément (Figure 17). Cette zone est parfois appelé « miR-17-92 polycistronique » [108].

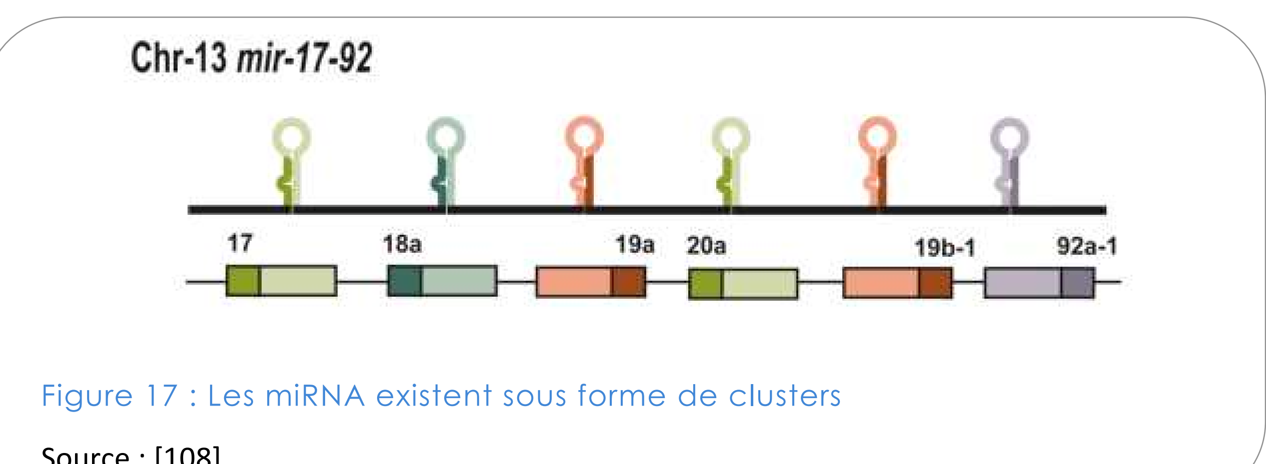

Figure 17 : Les miRNA existent sous forme de clusters

Source : [108]

Les miR tout juste transcrits ne sont pas encore matures, ce sont alors des miRNA primaires (pri-miRNA).

Les pri-miR sont constitués d'une séquence ou d'un cluster de séquences de miR, d'une séquence boucle, d'une "base stem sequence" avec des mésappariements, de segments d'encadrement simple brin et d'une queue polyA [109]. Ils sont pris en charge par le complexe microprocesseur, composé de DROSHA et DGRC8 qui va les modifier en pre-miR, en clivant la « base stem sequence », les segments d'encadrement simple brin de la queue polyA. Ces pre-miR sont exportés dans le cytoplasme via l'exportine 5, puis pris en charge par DICER1 (double-stranded RNA-specific endoribonuclease) et TRBP (trans-activation responsive RNA-binding protein), qui va couper la boucle et créer un duplex de miRNA matures [110] (Figure 18).

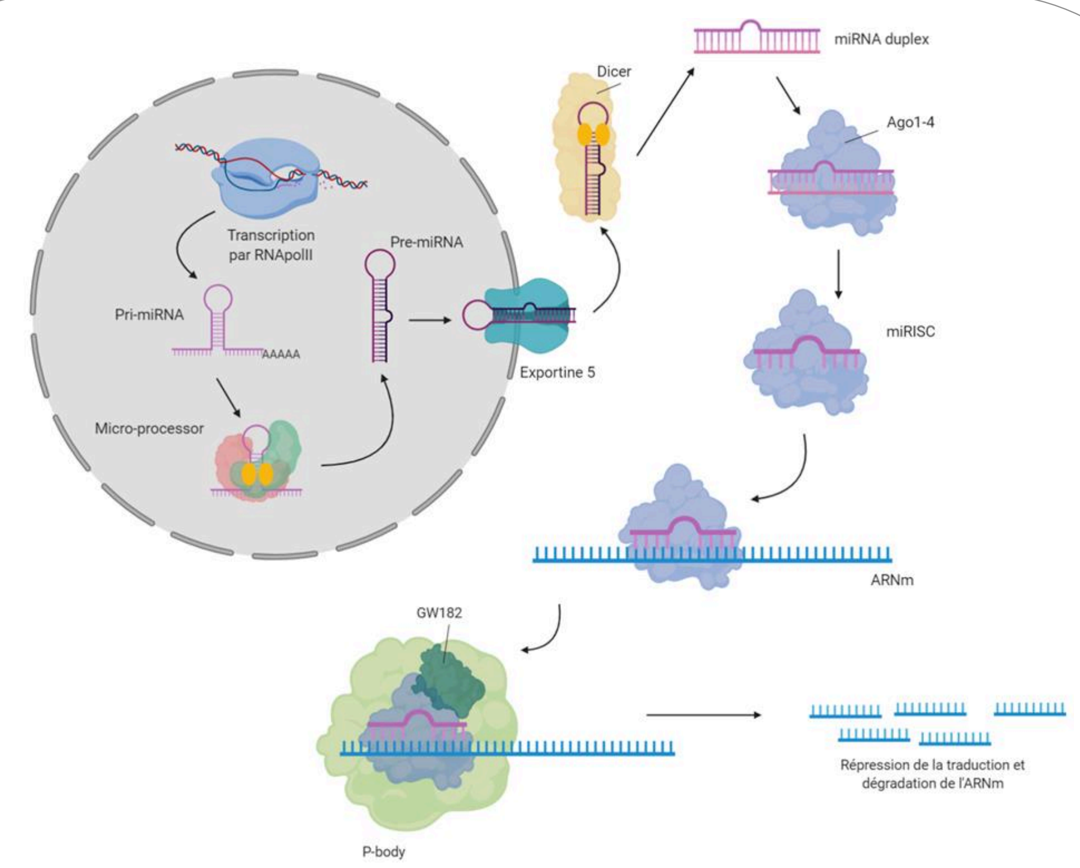

Figure 18 : Biogenèse des microARN

Un primary-miRNA est transcrit à partir de l'ADN par l'ARN polymérase II. Il est alors pris en charge par le complexe microprocessor, qui va cliver les extrémités non complémentaires et induire la formation d'un pré-miRNA. Ce dernier sera exporté dans le cytoplasme par l'exportine 5, et pris en charge par Dicer, qui va cliver la boucle et former un duplex de miRNA. Ce duplex sera pris en charge par les protéines de la famille Ago (1 à 4) et un seul brin du duplex sera conversé : le 3p ou le 5p ; c'est la formation du complexe miRISC. Un ARNm complémentaire sera alors reconnu par le complexe miRISC, recrutant GW182 et le P-body, induisant la répression de la traduction et/ou la dégradation de l'ARNm. Adapté de [110]

1.2.2) Mode d'action

Les protéines de la famille argonaute (AGO) viennent alors se lier à un brin du duplex de miR pour former le complexe miRISC (miRNA-induced silencing complex). L'autre brin du duplex est éjecté de miRISC et dégradé. Le complexe miRISC est capable de reconnaître un ARNm de façon complémentaire, de s'y lier et d'inhiber sa traduction voire d'entrainer sa dégradation [111]. GW182 (TNRC6A, trinucleotide repeat-containing gene 6A protein) a un

rôle de protéine « scaffold » du complexe miRISC [112]. Le tout a lieu au sein d'un « P-body » (processing body), dans lequel a lieu notamment la dégradation des ARN [113] (Figure 18).

Le point crucial dans ce mode d'action reste la complémentarité de bases entre le microARN et l'ARNm cible. Plusieurs règles d'appariement ont été décrites au cours du temps (Figure 19).

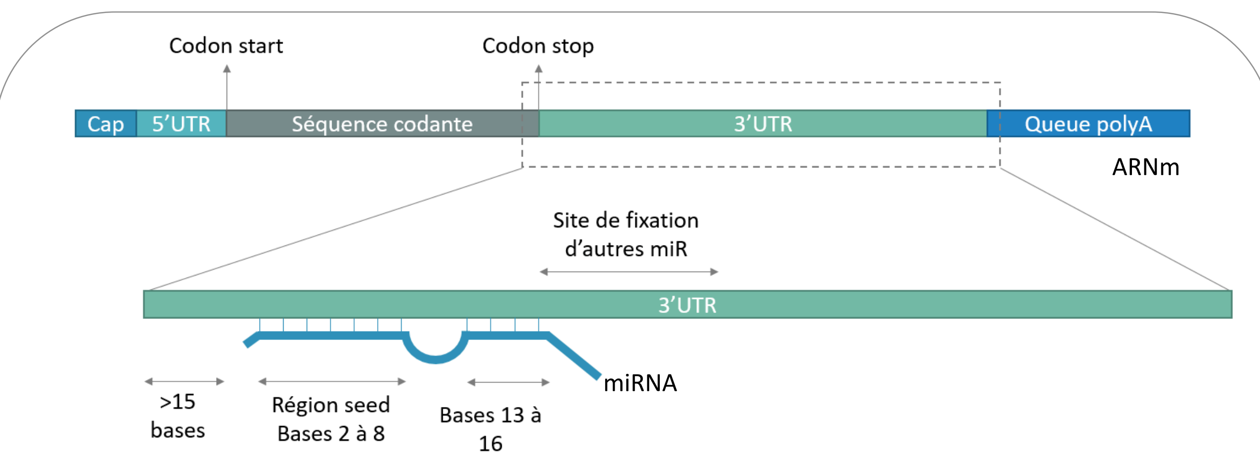

Figure 19 : Règles d'appariement miRNA/ARNm

L'appariement doit avoir lieu au minimum 15 bases après le codon stop, être parfait entre les bases 2 à 8 du miR, et améliore la stabilité du duplex entre les bases 13 à 16. La présence de sites de fixation d'autres miR augmente l'efficacité de répression transcriptionnelle.

Premièrement, l'hybridation doit être parfaite dans la région « seed », c'est-à-dire entre les nucléotides 2 et 8 du miR à partir de l'extrémité 5' [114]. En effet, un mésappariement dans cette zone (qui peut être dû à une liaison à la mauvaise cible) induit une diminution voire une perte de l'efficacité [115, 116]. Au contraire, l'efficacité de répresseur traductionnel peut être augmentée via la présence d'une adénosine de chaque côté de la région seed [117].

Deuxièmement, une complémentarité des bases 13 à 16 du miRNA avec l'ARNm stabilise le complexe miRNA/ARNm [118].

Troisièmement, le site de liaison du miR doit être à plus de 15 bases du codon stop sur le 3' UTR, ce qui induit une meilleure accessibilité du complexe miRISC [118].

Enfin, la présence de sites de fixation d'autres miRNA entraine un phénomène répressif coopératif et augmente l'efficacité de répression traductionnelle des miRNA [118].

1.2.3) Régulation des miRNA

Plusieurs facteurs régulent les miRNA tant au niveau de leur expression que de leur activité. Au regard des résultats présentés dans ce manuscrit, seuls trois d'entre eux seront ici exposés, même si d'autres existent (Figure 20).

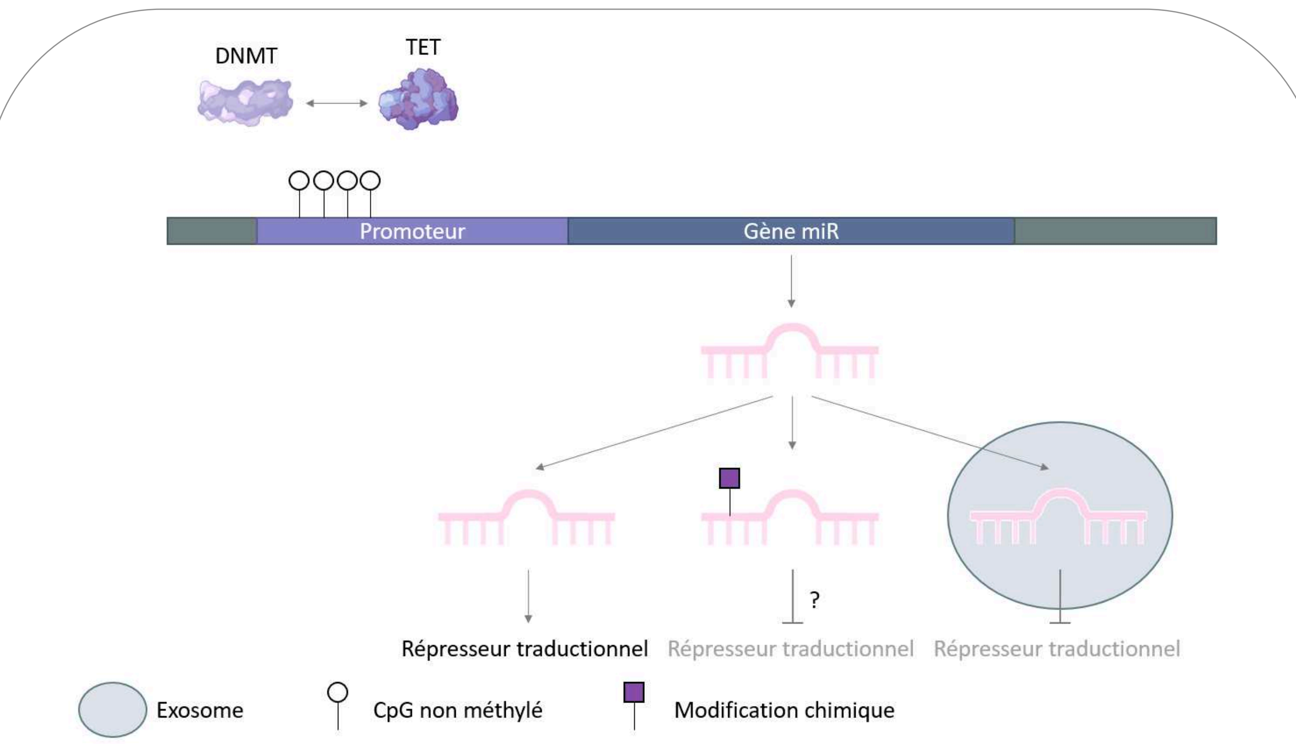

Figure 20 : Mécanismes de régulation des miRNA

L'expression des miR peut être régulée épigénétiquement, notamment via la méthylation des promoteurs. Leur action peut aussi être modulée, soit en les modifiant chimiquement (m6A, m5C, m7G) ou en les exportant dans les exosomes.

1.2.3.1) Régulation épigénétique de l'expression des miRNA

Les miRNA étant issus, comme tous les ARN, de la transcription de l'ADN par l'ARN polymerase II, ils sont eux aussi régulés par des mécanismes épigénétiques tels que la méthylation de l'ADN (Figure 20). Par exemple, la liaison de MBD2 sur le promoteur du

miR-373 induit la répression de sa transcription [119]. L'expression des miR-130b et 7-5p est aussi régulée par méthylation [120, 121].

Les protéines des familles DNMT et TET sont donc impliquées dans la régulation de leur expression. En effet, la surexpression de TET1 augmente l'expression du miR-34a [122], et le déséquilibre de la balance DNMT1/TET2 induit l'hyperméthylation du promoteur du miR-145 [123].

1.2.3.2) Régulation par modification chimique des nucléotides des miRNA

Au-delà de la régulation bien décrite de l'ADN par sa méthylation, les miRNA peuvent eux aussi être modifiés chimiquement, ce qui induit un changement dans leur efficacité biologique (Figure 20). De nombreuses méthylation ont été décrites dans les ARN (notamment m5C, m6A, m7G) [124], mais nous nous concentrerons ici uniquement sur les miRNA.

Plusieurs articles ont décrit des modifications chimiques de différents stades de maturation des miRNA. Par exemple, Berulava *et al.* ont démontré que les miRNA pouvaient être adénosine méthylés [125] et confirment la séquence consensus préalablement décrite par Harper *et al.* : RRACH (avec R=G ou A et H=A, C ou U) [126]. Cependant, la fonction de cette modification chimique reste peu décrite, bien qu'elle semble affecter la biogenèse et/ou la stabilité des miRNA [127].

D'après Xhemalce *et al.*, les précurseurs de miRNA peuvent être O-méthylés sur leur groupe monophosphate terminal par BCDIN3D (Bicoid interacting 3 domain containing RNA methyltransferase), et que cela réduit leur biogénèse en miR matures [128].

Enfin d'après Pandolfini *et al.* les miRNA peuvent être méthylés sur leur guanosine (m7G) [129].

Cependant, ces trois articles ne se sont pas intéressés à l'impact de la méthylation des miR sur leur fonction biologique de répresseur transcriptionnel. Mais récemment, Konno *et al.* sont parmi les premiers à vraiment s'intéresser aux rôles de ces modifications chimiques

en démontrant qu'elles modifient la stabilité et la reconnaissance de la cible. Il associe également la méthylation du miR-17-5p au stade précoce d'un cancer pancréatique [130].

L'étude des modifications chimiques microARN est incluse dans la notion d'épitranscriptomique, c'est-à-dire les modifications épigénétiques du transcriptome, des ARN [131, 132]. Cette notion sera plus détaillée/discutée dans la suite du manuscrit.

Tout comme pour la méthylation de l'ADN, il existe des "writers", "readers" et "erasers" pour la méthylation des miRNA, différents selon le type de méthylation (Figure 21). METTL3 (Methyltransferase Like 3) méthyle les adénosines dans les miR [133], alors que FTO (Fat Mass And Obesity-Associated Protein) les déméthyle (article 6). Les protéines avec un YTH domain vont alors lire la m6A [134].

La m5C dans les miR est encore une modification peu décrite, mais un article est en préparation dans le laboratoire à ce sujet. Nous avons identifié DNMT3A comme acteur de la m5C des miRNA, mais à ce jour nous n'avons pas caractérisé l'enzyme impliquée dans la déméthylation. Nous suspectons cependant TET2, étant donné que c'est la déméthylase que nous retrouvons le plus au niveau cytosolique dans les A172, une lignée de GBM. Des études sont en cours pour confirmer ces résultats.

	m6A	**m5C**
Writers	METTL3	DNMT3A/AGO4
Erasers	FTO	TET ?
Readers	Protéines avec un YTH domain	MDB ?

Figure 21 : "Writers", "readers" et "erasers" des méthylations dans les miRNA

1.2.3.3) Régulation par exportation des miRNA dans les exosomes

1.2.3.3.1) Généralités

Il existe plusieurs types de vésicules extracellulaires, généralement discriminées par leur taille, alors que leur biogénèse est différente [135]. Même si toutes ne portent pas les mêmes informations (notamment les corps apoptotiques, de par leur nature intrinsèque), les microvésicules et les exosomes ont tous deux un rôle de communication intercellulaire [136].

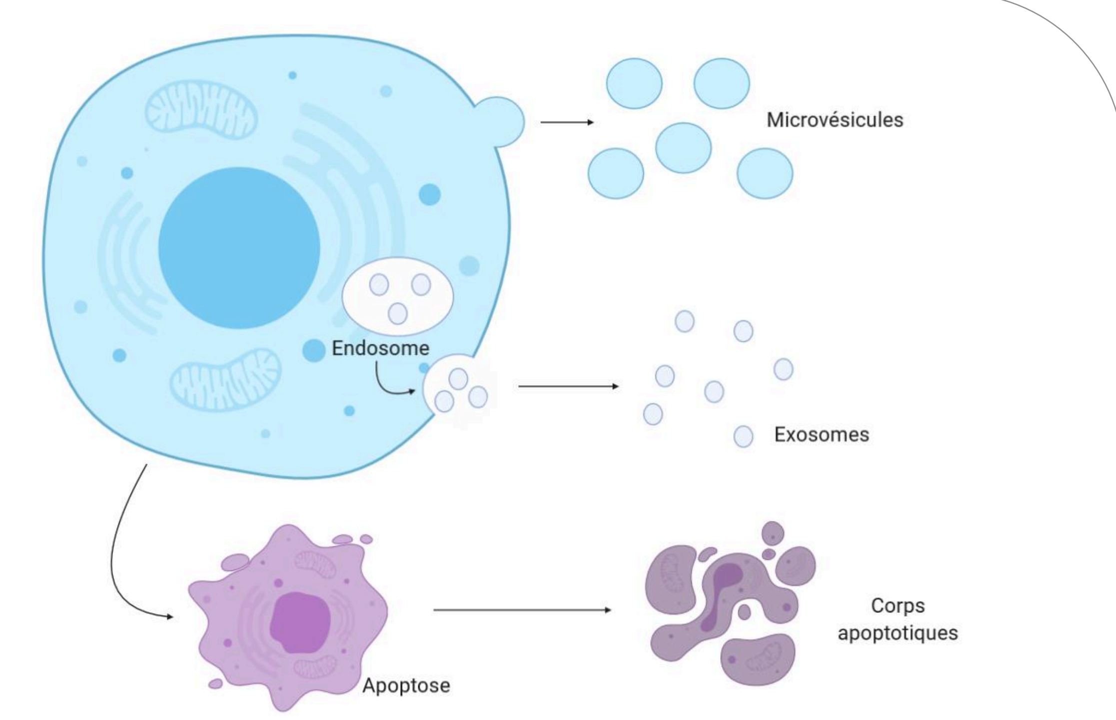

Figure 22 : Biogenèse des exosomes, microvésicules et corps apoptotiques

Les microvésicules sont issues d'une déformation de la membrane extracellulaire alors que les exosomes sont formés dans les endosomes puis exportés. Les corps apoptotiques quant à eux sont formés suite à l'apoptose cellulaire.

Commençons par les plus petites de ces vésicules : les exosomes. Ils sont formés dans les endosomes et chargés de leur contenu (ARN, ADN, protéines…), puis exportés à l'extérieur de la cellule (Figure 22). Leur taille est autour de 100nm (Figure 23) [137].

Les microvésicules, quant à elles, sont issues d'une déformation de la membrane extracellulaire, conduisant à leur formation (Figure 22). Leur taille est supérieure à celle

des exosomes mais inférieure à 1µm (Figure 23) [138]. Elles contiennent aussi les trois types de molécules biologiques : ADN, ARN et protéines.

Enfin, les corps apoptotiques sont, comme le laisse supposer leur nom, de petits fragments de cellules ayant subi l'apoptose. Contrairement aux exosomes et aux microvésicules qui reflètent le contenu d'une cellule vivante, ces derniers correspondent au contenu d'une cellule morte et ne donnent donc pas les mêmes informations (Figure 22) [139]. Cependant ils sont facilement distinguables des autres types de vésicules extracellulaires de par leur taille importante, comprise entre 1 et 5µm (Figure 22).

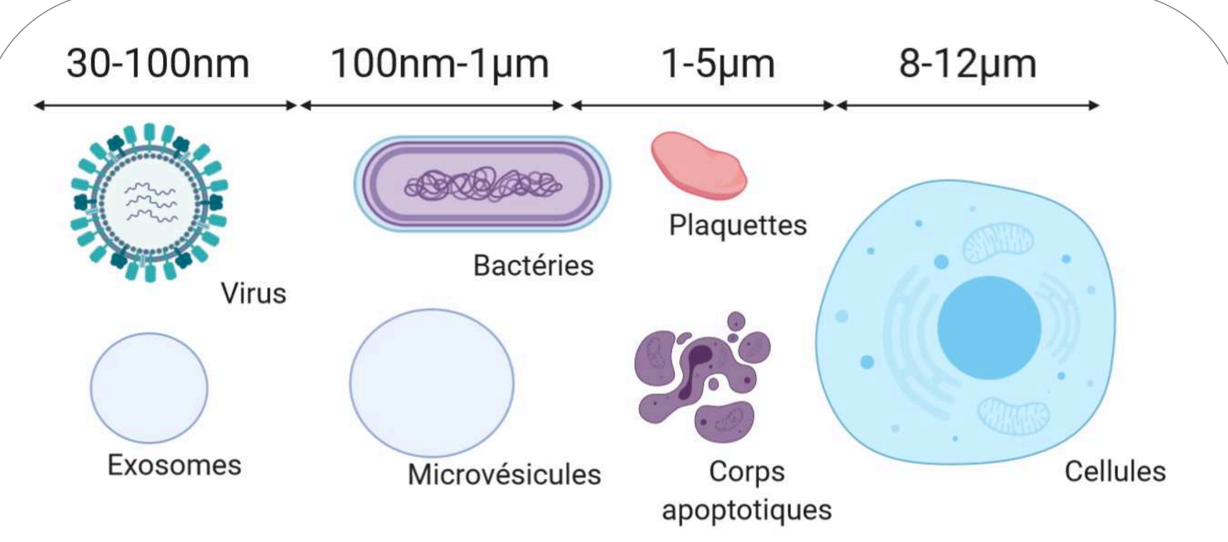

Figure 23 : Comparaison des tailles des différentes vésicules extracellulaires

En taille, un exosome est comparable à un virus, une microvésicule à une bactérie et un corps apoptotique à une plaquette.

1.2.3.3.2) MicroARN et exosomes

Les travaux de Valadi *et al.* et de Skog *et al.* sont parmi les premiers à démontrer la présence de miRNA dans les exosomes de mastocytes et de cellules de glioblastome multiforme (GBM) respectivement [140, 141].

Initialement, les exosomes étaient considérés comme servant à éliminer des protéines ou tout autre matériel biologique indésirable des cellules, y compris les miRNA. Avec le temps, un vrai rôle de communication intercellulaire a été mis en évidence [142, 143].

Étant donné que les exosomes ne sont pas juste une manière pour la cellule d'éliminer les miRNA en surnombre, l'exportation de ces derniers est régulée par différents mécanismes moléculaires.

En premier lieu, certains composants du complexe miRISC tels que GW182 ou AGO2 co-localisent avec les multivesicular bodies (MVB) et/ou les endosomes [144]. De plus, Guduric-Fuchs *et al.* ont montré que la perte d'AGO2 diminue le présence des miR-150 et 142-3p dans les exosomes des cellules [145]. Enfin, Kosaka *et al.* montrent que le niveau d'expression de la protéine nSMase (neutral sphingomyelinase) est corrélé avec le nombre de miRNA exosomaux, en particulier pour le miR-210 [146].

Par la suite, plusieurs travaux ont démontré que des RNA-binding proteins étaient responsables de l'export des miR dans les exosomes. Par exemple, les travaux et Lu *et al.* montrent que la protéine MEX3C (MEX3 RNA-binding family member 3) est impliquée dans l'exportation exosomale du miR-451 [147].

Enfin, des chercheurs se sont penchés sur les séquences consensus d'exportation des miRNAs dans les exosomes, appelées exomotifs, par les protéines RNA binding proteins SYNCRIP (hNRQ heterogeneous nuclear ribonucleoprotein Q), hnRNPA2B1 (hnRNPQ heterogeneous nuclear ribonucleoprotein A2/B1) et YBX1 (Y-box-binding protein) (Figure 24). De plus, des protéines comme hnRNPA2B1 ont été décrites comme étant des « readers » de la 6mA dans les ARNm [148]. Cependant, ces différents travaux ne prennent pas en compte les différentes méthylations des miRNA. Ce point sera abordé dans la discussion.

Nom	Taille (kDa)	Séquence	Référence
SYNCRIP	69.6	GGGCA/UG/C	[149] [150]
hnRNPA2B1	37.4	GGAG	[151]
MEX3C	69.3	CCCT/G	[152]
		Non décrite	[147]
YBX1	35.9	UC/TAUC	[153–155]

Figure 24 : Récapitulatif des différentes protéines impliquées dans l'export exosomal et de leurs séquences consensus

Le dernier mécanisme décrit comme participant à l'export des miRNA dans les exosomes est l'uridylation de l'extrémité 3' des miRNA par les uridityl transferases, alors que cette extrémité est plutôt adénylée dans les miR intracellulaires [156].

Après la description des différents mécanismes de régulation de l'expression des gènes, nous allons nous intéresser à l'influence de l'environnement sur ceux-ci.

1.3) Méthylation de l'ADN, microARN et environnement

D'après le dictionnaire de l'Académie française, l'environnement est défini comme «l'ensemble des agents chimiques, physiques, biologiques, et des facteurs sociaux exerçant, à un moment donné, une influence sur les êtres vivants et les activités humaines».

Sur la base de cette définition, plusieurs modifications du méthylome et/ou du miRome ont été décrits.

1.3.1) L'alimentation comme facteur biologique

Plusieurs phénomènes épigénétiques ont été décrits comme directement modifiés par l'alimentation.

Chez les abeilles par exemple, c'est grâce à des mécanismes épigénétiques qu'une abeille avec le même patrimoine génétique que les autres individus de sa ruche devient reine,

simplement en se nourrissant de gelée royale. En effet dans le cerveau des abeilles, la méthylation de plus de 550 gènes conservés est différente entre les reines et les ouvrières, et l'inhibition artificielle de la DNMT3 induit le développement de reines [157, 158]. Dans la gelée royale, il existe un HDAC inhibiteur, 10HDA, qui agit en synergie avec la 5-azacytidine mais n'est pas capable de déméthyler l'ADN seul. 10HDA serait le principal élément modulant l'épigénétique dans la gelée royale [159].

Chez les souris, les effets d'une mutation transmise de générations en générations peuvent être inhibés par une alimentation riche en vitamine B9 (également appelée folate ou acide folique), donneur de groupements méthyl. A l'origine de cette découverte, le gène *agouti*. Ce gène code pour la protéine agouti-signaling protein (ASIP), responsable de la distribution de la mélanine chez les mammifères en modifiant la production de phaeomélanine, de couleur jaune à rouge, et d'eumélanine, de couleur marron à noire. Chez la souris, les allèles non mutés (A) et (a) induisent un phénotype de poils gris et noir respectivement, alors que l'allèle « jaune viable » (A^{vy}) induit le syndrome « jaune obèse » avec des souris qui présentent une obésité, une hyperinsulinémie et un développement de tumeurs [160]. Un troisième allèle existe, l'allèle « jaune létal » qui est létal pour les individus homozygotes. Avec le même génotype (a/A^{vy}) un panel de phénotypes est observé (Figure 25), et cette variation est due à la méthylation du gène *agouti* : plus le gène est méthylé plus le phénotype est proche du phénotype non muté. Le plus intéressant est qu'il est possible d'inverser les effets du gène A^{vy} simplement en modifiant l'alimentation des souris. En effet, enrichir l'alimentation des mères en vitamine B9, donneur de groupements méthyl, oriente la descendance plutôt vers un phénotype agouti [161].

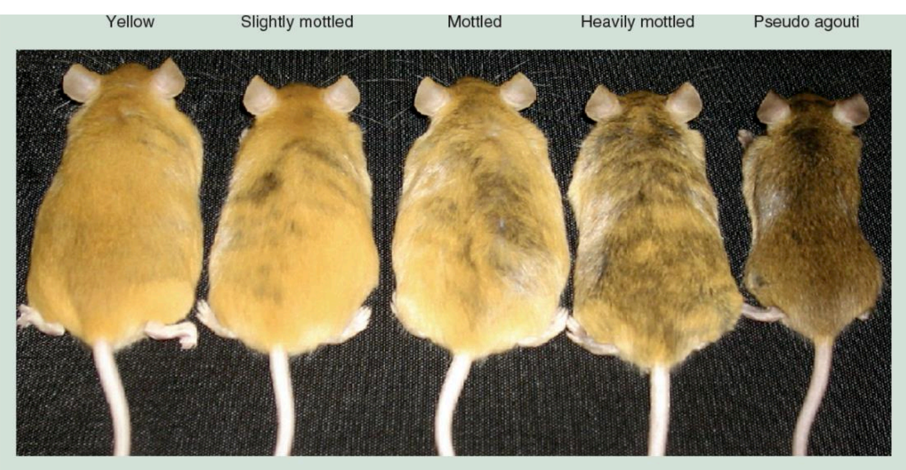

Figure 25 : Les différents phénotypes de souris agouti avec le même génotype

De gauche à droite, les souris expriment de moins en moins le phénotype agouti jaune obèse. Cette différence de phénotype est due à une méthylation différentielle du gène. Source : [162]

Un dernier exemple est celui de la famine de 1944/1945 en Hollande, où les enfants nés de femmes enceintes à cette période présentent plus de pathologies telles que le diabète, l'obésité ou des maladies cardiovasculaires que la normale [163]. Ils sont aussi plus petits que la moyenne, et leurs enfants eux-mêmes sont plus petits que la moyenne. Ce phénomène est notamment dû à une hypométhylation transmise du gène IGF2 (insulin-like growth factor), un facteur de croissance important du développement. Un évènement peut donc avoir des répercussions pendant plusieurs générations d'individus.

Au-delà des effets directs de l'alimentation, cette dernière peut être contaminée par différents polluants, notamment les herbicides et pesticides. Ce sujet sera abordé dans une prochaine partie.

1.3.2) Les facteurs chimiques : l'exposition aux polluants

L'exposition aux polluants peut aussi bien être volontaire (le tabac ou l'alcool par exemple) qu'involontaire (comme la pollution de l'air ou les pesticides). De plus, l'exposition peut être chronique ou aigüe, et lorsqu'elle est chronique elle peut être plus ou moins

importante, selon les conditions de travail par exemple. En effet, un agriculteur sera exposé de manière chronique à des doses plus élevées de pesticides qu'une personne n'utilisant pas ces produits dans le cadre de son activité professionnelle.

Étant donnée la constante augmentation de l'utilisation des pesticides dans le monde, il nous semblait pertinent de s'intéresser à ces derniers, et plus particulièrement de se concentrer sur leurs effets épigénétiques, peu décrits.

1.3.2.1) Les herbicides : revue "Herbicides exposure: the epigenetic consequences still poorly explored"

Dans cette première revue écrite par le réseau EpiSAVMEN, nous nous sommes attelés à décrire les effets épigénétiques des herbicides décrits dans la littérature, sur différents organismes impactés lors du cycle de vie des herbicides. Le réseau EpiSAVMEN (Epigénétique - Santé - Agroalimentaire - Végétal - Mer - Environnement - Nutrition) regroupe 10 équipes de recherche et est soutenu et financé par la région Pays de la Loire pour une durée de 5 ans. Ses objectifs sont de structurer la recherche en épigénétique dans la région Pays de la Loire, promouvoir de manière durable l'enseignement de l'épigénétique et diffuser auprès du grand public les recherches en épigénétique menées dans les laboratoires de la région en organisant des rencontres.

Epigenetic consequences of herbicides exposure: a field yet to be fully explored

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Running title: Herbicides exposure: epigenetic point of view.

Keywords: Herbicides, epigenetics, cancer, immunity, environment

ABSTRACT

Herbicides are molecules used in crops, railways, roadsides or boat hulls in order to kill unwanted weeds. Aside from their efficient weed-killing properties, these chemicals are carried by wind, water or other organisms away from their initial spreading areas, leading to exposure of non-targeted species. Humans, rodents, fishes or algae, many organisms are exposed to herbicides through their living environment or food chain. Beyond the simple warning of the phytosanitary industry on herbicides' toxicity, long term epigenetic phenomena have been described to be modified by environmental factors such as exposure to pollutants and are involved in several pathologies, especially cancers. Epigenetics is a trending domain in science and has been described as reversible changes of gene activity that do not involve change in the DNA sequence and that can be transmitted to progeny through cell division. These Despite growing use of herbicides and increasing importance given to epigenetics in pathological mechanisms, only few studies evaluated impacts of these chemicals on epigenetics regardless of the organism studied. Therefore, it seems important to give a clear and updated picture of the highlighted epigenetics modifications following exposure to herbicides in different species. In this review, we recall fundamental epigenetic specificities of organisms and gather information available regarding modifications induced by three different herbicides: atrazine, diuron and glyphosate.

I- INTRODUCTION

1) A global issue due to the extent of their use

Between 1950 and 2018, global human population increased from 2.54 to 7.63 billion, which represents an increase of 300% (United Nations, 2019). As a result, the intensity of agricultural production has increased dramatically as well as the use of pesticides. If the trend continues, by 2050 world production and trade in pesticides would be 2.7 times the current amount, which means that humans and many other living organisms will be exposed to increasing levels of pesticides (Tilman, 2001). Pesticides are chemical substances commercialized for pest control, these include phytosanitary products and biocides, and there are hundreds of active molecules on the market. They can be divided into several categories, targeting different unwanted organisms such as insecticides, rodenticides, bactericides, fungicides, larvicides and finally herbicides, employed to destroy weeds.

Herbicides are molecules widely used over crops, roads, railways or boat hulls all around the world. Some of them are selective, able to control specific weed species growth, others are total weedkillers, especially used to clear farmland, roadsides or railways. The 2,4-Dichlorophenoxyacetic acid is the first developed herbicide (Hamner & Tukey, 1944), and in 2010 the Herbicide Resistance Action Committee (HRAC) listed about 300 herbicides classified into 22 major families depending on their action mode (Figure 1). More than 23 000 tons of herbicides were sold in 2013 in France ranking it to the top of European market for phytosanitary products use (French Ministry of Ecological Transition). According to USEPA (United States Environmental Protection Agency) data, in 2012, approximately 1.3 million tons of herbicide active ingredient were used all over the world, including 307 000 tons in the US. Nowadays, this worldwide and massive use of pesticides becomes more and more controversial especially due to emerging and increasing number of studies describing impacts on human health.

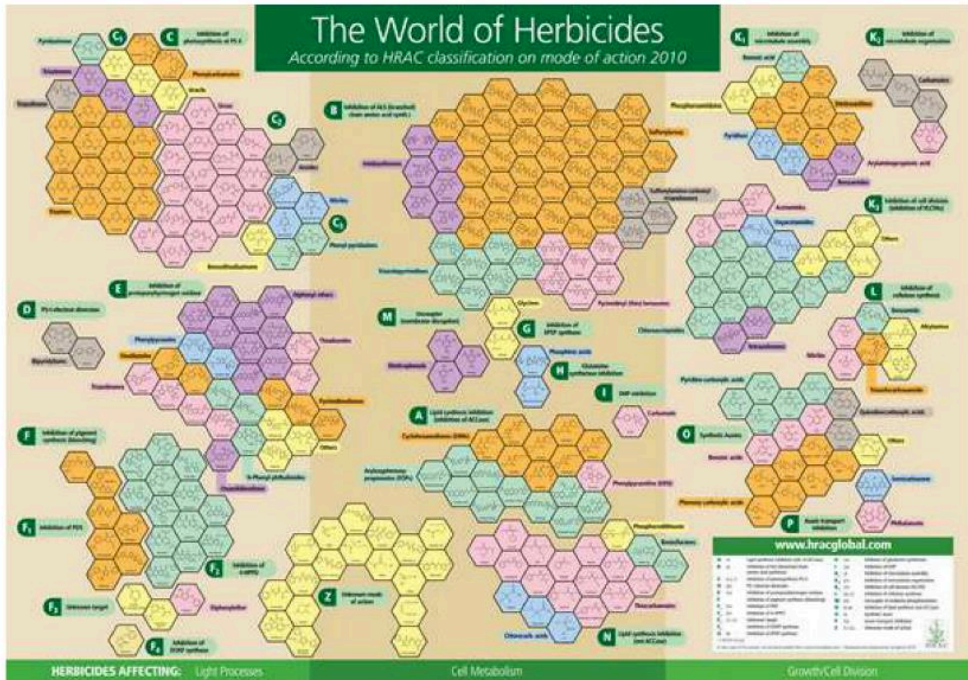

Figure 1. Poster of about 300 herbicides listed by the Herbicide Resistance Action Committee (Heap, 2018)

2) Tangible examples

There are several hundred herbicides, classified into different types depending on timing of herbicide application (post-emergent and pre-emergent), targeted weeds, persistence in soils (residual and non-residual), etc. It would be too tedious to draw up an exhaustive list, but the next three examples are fully representative of how herbicides have been used since their introduction. In this review, we will focus on effects induced by glyphosate, atrazine and finally, diuron. Focus on these herbicides appears to be relevant as they are used as models for their chemical families in many scientific studies.

Glyphosate (N-(phosphonomethyl) glycine), is an organophosphate compound. It is the most common herbicide, with over 9 million tons spread since the 1970s (Benbrook, 2016). It is a non-selective and post-emergent herbicide which inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity, responsible for the synthesis of aromatic amino acids (Herrmann & Weaver, 1999; Tsui & Chu, 2003). This inhibition degrades chloroplasts and therefore, reduce photosynthesis (Vera *et al.*, 2010; Wang *et al.*, 2016; Wood *et al.*, 2016). The best known formulation containing glyphosate is the Roundup, in which it is present under the isopropylamine salt (IPA) form, with the polyoxyethylene amine (POEA), a surfactant added to amplify herbicide's activity (Tsui & Chu, 2003). Recently, investigations started to highlight glyphosate dangerousness on human health. In 2018, Dewayne Johnson was the first to obtain the condemnation of Monsanto, judged responsible for his cancer due to glyphosate exposition. Despite this case and the report of the International Agency for Research on Cancer (IARC) monographs program, classifying glyphosate as probably carcinogenic to humans (group 2A), still no interdiction of this substance has been applied to this date.

Another well-known herbicide is atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) still widely used, especially in the United States, but banned in the European Union since 2003. Atrazine belongs to the simazine family and is mostly spread over corn, sorghum, sugarcane, and wheat crops (Wirbisky-Hershberger *et al.*, 2017) to control broadleaf as well as fatty leaf plants (Kumar *et al.*, 2014). This herbicide acts on different plant molecular mechanism, such as hormonal regulation, cell cycle, pigment synthesis or photosynthesis (Radosevich, Holt, & Ghersa, 2007). Marketed under the name Primextra®, its half-life in soils is 40 days (Filimonova *et al.*, 2016).

Finally, diuron is a phenylurea compound used for weed control in soy, fruits, cotton and sugarcane cultures particularly in developing countries (Roubeix *et al.*, 2011; Domingues *et al.*, 2011). Previously used for agricultural purposes, it has been banned in France since 2003 for its toxicity on organisms (Moisset *et al.*, 2015). However, in France, it is still found in trace amounts in 38% of surface water (Pesce *et al.*, 2006), at concentrations ranging from a few ng/L to 4 µg/L (Legrand *et al.*, 2006), due to its use as anti-algae on boat hulls (Khanam *et al.*, 2017; Silkina *et al.*, 2009).

3) Herbicides exposure: what health outcomes?

After spreading, all herbicides can be leached below the root zone. Different factors affect leaching such as soil structure, moisture, presence of microorganisms able to degrade the herbicide, type of irrigation system, amount of rainfall (Flury, 1996) and physical properties such as slope and depth. Obviously, the amount of herbicides found in groundwater also depends on the herbicides physicochemical properties such as solubility, persistence and adsorption coefficient (K-oc). For example, atrazine is a highly leachable compound, inversely to glyphosate. Because of these phenomena, glyphosate, diuron, atrazine and their metabolites are found in groundwaters, lakes, generally near fields (Castro Berman *et al.*, 2018; Thurman, Bastian, & Mollhagen, 2000; Kapsi *et al.*, 2019; Masiol, Giannì, & Prete, 2018), but also in urban and rural wastewater treatment plant effluents (Münze *et al.*, 2017; Nitschke & Schüssler, 1998). Molecules found in water are from herbicides commonly used but also those banned several years ago (e.g. diuron and atrazine in France). This raises the question: What impacts do herbicides have on the health of all organisms, even far from treatment sites and several years after exposure?

Several herbicides are controversial, and it seems difficult to find a consensus on their use. On one hand, farmers claim advantages of herbicides on increasing efficiency and facilitating work in fields. On the other hand, an increasing body of experimental studies demonstrate that herbicides could have impacts on both environment and health, which raises concerns on the need of clinical studies. Indeed, in the past decades, increasing evidence show that herbicides might be harmful for plants, animals and humans (Figure 2). A review of recent literature over the last 10 to 15 years shows that although not all of them are in harmony, some articles report the negative effects of herbicides use. It seems that all organisms are impacted in various manners, for instance development disorders, reduced photosynthetic abilities or immune system alterations. Several reviews discuss the link between pesticides exposure and cancer occurrence (Alavanja *et al.*, 2004; Bassil *et al.*, 2007; Chiu & Blair, 2009; Damalas & Eleftherohorinos, 2011; Dich *et al.*, 1997) but only a few of them raise the epigenetic consequences. Epigenetics has been defined as the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence. Environment and nutrition are well known to modify epigenetics (Bollati & Baccarelli, 2010; Syed *et al.*, 2013). The clearest example are bees: the honeybee queen has the same genome than other workers in the hive, but she has been fed with royal jelly. This different diet induces epigenetic modifications like DNA methylation modifications and a very different phenotype (Chittka & Chittka, 2010). In humans, it has been shown that environment can modify epigenetics, even in twins (Wong *et al.*, 2010) and, surprisingly, only a few studies have linked herbicide exposure and epigenetics. Baccarelli and Bollati reviewed the epigenetics impacts on mammals of environmental chemicals including Persistent Organic Pollutants (POP) some of which are pesticides (Baccarelli & Bollati, 2009). Later, in 2013, Collota et al. summarizes epigenetic impacts of various pesticides (including herbicides, fungicides, and insecticides) on mammals (Collotta, Bertazzi, & Bollati, 2013). Only two herbicides are mentioned, paraquat and dieldrin, able to induce histone modifications. Moreover, mammals are not the only

organisms to be in contact with environmental pollutants such as herbicides. Indeed, other species are regularly in contact with these molecules and can therefore be affected at the epigenetic level.

Therefore, it seems important to give a clear and updated picture of the highlighted epigenetics modifications following exposure to herbicides in different species. Even if there is always a gap between different study models, to collect information reflecting the entire ecosystem appears a necessity. In this context, this review recalls main epigenetic particularities in different organisms and gather information about described epigenetic impact of herbicides.

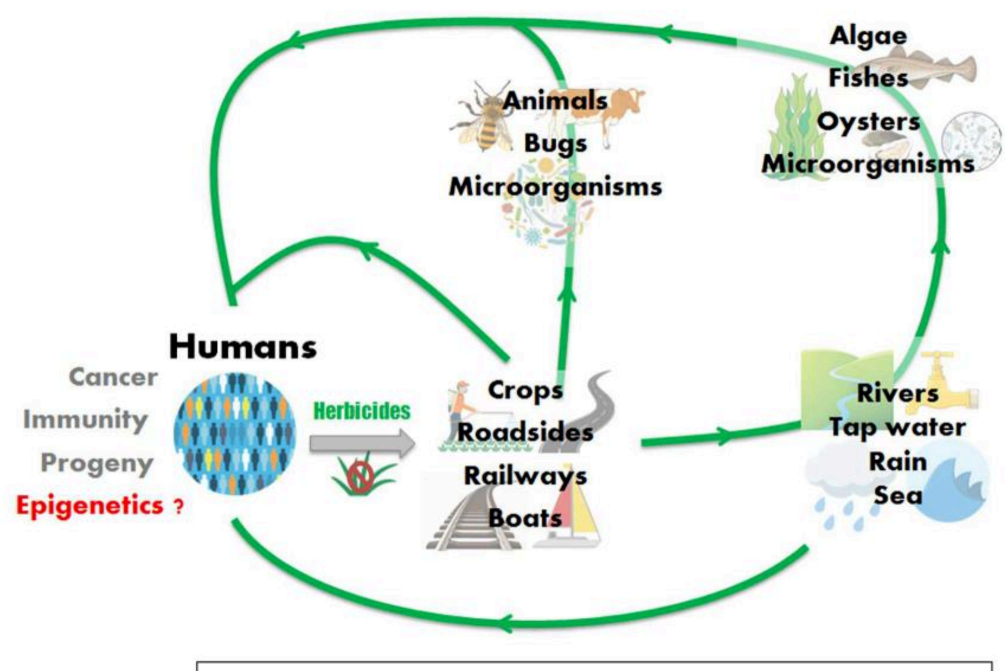

Figure 2. Herbicides life cycle and their potential off-target effects.

II- HERBICIDE EXPOSURE: EPIGENETIC ISSUES

As mentioned, herbicides may impact several organisms along their way from spreading to total degradation. Organisms may be subject to bioaccumulation by feeding on contaminated food and absorbing polluted water. The substance is absorbed faster than it is excreted, and the concentration increases in the tissues. In addition to bioaccumulation, the biomagnification phenomenon is linked to the persistence of the pollutant. It refers to the transfer of the substance through the food chain which is faster than its elimination, causing an increase in the concentration of the chemical when moving up in the food chain (Langenbach, 2013). Whether it is on mammals, plants or other organisms, described effects of herbicides exposure are various and it seems that the intrinsic importance of most of them is still to be elucidated. Among observed effects, most of them concern immunity, progeny, growth, cancer risk, but very few studies are interested in epigenetic modifications. Epigenetics is one of the only fields of molecular biology in which the use of various models,

representing all kingdoms of life, is so widespread. In this context, we have decided to distinguish between the epigenetic modifications highlighted in humans and in other animals and plants. In the first part, main mechanisms of human epigenetics will be described, and studies conducted on human study models such as mice and rats will be considered. Then, epigenetic specificities of organisms as plants, algae, fish and invertebrates will be briefly described and herbicides-induced epigenetic modifications will be recounted.

1) Mammals

Recently, epigenetics has been defined as the sum of the modifications to the chromatin template that collectively establish and propagate different patterns of gene expression and silencing from the same genome (Allis *et al.*, 2015). In fact, more than 200 different cellular phenotypes naturally occur in one person from the fertilized egg, starting with a single genome that becomes multitude of distinct epigenomes. In this context, DNA is submitted to lots of modifications, and nucleosomes also carry covalent and noncovalent variations, which together constitute the epigenetic language. Currently, we know three types of epigenetic mechanisms: DNA methylation, histone marks modification and non-coding RNAs expression. These three operations all contribute to the same end effect, gene transcription regulation.

a) Epigenetic mechanisms

DNA methylation corresponds to an addition of a methyl group (CH_3) on CpG dinucleotide cytosine, which becomes a 5-methylcytosine (5mC). Main actors of DNA methylation are DNA methyltransferases (DNMTs). DNMT1 is implicated in maintaining methylation during cell division, whereas DNMT3A and 3B make *de novo* methylation (Jurkowska, Jurkowski, & Jeltsch, 2011). DNA demethylation can occur from two different mechanisms. The first one is passive DNA demethylation: after cell division, if DNMT1 does not remethylate DNA, CH_3 groups will be lost after a few cell cycles (Wu & Zhang, 2014). The other mechanism is active DNA demethylation, processed by TET (Ten-Eleven-Translocation) enzymes which will convert 5mC to 5-hydroxymethylcytosine (5hmC), then to 5-formylcytosine (5fC) and finally to 5-carboxylcytosine (5CaC). These modified cytosines will be replaced by a non-modified cytosine by Thymine-DNA glycosylase (TDG) and Base excision repair (BER) proteins (Kohli & Zhang, 2013). DNA methylation is implicated in transcription regulation: when a gene promoter is methylated, the gene is very often repressed. Indeed, methylation will cause a steric hindrance preventing transcription factor binding, directly or after Methylated DNA Binding (MDB) protein recruitment (Fatemi, 2006).

In eukaryotic cells, DNA is compacted into chromatin which consists in several histone octamers called nucleosomes. The nucleosome is a 146 bp of DNA wrapped around 2 sets of histones H2A, H2B, H3 and H4 (Luger *et al.*, 1997). Histone post-translational modifications (PTMs) refer to chemical events including acetylation, methylation, phosphorylation (Rossetto, Avvakumov, & Côté, 2012), and ubiquitination (Weake & Workman, 2008) on amino acids at the N-terminal tails of core histones. It turns out that some histone PTMs are related to gene activation or silencing through altering chromosomal relaxation or

compaction, respectively. The two most common histone marks are acetylation (Ac) and methylation (Me).

Acetylation of histones may have direct effect on nucleosomal architecture and influence chromatin structure by affecting histone–histone and histone–DNA interactions (Tessarz & Kouzarides, 2014). This is a very dynamic process during which histone acetyltransferases (HATs) and histone deacetylases (HDACs) act in concert to add and remove acetylation marks, often on histone-tail-lysines. When acetylated, DNA is less condensed and then, gene expression is increased. These marks are very transient and are believed to be vital for precise temporal transcriptional control (Chen, Zhao, & Zhao, 2015). Hyperacetylation of oncogenes or proto-oncogenes have been described in various cancers such as hepatocellular carcinoma (Bai *et al.*, 2008), and acetylation of a specific lysine on histone H3 (H3K18) was correlated with prostate cancer recurrence (Bianco-Miotto *et al.*, 2010).

Histone methylation regulates gene expression through recruiting histone-tail binding proteins. Depending on the location of the methylation, the effects on gene transcription are various (Dong & Weng, 2013). On one hand, methylation on H3 histone or on Lysine 4 (H3K4), H3K36, and H3K79 results in transcription of genes, on the other hand, methylation of H3K9, H3K27, and H4K20 often leads to the silencing of transcription. The number of methyl group added (mono, di or tri-methylated) can also impact the output of the modification, for example, H3K9 mono-methylated leads to the activation of the transcription whereas its tri-methylated form causes transcriptional inhibition (Fardi, Solali, & Farshdousti Hagh, 2018).

In humans, a very large majority of DNA does not code for proteins (ENCODE Project Consortium *et al.*, 2007), whereas 80% are transcribed into RNAs (Djebali *et al.*, 2012). In other terms, the most of transcribed DNA sequences are non-coding RNAs, with different roles. These non-coding RNAs has been split in two main families, using a size criterion: long non-coding RNA (>200nt) and short non-coding RNA (19nt<size<200nt). The long non-coding RNAs family is very miscellaneous and not well classified, but in the second family different subtypes have been described. For example, small nuclear RNA (snRNA) are implicated in splicing, piwiRNA (piRNA) have a role in transposons silencing, microRNA (miRNA) target mRNA in order to induce their degradation and inhibit transcription and small nucleolar RNA (snoRNA) participate in ribosomal RNA maturation (Esteller, 2011). Non-coding RNAs play a crucial role in all cell types and are a big part of epigenetic players.

b) Epigenetic consequences of herbicide exposure

Epidemiological studies of the impact of herbicides on humans are rare and difficult to implement. They should incorporate the duration, the frequency of exposure but also the formulation of the herbicide. The few articles available focus on the immune system and cancer, and for the most part they do not provide convincing evidence of the link between herbicide exposure and cancer in humans (Sathiakumar *et al.*, 2011; Mink *et al.*, 2012). Surprisingly, almost none evoke epigenetic alterations while they sometimes constitute a significant keystone of several diseases. There are nevertheless some exceptions. First, some

articles mention the ability of herbicides to hyper or hypomethylated the DNA template. This is the case of a study published in 2017, which reports for the first time that a decrease of global DNA methylation was observed in human peripheral blood mononuclear cells exposed to 0.25 mM of glyphosate (Kwiatkowska *et al.*, 2017). High concentrations of glyphosate are also responsible for hypermethylation of the tumor suppressor p53 promoter. The authors emphasize that it would be necessary to confirm these results in other cell types and *in vivo*. Glyphosate toxicity on PBMC also has been demonstrated (Woźniak *et al.*, 2018). Also concerning the immune system, diuron has been shown to reduce pDC activity by demethylating ILT7 and TRAIL promoter after TET2 overexpression (Briand *et al.*, 2019). Another study evaluated the link between "high pesticide exposure events" and DNA methylation in a cohort of pesticide (including herbicides) users (Rusiecki *et al.*, 2017). After DNA extraction from whole blood, they used pyrosequencing to quantify methylated DNA. They showed that pesticides may contribute to decrease DNA methylation in repetitive element LINE-1, an emerging biomarker for neoplasia (Ardeljan *et al.*, 2017). Among older men, pesticide users carried some CpG sites in MGMT promoter less methylated than in controls. MGMT is a protein involved in DNA repair and has a major role in tumor development (Sharma *et al.*, 2009) and chemoresistance (Sarkaria *et al.*, 2008). Very recently, a study reveals that glyphosate triggered TET3-dependent DNA global hypomethylation, and participate in mammary cells carcinogenesis (Duforestel *et al.*, 2019).

miRNAs are another epigenetic mechanism which can be modified by herbicides. Wirbiski et al. observed a dose-dependent alteration of human miR expression following an embryonic atrazine exposure in zebrafish (Wirbisky *et al.*, 2016). The only downregulated miRNA at the lowest atrazine concentration (*e.g.,* 0.3 ppb) is miR-126-3p. In a physiological context, miR-126-3p is able to limit proliferation and metastatic progression in different cancers by targeting a subunit of PI3K (Du *et al.*, 2014; Wu *et al.*, 2016). It can thus be hypothesized that the decrease in the expression of this miR following exposure to the herbicide may, in addition to other oncogenic hits, potentiate the phenomenon of tumorigenesis. Other human miRs have seen their concentration changed when exposed to higher doses of atrazine. Two other studies have shown that paraquat, an herbicide already known for its acute and chronic toxicity, is responsible for mRNAs and miRNAs changes in human neural progenitor (Huang *et al.*, 2016; Yan *et al.*, 2017). More particularly, Huang et al. (2016) report that among the 43 differentially expressed miRNAs following paraquat exposure, the mir-200a is downregulated. The decrease in the expression of this miR is responsible for a loss of differentiation ability in neural cells.

For reasons mentioned above, it is easier to work on study models due to the possibility of controlling the environment and avoiding some biases. Male mice have been exposed to atrazine, which revealed to induce effects on epigenetic processes of meiosis (Gely-Pernot *et al.*, 2015). In rats exposed to glyphosate, an epigenetic disruption of ERα has been shown (Lorenz *et al.*, 2019). In addition, other studies demonstrated that effects of herbicides on epigenome can be transmitted to rat progeny, even if these descendants were not exposed

to herbicide (Kubsad *et al.*, 2019). Consistently, McBirney *et al*. have highlighted that atrazine exposure promotes sperm differential DNA methylation regions (DMRs) in rats and epigenetic transgenerational inheritance of various diseases such as testis dysfunctions and mammary tumors (McBirney *et al.*, 2017). These DMRs could provide potential epimarkers for those diseases. Atrazine has the same transgenerational effect in mice, inducing a decrease of trimethylation of histones in the third generation (Hao *et al.*, 2016).

Finally, some effects of glyphosate-based herbicides (GBH) on rats have been observed. First, GBH exposure during the developmental period alters mammary gland development and steroid hormone receptor expression involving methylation modifications (Gomez *et al.*, 2019). Then, it has been shown that GBH formulation induces epigenetic modifications in adult female rats following in utero and lactational exposure especially histone H3 modifications (Lorenz *et al.*, 2019). However, these last studies must be put into perspective, preparations tested are glyphosate-based and not pure, the mixture can have an influence on health without it being the same for glyphosate alone (Vandenberg *et al.*, 2017). Despite that, all these examples give us primary insight on the variety of effects herbicides can have on rodents and humans at the epigenetic level. Nevertheless, it seems clear that research in this area needs to be deepened to confirm these discoveries and avoid missing epigenetic consequences in mammals and their offspring that can lead to serious diseases.

2) Aquatic fauna

a) Epigenetic particularities

Most of what we know about epigenetics in animals comes from mammals, and even they are similarities we must not neglect taxa specific patterns. Among fish and shellfish, Zebrafish (*Danio rerio*) is considering as an excellent model to study pollutant-induced epigenetic changes as described in by Cavalieri and Spinelli. Indeed, in both fish and shellfish, the basic methylation machinery is comparable to humans. As example, Zebrafish gets all DNA methyltransferases identified in mammals, however, in contrast to mammals, which have three active DNMTs as mentioned earlier, the zebrafish genome contains multiple paralogs (for instance, they express 7 DNMT : one homolog of DNMT1 for maintenance methylation, 2 homologs of DNMT3A and 4 homologs of DNMT3B for *de novo* methylation) (Cavalieri & Spinelli, 2017). Among the differences with mammals, it seems that in fish, the global DNA methylation level is higher (Metzger & Schulte, 2016) and some environmentally-induced methylated regions can escape reprogramming in the early embryo (Shao *et al.*, 2014). In pacific oyster, as in vertebrates, the methylome dynamics plays a role in transcription regulation during the development (Riviere *et al.*, 2017). However, as in invertebrates in general, methylation profile is similar to a mosaic composed of methylated regions punctuated by unmethylated tracts of the same length (Suzuki *et al.*, 2007; Rivière, 2014). The percentage of methylated CpGs in Pacific oyster somatic tissues reached 15% versus 60 to 70% in mammals (Gavery & Roberts, 2013).

Studies on non-coding RNAs and histones in aquatic fauna are quite rare, moreover in non-model animals. Nevertheless, there is a large number and variety of non-coding RNA in fish and shellfish (long intergenic noncoding RNAs, miRNA, piRNA etc ...) whose exact properties are not always known. As in humans, these RNAs seem to be involved in different physiological processes, depending of the species: larval development, immune response, shell color diversity, salinity tolerance, transcription regulation (Houwing *et al.*, 2007; Zhao *et al.*, 2016; Yu, Zhao, & Li, 2016; Andreassen *et al.*, 2017; Wang *et al.*, 2018; Feng *et al.*, 2018). Concerning histones, variants and post-translational modifications are well conserved between taxons. Histones H3, H4, H2B present in humans have been found in oysters, some even in the cytoplasm as fragments with antimicrobial properties (Bouilly *et al.*, 2010; Dorrington, Villamil, & Gómez-Chiarri, 2011; Seo, Stephenson, & Noga, 2011). Valero et al, have characterized histone H1 and H2B in European sea bass and gilthead seabream (Valero *et al.*, 2016). Studies conducted on zebrafish have highlighted histone modifications created by methyltransferase such as Polycomb/Thritorax group proteins. Histone demethylation seems necessary for regeneration (Stewart, Tsun, & Belmonte, 2009). Among the known specificities in lower vertebrates, is the existence of unique histone variants in fish oocytes (Wu *et al.*, 2009), but this part of epigenetics needs further exploration to discover the particularities of each species.

b) Epigenetic consequences of herbicide exposure

In oysters (*Crassostrea gigas*), diuron exposure in parents leads to higher methylation levels in sperm and higher global DNA methylation in offspring from different genitor groups (Bachère *et al.*, 2017). In carp (*Cyprinus carpio L.*), two similar studies demonstrated that DNA methylation levels and most DNMT mRNA were decreased in liver, kidney and gill (Wang *et al.*, 2014) as well as in brain and gonads (Xing *et al.*, 2015) under atrazine treatment. In this same species, four circulating miRNAs were differentially upregulated by paraquat exposure during 7 days (Ma *et al.*, 2018). Exposure to environmentally relevant concentrations of glyphosate and Roundup in medaka (*Oryzias latipes*) leads to decrease of Dnmt1 mRNA and increase of Tet1 and Tet3 mRNA (Smith, Vera, & Bhandari, 2019). Concerning zebrafish, Atrazine exposure (*D. rerio*) causes the decrease of DNMTs activity and expression level and the diminution of global DNA methylation level (Wirbisky-Hershberger *et al.*, 2017). Embryonic atrazine exposure also alters 17 miRNAs in larvae especially miR-10 family, critical for several pathways such as embryonic development and angiogenesis signaling (Wirbisky *et al.*, 2016).

Obviously, knowledge of the epigenetic effects of herbicides on aquatic fauna image the general knowledge about epigenetics of these animals: few articles available. But overall, it is known that organisms require the correct amount of methylation to avoid changes in gene expression and/or genomic rearrangements. A priori less toxic changes in the environment such as water temperature (Varriale, 2014; Whitaker *et al.*, 2018) or captive rearing (Rodriguez Barreto *et al.*, 2019) are able to induce variations in DNA methylation, so why could not herbicides cause serious damages ? The same goes for histones and non-coding RNA. Their

broad involvement in various pathways, as mention above, and their sensitivity to environmental pollutants in general put us on the track of the role they might play in herbicide exposure consequences.

3) Plants and algae

a) Epigenetic particularities

Following what was previously described, plants also have epigenetics specificities. DNA methylation in plants and microalgae includes not just the CG methylation, but also CHG (where H = A, C or T) and CHH methylation (Henderson & Jacobsen, 2007; Zhang, Lang, & Zhu, 2018). There are also 3 DNA methyltransferases with different roles in plants (whether they are large plants or microalgae) : (i) DNMT1 allows methylation on the CG, (ii) DRMs (domain rearranged methyltransferase) are homologous to DNMT3 and will allow methylation to be maintained on CHH and (iii) CMTs (chromomethylases) which intervene in CHG methylation (Greco *et al.*, 2012). Another difference in plants' epigenome is that it remains mostly unchanged from one generation to the other in contrast to mammals, in which methyl marks are cleared during reproduction (Henderson & Jacobsen, 2007). This leads to so called "epialleles" encoded mainly because of methylation status and responsible for various phenotypes such as timing of flowering, disease resistance, biomass or fruit ripening (Bartels *et al.*, 2018).

In plants, histones are same than in mammals, but with more variants (Deal & Henikoff, 2011). Post-translational modifications and their effects are highly conserved between organisms, including plants (Fuchs *et al.*, 2006).

Investigated later in comparison with human studies, non-coding RNAs in plants begin to be puzzled out and seem to have an important role in stress response (Liu *et al.*, 2015; Wang *et al.*, 2017). For example, long non-coding RNA (lncRNA) has been described as implicated in plant defense against pathogens (Zaynab *et al.*, 2018), but they also are precursors to smRNAs in the RNA interference (RNAi) pathway and many other roles in smRNAs regulation or scaffolding chromatin (Wang & Chekanova, 2017). miRNA in plants and algae have pretty much same effects as in mammalian, with transcriptional repressor effect (Chung *et al.*, 2017; Liu *et al.*, 2017).

b) Epigenetic consequences of herbicide exposure

Global methylation in plants appears to be altered by herbicides exposure as in humans. Both glyphosate and atrazine have been responsible for DNA methylation alterations in rice and wheat (Lu *et al.*, 2016; Nardemir *et al.*, 2015). Using bisulfite sequencing, differentially methylated regions by glyphosate and their locations have been demonstrated in *Arabidopsis thaliana* (Kim *et al.*, 2017). In Canadian horseweed (*Conyza Canadensis)*, resistance to glyphosate has been shown to be related to hypermethylation of EPSP-synthase 1 gene (Margaritopoulou *et al.*, 2018). Few studies described implication of non-coding RNA in herbicide responses but glyphosate treatment of tall fescue (*Festuca arundinacea*) suggests downregulation of transcription factors induced by miRNAs (Unver *et al.*, 2010).

In a very logical way, plants are exposed and affected, whether they are directly targeted or just growing in the surrounding areas of an herbicide spreading. At first sight, it is a little less evident that microalgae might also encounter herbicides to some extent, through leaching and water pollution. Therefore, herbicides impacts are not limited to terrestrial organisms and their study has to be extended to seaweeds, seagrasses, and especially phytoplankton, which play a major role in marine ecosystems and food webs. Several algal studies described effects already known in plants due to their homology, such as photosynthesis breakdown induced by diuron in *Dunaliella tertiolecta* (DeLorenzo, Scott, & Ross, 2001). In aquatic photosynthetic organisms, whether they are unicellular like diatoms (*Skeletonema costatum*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana, Chaetoceros calcitrans*) or multicellular like seagrasses (*Halophila ovalis*, *Halophila universis* or *Zostera muelleri*), effects are variable depending on species and often lead to proliferation and/or photosynthesis alterations (Dupraz *et al.*, 2019; Flores *et al.*, 2013; Negri *et al.*, 2015; Wang *et al.*, 2016; Wilkinson *et al.*, 2017). Although some studies have evaluated the impacts of these herbicides on algae, none mentioned epigenetic dysregulations, it may be worth to investigate further. It is important to note that a similar conclusion could be drawn for other organisms such as insects or bacteria for which the few information available only mention effects on growth and proliferation without investigating epigenetic consequences.

III- CONCLUSION

Herein, we gathered available information about the epigenetic impacts of herbicides, which are the most used pesticides, all along their life cycle, and on their impacts on several organisms including plants, algae, aquatic vertebrates and invertebrates, humans and other mammals (**Figure 3**). This review complete and extends a previous work by Collota and colleagues on the epigenetic impacts of various pesticides (including herbicides, fungicides, and insecticides) on mammals and humans only (Collotta *et al.*, 2013). Nowadays, it appears obvious that atrazine, diuron, glyphosate and other herbicides are found everywhere, sometimes years after their use. The impacts of these compounds and their residues on organisms are not limited in space and time. Despite a growing attention paid towards herbicides used worldwide in the last decades, it seems surprising to have so few recent publications dealing with their impacts. To date, these few studies are usually not fully convincing and the conclusions for human health hazards are divergent. It is therefore obvious that there is a lack of strong evidences on this subject, on the long-term toxicity of herbicides, especially regarding genetics and epigenetics, whatever the model used. Certain species at the base of food chain or essential to maintain the good functioning of an ecosystem, such as algae and bees, respectively, are mostly absent from these studies, which is an ecological and ecotoxicological nonsense. Despite this lack of in-depth studies, it appears that data already available generally agree on the epigenetic consequences of herbicides exposure. As mentioned, epigenetic mechanisms are modified by environmental perturbations and gathered studies suggest that methylation, histones modification or non-coding RNAs might, at least partially, be altered in various species. However, these results are most of the time

obtained after acute exposures, at specific concentrations and using herbicides isolated from their chemical compounds, metabolites and residues.

It is therefore important to stress the need to multiply epigenetic studies, particularly in humans. Indeed, it is now known that cellular epigenetic processes are diverse and very frequent, crucial for gene expression and cell functions, and involved in many pathological phenomenon such as cancers, neurodegenerative and psychological disorders or autoimmune diseases. In this context, it also seems important to point out that most human diseases are multifactorial. For example, cancer involves several oncogenic hits, which suggests that genetic and epigenetic modifications induced by herbicides that would be phenotypically invisible alone, could be at the origin of the carcinogenesis process associated with another spontaneous or induced oncogenic hit. The emergence of therapies targeting epigenetics and the growing interest in circulating epigenetics marks are reasons to consider the epigenetic changes caused by herbicides. Indeed, it could then be possible in humans to highlight epigenetic changes caused by herbicides through circulating DNA in the blood.

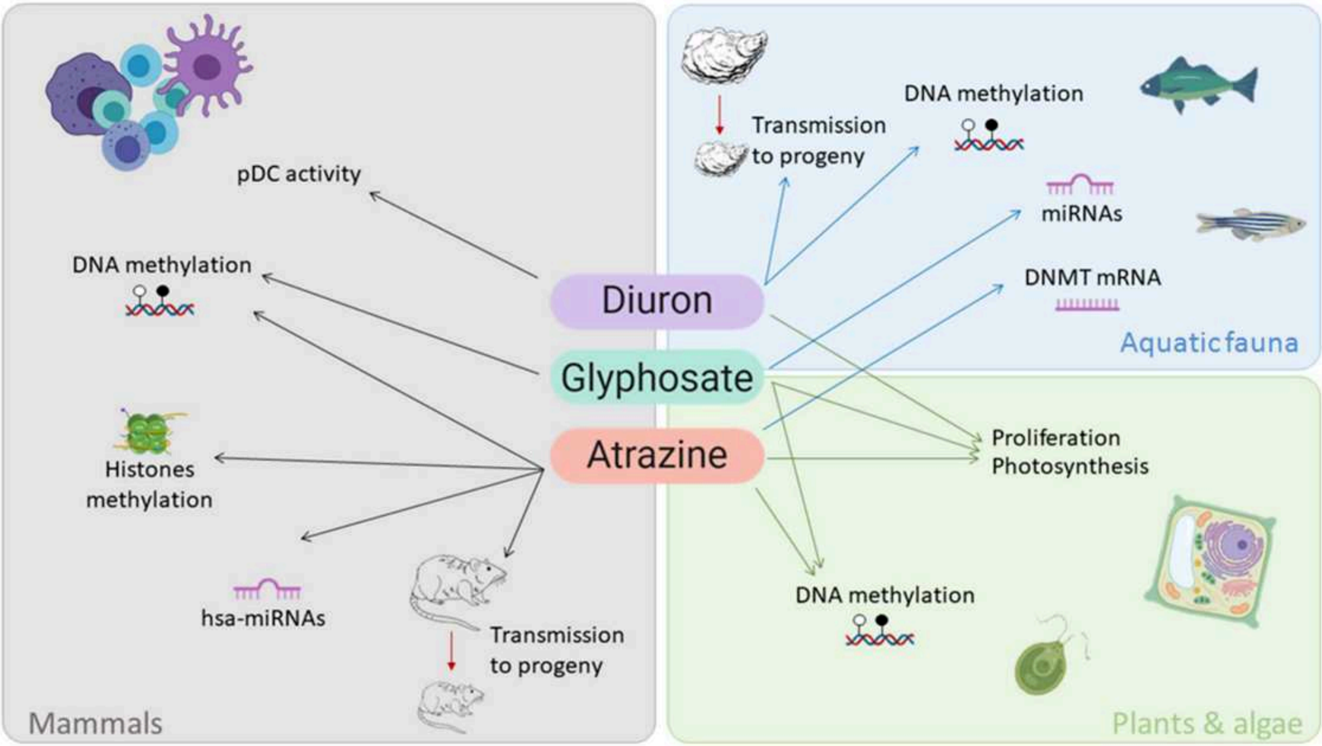

Figure 3. Described impacts of herbicides on organisms.

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En ces temps de crise écologique, il semble crucial d'approfondir nos connaissances sur l'impact des pesticides sur la santé, mais aussi d'élargir les connaissances à l'ensemble des organismes touchés. Les mécanismes et profils épigénétiques étant directement impactés par l'environnement, comme le montre l'étude sur les abeilles [158] ou celle sur la famine de 1944 en Hollande [163, 164]. Dans cette revue, nous avons démontré que les études épigénétiques dans les différents organismes impactés par les herbicides étaient encore rares.

Comme décrit dans la revue, les études épidémiologiques chez l'Homme sont rares et souvent biaisées. En effet, pour avoir une preuve irréfutable de la toxicité d'un pesticide donné, il faudrait que l'exposition à ce dernier soit le seul paramètre qui soit modifié dans l'ensemble de la cohorte. En d'autres termes, tous les individus de la cohorte devraient avoir le même mode de vie : alimentation similaire, activité physique comparable, emploi équivalent, etc… Par conséquent, l'étude mettant en évidence les bénéfices de l'alimentation biologique sur l'apparition de cancers, dus à une quantité de pesticides moins élevée reste à confirmer [165]. En effet, d'un point de vue sociologique, les individus se nourrissant exclusivement avec une alimentation biologique sont plus attentifs à leur mode de vie global : alimentation équilibrée, activité physique, moins de tabac…

C'est pourquoi des modèles animaux sont utilisés pour tester l'impact des pesticides, car leur environnement est plus facilement contrôlable. Ici cependant, le problème qui se pose est celui de la cohérence de l'administration avec la réalité. En effet, dans la population générale les pesticides sont généralement ingérés de façon chronique de par leur présence dans l'alimentation et dans l'eau de boisson. Mais les agriculteurs souffrent plutôt d'une exposition par inhalation, ces derniers étant majoritairement exposés au moment de la préparation des pesticides et du traitement de leurs surfaces agricoles. De plus, les doses font toujours débat : est-il plus pertinent de traiter régulièrement à de petites doses pour mimer une exposition chronique, ou une fois à une dose plus importante pour mimer une exposition aigüe ? Aussi, lorsqu'un individu est exposé à un pesticide, il l'est de façon très probable à d'autres. Dans ce cas, traiter avec un cocktail de pesticides n'est-il pas plus pertinent [166–168] ? Mais dans ce cas, difficile de savoir si l'impact vient de la synergie entre eux, ou d'un polluant en particulier.

Pour conclure, la question de la toxicité des pesticides voire des polluants au sens large du terme reste une question compliquée, de par la difficulté aussi bien à étudier qu'à mimer la réalité. Il est normal de ne pas se précipiter pour décréter la toxicité d'un produit, mais il est aussi important de protéger et d'informer au mieux les populations.

1.3.2.2) Autres polluants

Le tabac, bien que son exposition soit la plupart du temps volontaire, peut être considéré comme un polluant de l'environnement. Il provoque une modification de la méthylation de l'ADN [169, 170] et modifie la signature de l'expression des miRNA [171]. De plus, ses effets sont aussi délétères chez la descendance des mères exposées au tabac [172], de manière active ou passive : la méthylation de l'ADN est globalement altérée [173], les miR-155-5p, -21-3p et 18a-5p (décrits comme oncogéniques et impliqués dans l'allergie) voient leur expression augmenter [174].

Les pesticides sont une classe très large de polluants de l'organisme. En effet, sous ce nom générique sont inclus les « produits chimiques destinés à lutter contre les parasites animaux et végétaux des cultures », soit les insecticides, les fongicides, les rodenticides, les herbicides déjà mentionnés plus haut. Pour citer quelques exemples de l'impact des pesticides sur les mécanismes et profils épigénétiques : le propiconazole modifie l'expression des microARN dans le foie de souris [175], l'arsenic induit des changements dans la méthylation de l'ADN dans des leucocytes chez l'Homme [176], pour ne citer qu'eux.

La pollution de l'air, notamment via les particules et l'ozone induit elle aussi des modifications au niveau de la méthylation de l'ADN [177, 178].

Bien sûr, de nombreux autres polluants existent, mais le but ici n'est pas de faire une étude exhaustive. L'exposition au polluants au sens large est en partie conditionnée par le mode de vie.

1.3.3) Les facteurs sociaux

Le mode de vie regroupe un nombre de facteurs très large : chez l'Homme, cela comprend par exemple la qualité de l'alimentation, le fait de fumer ou de faire du sport, et chez les

animaux cela peut être la température d'incubation des œufs ou le fait de prendre soin de la progéniture.

Chez les rats, l'attention portée à la descendance notamment via le toilettage modifie le profil épigénétique dans le cerveau, dont les profils de méthylation et d'acétylation d'H3K9, induisant la modification d'expression de nombreux gènes, dont notamment NR3C1 (GCR, glucocorticoid receptor) ou la protocadhérine [179].

Chez les jumeaux monozygotes, Fraga *et al.* ont montré que le paysage épigénétique (notamment au niveau de la répartition des 5mC) est similaire lors des premières années de la vie, mais que la différence se créait au fur et à mesure du vieillissement et des modifications du mode de vie [180].

De nombreux facteurs peuvent donc modifier notre profil épigénétique de plusieurs façons, et favoriser le développement de pathologies telles que le cancer.

1.4) Méthylation de l'ADN, microARN et cancer

1.4.1) Le glioblastome

Le glioblastome multiforme est un type de tumeur cérébrale très agressive, avec une médiane de survie inférieure à 15 mois. Dans ce travail, nous nous sommes donc intéressés à cette pathologie pour tenter d'améliorer sa prise en charge.

1.4.1.1) Généralités

Le glioblastome multiforme (GBM) est une tumeur du cerveau issue de la transformation tumorale d'une cellule progénitrice de la glie. La glie correspond aux cellules de soutien des neurones et est composée d'un ensemble de types cellulaires : les astrocytes, les oligodendrocytes, les cellules de Schwann, la microglie et leurs cellules progénitrices. Les GBM font donc partie d'un ensemble de tumeurs nommée gliomes ; le terme « blast » faisant référence à la notion de cellule indifférenciée alors que le terme « multiforme » renvoie à l'hétérogénéité des cellules composant ce type de tumeur, même s'il est admis que les GBM se composent majoritairement d'astrocytes.

1.4.1.2) Le glioblastome multiforme en quelques chiffres

Les études de l'INCa indiquent qu'en France et chez l'adulte, les GBM sont les tumeurs primitives cérébrales les plus fréquentes, avec une incidence de 4 nouveaux cas par an pour 100 000 habitants, soit 2600 nouveau cas chaque année. L'âge moyen des patients atteints de GBM est de 58 ans, 70% des patients atteints de GBM ont entre 45 et 70 ans et deux tiers d'entre eux sont des hommes.

1.4.1.3) Diagnostic et traitement

Étant une tumeur cérébrale, les signes cliniques annonciateurs des GBM sont la conséquence du développement d'une masse tumorale dans un espace non extensible : la boîte crânienne. Les fonctions cérébrales sont alors endommagées, et le patient présente les signes cliniques suivants : déficit moteur, sensitif, visuel ou encore auditif, aphasie, crises d'épilepsie, troubles psychiatriques, maux de tête, hypertension intracrânienne.

La confirmation du diagnostic a ensuite lieu *via* des examens radiologiques et histologiques. Que ce soit au niveau de l'IRM ou au niveau de l'analyse histologique, la tumeur n'est pas homogène et montre aussi bien des zones de nécrose que des zones hémorragiques. Génétiquement, de nombreuses délétions, amplifications et mutations sont observables [181].

La prise en charge standard des patients atteints de GBM repose sur une exérèse de la tumeur suivie d'un protocole de radio-chimiothérapie simultanée, composé de deux phases séparées par une période d'un mois. Lors de la première phase, dite d'attaque, le patient est traité durant 6 semaines avec 30 séances de 2 Gy de radiothérapie (IR), couplées à la prise de témozolomide (TMZ) par voie orale (70mg/m^2/jour). La seconde phase du traitement, dite de consolidation, équivaut à la prise de temozolomide par voie orale (200mg/m^2/jour) 5 jours par semaine pendant 28 jours. Ce protocole de première ligne pour les glioblastomes nouvellement diagnostiqués est également connu sous le nom de protocole Stupp, du nom du médecin suisse ayant mené l'essai clinique en Europe qui a conduit à sa validation. Avec ce protocole, la survie médiane est d'environ 15,7 mois et le taux de survie à 1 an est de 64% (European revueOrganisation for Research and Treatment of Cancer EORTC). À 5 ans, le taux de survie est inférieur à 5%.

La récidive survient en moyenne entre 6 à 10 mois après la chirurgie initiale, de par le caractère très invasif de la tumeur, associé au fait que les cellules tumorales ont souvent acquis des résistances à la radiothérapie et/ou à la chimiothérapie.

1.4.1.4) Oncogènes dans la gliomagenèse

1.4.1.4.1) Origine des glioblastomes multiformes et altérations génétiques

La plupart des GBM (90%) se développent *de novo*, alors que les 10% restants correspondent à l'évolution d'un gliome de bas grade [182].

Comme l'illustre la Figure 26, les voies de transformations tumorales se caractérisent par de nombreuses altérations moléculaires, parmi lesquelles la perte du chromosome 10q, l'amplification du gène codant pour EGFR, l'amplification du gène PDGF-β, l'inactivation de CDKN2A/p16 sur le chromosome 9p, l'inactivation de PTEN et l'activation de Ras/Akt [182, 183].

L'origine cellulaire des GBM reste aujourd'hui incertaine. Plusieurs hypothèses ont été avancées mais aucune d'entre-elles n'identifie avec certitude les cellules souches neurales (NSC), les progéniteurs d'astrocytes (APC) ou encore les cellules différenciées comme seule cellule à l'origine des GBM (Figure 26). Différents cas sont possibles : soit plusieurs cellules distinctes évoluent respectivement en GBM, soit différentes altérations génétiques d'un seul type de cellule mènent aux différentes manifestations pathologiques, ou encore les deux à la fois [184].

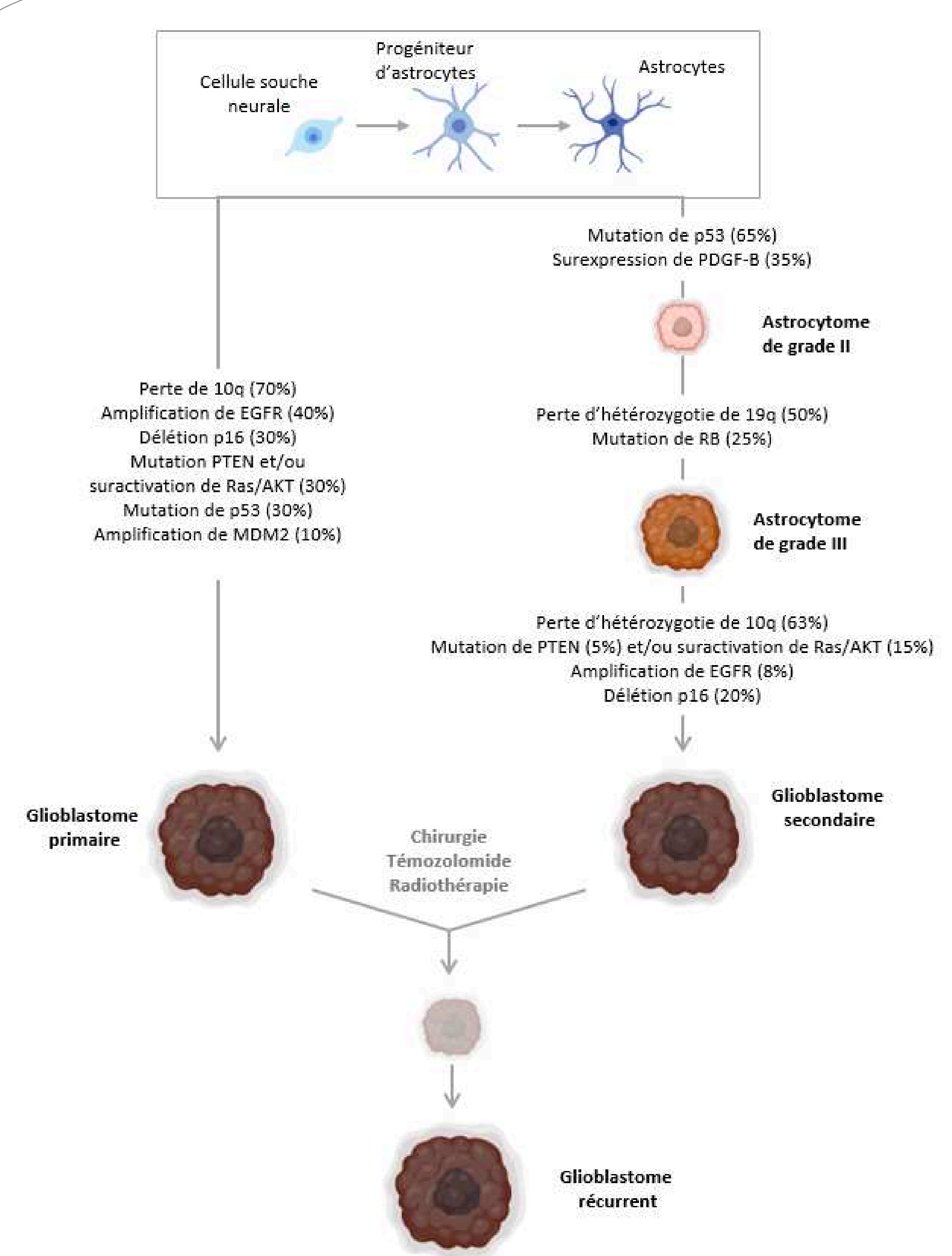

Figure 26 : Origines génétiques des glioblastomes

Pour se former, les glioblastomes multiformes passent par plusieurs altérations génétiques. Adapté de [183, 184]

La mise en évidence de certaines altérations génétiques énoncées ci-dessus et la considération d'une origine cellulaire plutôt qu'une autre a été à l'origine du développement de modèles de gliomagenèse induite. En effet, les travaux du laboratoire de EC Hollande montrent, au sein de souris transgéniques dite RCAS (replication-competent ASLV), que la double surexpression de Ras et Akt induit la formation de GBM [181].

1.4.1.5) Classification moléculaire des GBM hors considérations épigénétiques

Favorisées par le développement des outils de séquençage, plusieurs classifications moléculaires des GBM ont été proposées. Parmi ces classifications, celle de Verhaak les classe en quatre sous-types : proneural, neural, classique et mésenchymal [185] (Figure 27).

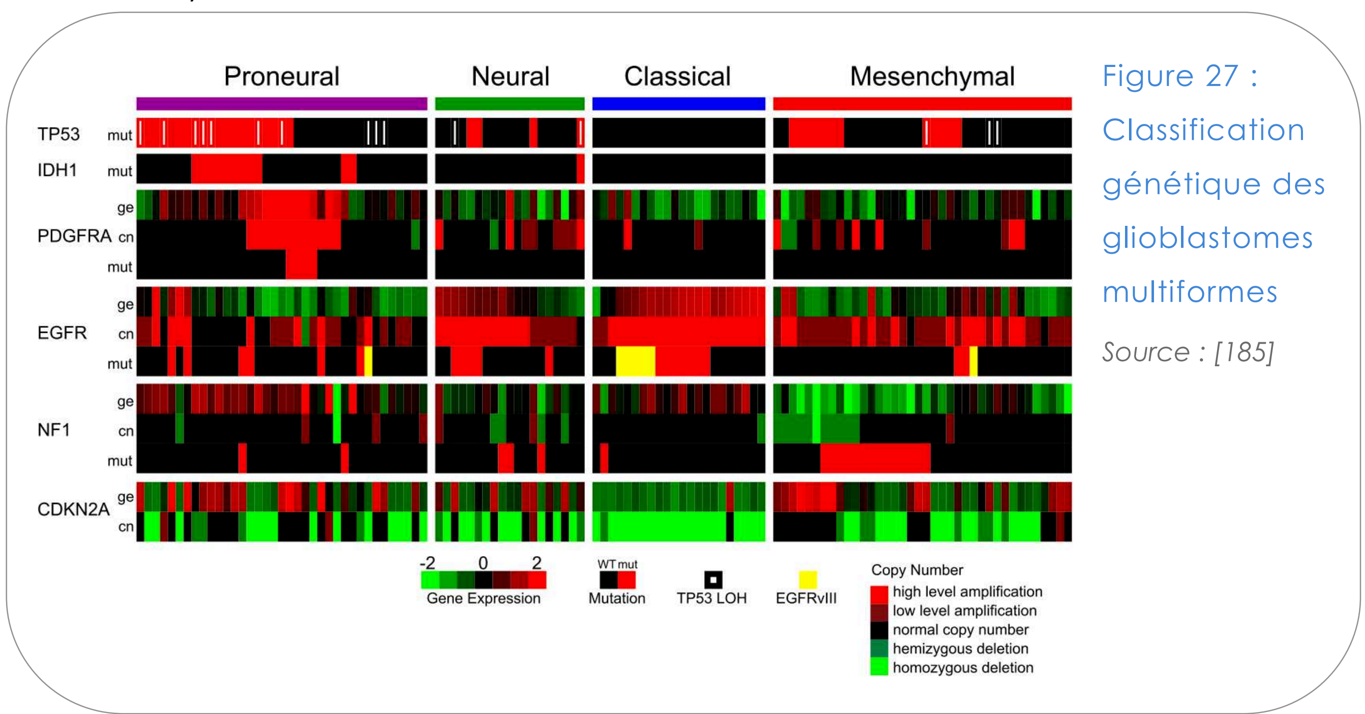

Figure 27 : Classification génétique des glioblastomes multiformes

Source : [185]

Une autre classification propose de stratifier les GBM en se basant sur la mutation ou non d'IDH [186] (Figure 28).

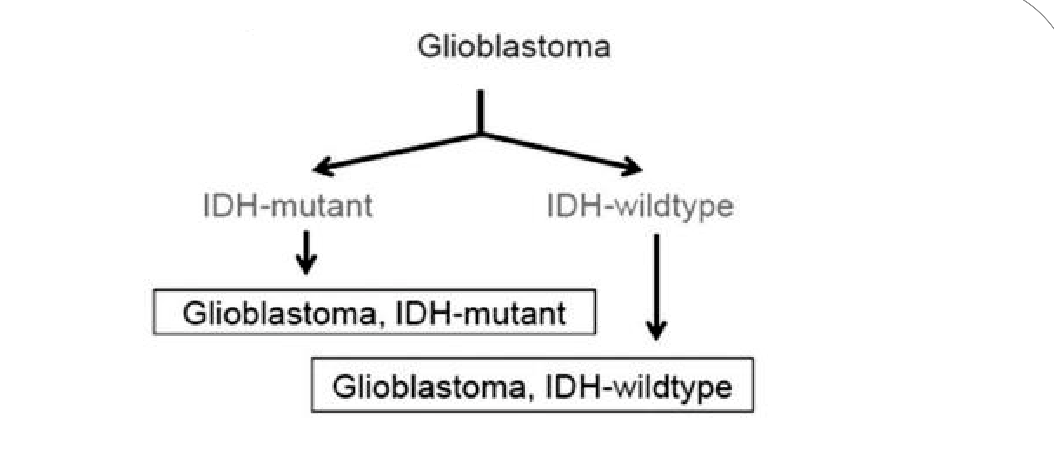

Figure 28 : Stratification des patients atteints de GBM en fonction de la mutation IDH

Source : [186]

1.4.1.6) Méthylation de l'ADN et glioblastome multiforme

Comme nous le verrons dans les prochaines parties, la méthylation de l'ADN peut avoir un lien avec le glioblastome multiforme dans plusieurs cas. Tout d'abord, ses modifications sont impliquées dans la glioblastomagenèse, mais peuvent aussi être utilisées comme marqueur de la survie et/ou de la réponse au traitement. Enfin, la méthylation de l'ADN et ses modifications dans le GBM pourraient être utilisées comme outils de stratification des patients, afin de proposer une thérapie aussi personnalisée et adaptée que possible.

1.4.1.6.1) Méthylation de l'ADN comme causes à la glioblastomagenèse

Dans un premier temps, la rupture induite par un peptide synthétique du complexe de maintien de la méthylation DNMT1/UHRF1/PCNA a été démontré comme induisant la gliomagenèse [59].

De plus, dans un article de cette thèse (Article 1) nous avons démontré la surexpression d'AKT couplée à l'exposition au diuron, un herbicide, induisait une hypométhylation globale de l'ADN et le développement de GBM.

1.4.1.6.2) Méthylation de l'ADN comme biomarqueur de survie ou de pronostic à une thérapie donnée

Comme de très nombreuses tumeurs, les GBM se caractérisent par une hypométhylation globale de l'ADN [187]. De plus, il a été montré que l'hypométhylation globale de l'ADN était liée à une plus grande agressivité de la tumeur, cet élément pourrait donc être pris en compte dans l'estimation de l'agressivité tumorale [59].

Les travaux d'Esteller et de Hegi montrent que l'état de méthylation du gène MGMT (O-6-Methylguanine-DNA Methyltransferase) est pronostique de la réponse au traitement standard du GBM : les patients dont la tumeur possède un génome méthylé au niveau du gène MGMT répondent favorablement au traitement standard du GBM [188, 189].

1.4.1.6.3) Méthylation de l'ADN comme outil d'aide à la stratification des patients afin d'affiner le prognostic de survie ou la prescription thérapeutique

Trois cas : Prise en compte de l'hypométhylation globale et locale de MGMT

Comme décrit précédemment, la méthylation de MGMT est pronostique de la réponse au traitement. Sur la base de ce constat, les patients atteints de GBM pourraient être subdivisés en deux sous-groupes : MGMT méthylé et MGMT non-méthylé.

De plus, une piste thérapeutique intéressante est de reméthyler MGMT chez les patients du groupe « MGMT non-méthylé ». Notre laboratoire a démontré que cela était possible via l'utilisation de l'acide folique et que cela était couplé à un gain de sensibilité à l'induction de l'apoptose induite par le traitement standard du GBM et à une diminution de la croissance tumorale [190].

La méthylation de *MGMT* pourrait donc être utilisée comme outil de stratification et comme levier thérapeutique. À partir de ces éléments, un essai clinique de phase I nommé FOLAGLI a été mis en place à l'Institut de Cancérologie de l'Ouest (ICO) (NCT01700569). Parmi les objectifs de cet essai clinique figure l'analyse du niveau de méthylation de MGMT dans l'ADN tumoral circulant des patients présentant le gène MGMT non-méthylé au moment de leur inclusion et la corrélation avec la survie sans progression de ces patients.

Bien qu’étant toujours en cours, cette étude montre déjà que la méthylation du gène MGMT augmente suite au traitement avec le folate (Figure 29).

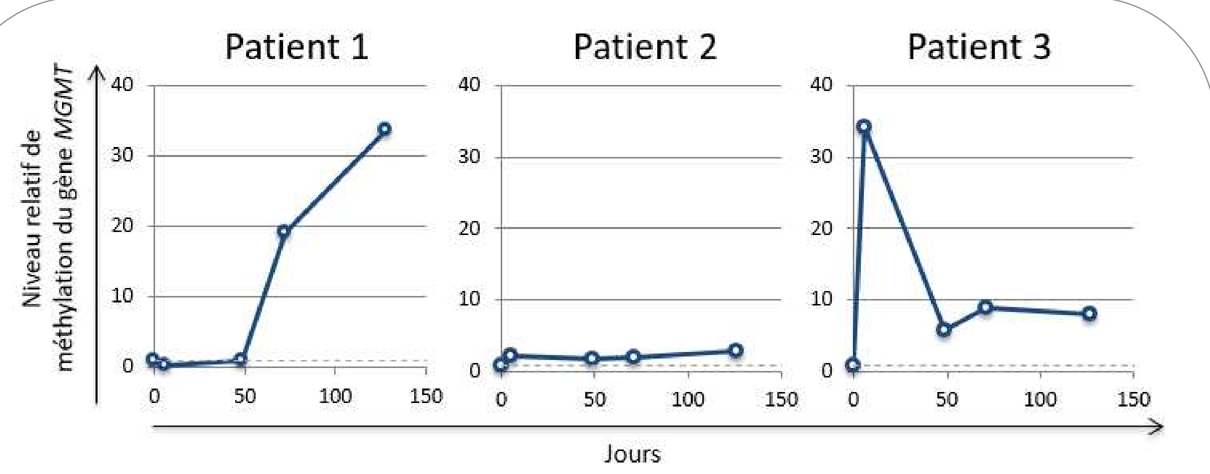

Figure 29 : Résultats préliminaires de l'essai clinique FOLAGLI

Ces graphes présentent le niveau de méthylation du promoteur de MGMT chez trois patients ayant reçu de l’acide folique en plus du traitement standard du GBM. Ces résultats montrent les différents profils de méthylation obtenus. Données non publiées.

Cas de la stratification des patients sur la base du G-CIMP

De la même manière que pour la détection des anomalies génétiques, le développement technologique a aussi permis l’émergence d’une classification basée sur l’analyse de la méthylation de l’ADN. En 2010, Noushmehr *et al.* décrivent l’existence de la signature dite « glioma-CpG island methylator phenotype (G-CIMP) », définie comme étant l’hyperméthylation spécifique des CpG islands d’un sous-ensemble de gènes dans le GBM [191]. En 2012, Turcan *et al.* montreront que la présence de G-CIMP est associée à la présence de mutation IDH1 [192].

Basé sur la prise en considération de plusieurs classifications moléculaires des GBM, Malta *et al.* proposent une nouvelle classification des GBM [193] (Figure 30).

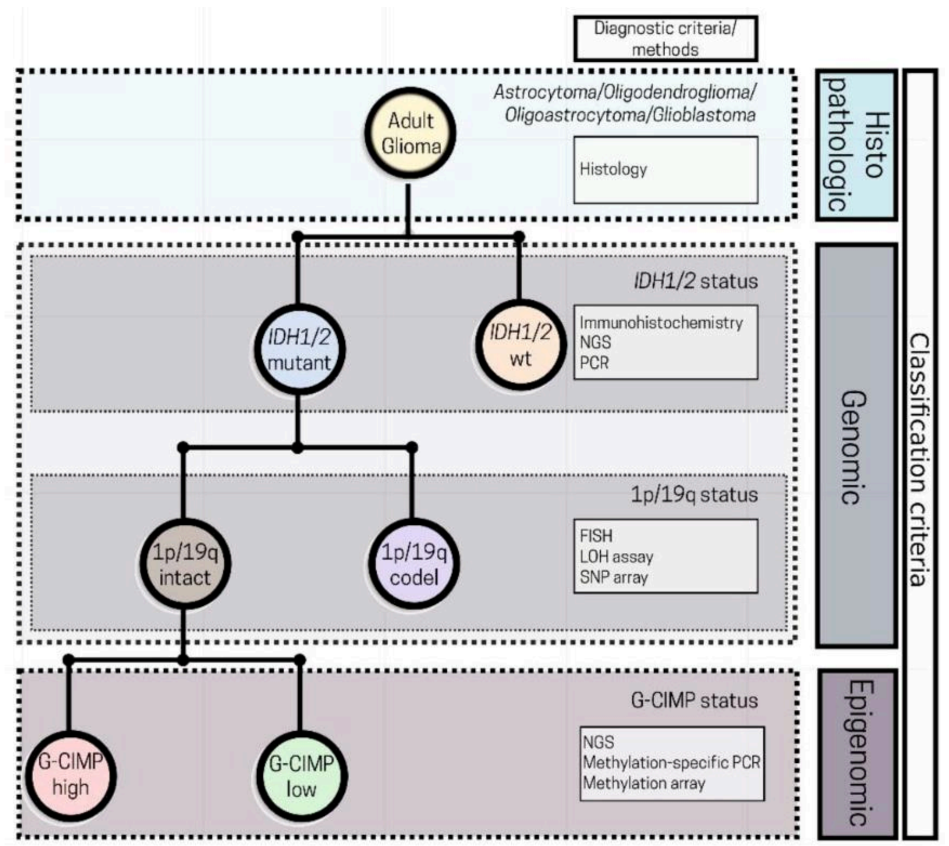

Figure 30 : Hiérarchie des glioblastomes multiformes en fonction des mutations

Source : [193]

Toutefois, malgré ces stratifications de plus en plus précises, le schéma de choix pour traiter les GBM n'a pas changé et équivaut toujours à celui redéfini par Wick *et al.* [194] (Figure 31).

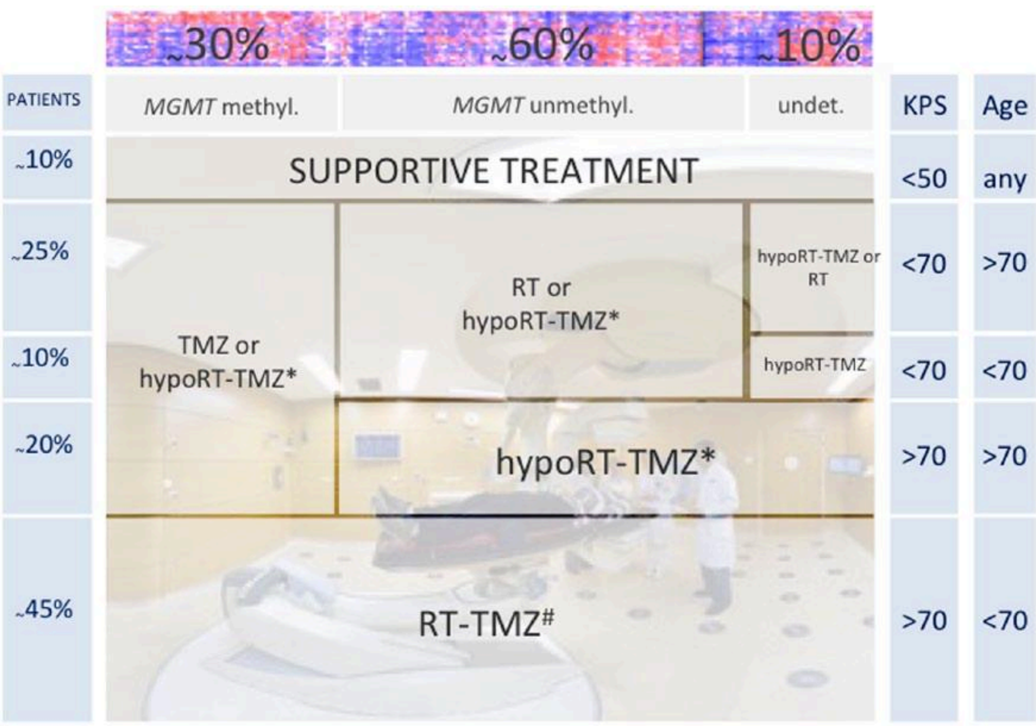

Figure 31 : Stratification des GBM et traitement

Quelle que soit la stratification, le traitement du GBM ne varie que peu. Source : [194]

Cas des interactions DNMT3A/ISGF3γ

Dans le cas des complexes DNMT3A/ISGF3γ (Interferon-Stimulated Transcription Factor 3, Gamma), ce n'est pas directement la méthylation de l'ADN qui est utilisée pour stratifier des patients, mais l'interaction d'une DNMT avec un facteur de transcription. L'article de Cheray *et al.* ne tient pas compte de la méthylation globale des tumeurs, mais du nombre de complexes présents dans la tumeur et le corrèle avec une augmentation de l'agressivité tumorale. De plus, l'utilisation d'un peptide capable de casser cette interaction augmente la sensibilité au traitement TMZ/IR (témozolomide/irradiation) [195].

1.4.1.7) miRNA et glioblastome multiforme

Comme pour de nombreux autres types de cancers, plusieurs miRNA ont été décrits comme jouant un rôle clé dans la glioblastomagenèse, à travers leur fonction oncogénique ou tumeur suppressive. En effet, un miR peut être qualifié de pro-tumoral s'il induit la

transformation tumorale d'une cellule saine ou s'il promeut l'acquisition ou le renforcement d'un phénotype pro-tumoral.

Ainsi, la surexpression des miR-132 et -10b promeuvent la prolifération et l'agressivité des GBM [196, 197].

Le miR-504 quant à lui aurait un rôle suppresseur de tumeur en supprimant la signature moléculaire dit mésenchymateux sous-type de GBM le plus agressif, alors que le miR-370 augmenterait la réponse au traitement en ciblant MGMT [198, 199].

D'autres travaux ont essayé d'élargir le panel de miRNA, afin de dégager des signatures d'expression. Ainsi, les travaux de Matos *et al.* suggèrent qu'il est possible de différencier les GBM primaires des GBM récurrents sur la base du différentiel de niveau d'expression d'un ensemble de miRNA (miR-7, miR-9, miR-21, miR-26b, mirR-124a, miR-199a et let-7f) [200].

Les travaux de Li *et al.* et de Marzziali *et al.* indiquent que les niveaux d'expression de groupes de miRNA permettent de pronostiquer la survie de patients atteint de GBM déjà catégorisés en fonction de la classification de Verhaak [201, 202].

Afin de caractériser au mieux les tumeurs, s'intéresser à des signatures plutôt qu'à des facteurs isolés semble être un axe prometteur.

1.4.1.8) Mutations des acteurs de méthylation et de déméthylation de l'ADN et de la machinerie des miRNA

La fonctionnalité des acteurs de la méthylation/déméthylation de l'ADN et de la machinerie de biogenèse des miRNA peut être affectée par des mutations, d'où leur recherche en cancérologie, et ici plus particulièrement dans les GBM. Il est aussi à noter que ces mutations peuvent avoir un rôle dans les maladies génétiques telles que l'ICF syndrome [183].

L'utilisation de la banque de donnée GRCh38 · COSMIC v90 montre que les GBM présentent peu de mutations des acteurs de la méthylation/déméthylation de l'ADN et de la machinerie de biogenèse des miRNA, en comparaison avec d'autres mutations

caractéristiques des GBM comme les mutations de p53 (30%) ou celle de PTEN (30%) (Figure 32).

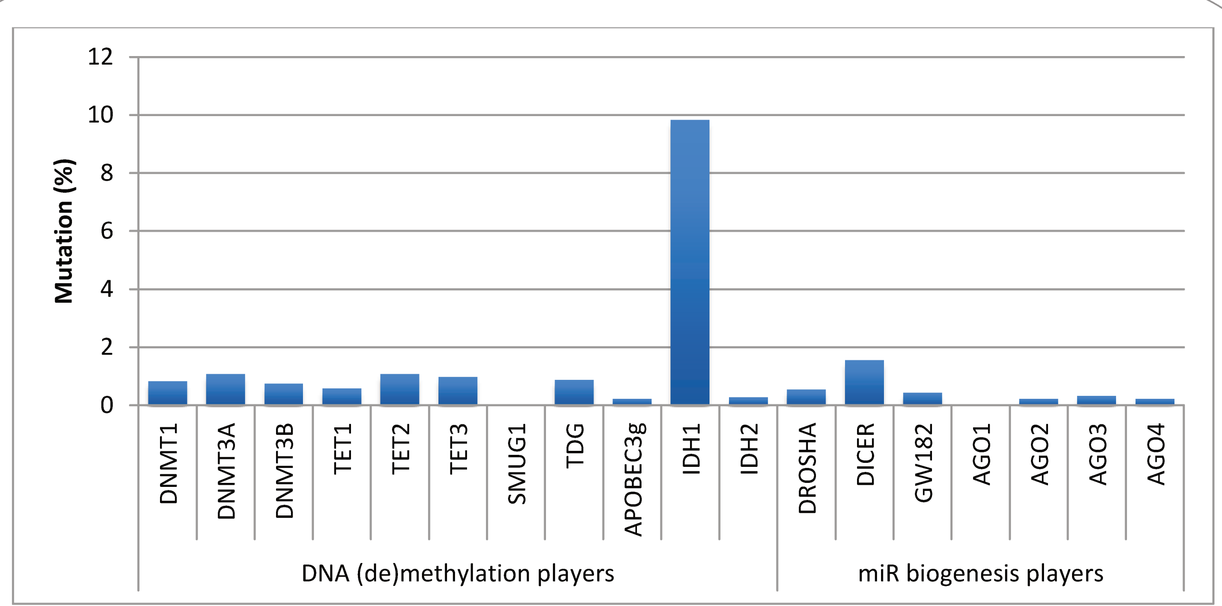

Figure 32 : Mutations des acteurs de la méthylation/déméthylation de l'ADN et de la biosynthèse des miRNA dans le glioblastome multiforme

Le graphique représente le pourcentage de mutations retrouvées pour différents acteurs épigénétiques dans le GBM, d'après la base de données GRCh38 · COSMIC v90.

Comme le montre le tableau de la Figure 33, certains acteurs de la méthylation/déméthylation de l'ADN et de la machinerie de biogenèse des miRNA sont mutés au sein de différents cancers ou pathologies.

	Protéine	Type de mutation	Pathologie	Référence
Acteurs de la méthylation Déméthylation de l'ADN	DNMT1	Perte de fonction	Cancer colorectal	[203]
		Faux sens	Cancer du sein	[204]
	DNMT3A	Perte de fonction	Tumeurs myéloïdes	[205]
		Perte de fonction	Leucémies	[206]
		Gain de fonction	Paragangliome	[207]
	TET2	Perte de fonction	Leucémie myéloïde aigüe, syndrome myélodysplasique	[208]
		Faux sens, de bénigne à possiblement dommageable	Gliomes	[209]
Acteurs de la biogenèse des miRNA	DICER	Décalage du cadre de lecture ou non sens	Blastome pleuropulmonaire	[210]
	Drosha	Perte de fonction	Tumeur de Wilms	[211]
	Dicer et Drosha	Perte de fonction		[212]

Figure 33 : Exemple de mutations de certains acteurs de la méthylation/déméthylation de l'ADN et de la biogenèse des miRNA dans différents cancers

1.4.2) Causes d'échappement à la thérapie

Une tumeur évolue au cours du traitement et des phénomènes de résistance apparaissent, induisant une rechute chez le patient. Deux mécanismes sont soupçonnés : premièrement, le traitement induirait des modifications génétiques et/ou épigénétiques, entrainant l'acquisition d'un phénotype résistant au traitement. Ces cellules seraient alors plus aptes à survivre et seraient sélectionnées selon un mécanisme Darwinien : cela correspond à la résistance acquise. Le deuxième mécanisme est celui de la résistance innée : dans la tumeur il existe une hétérogénéité tumorale, et parmi les cellules certaines sont intrinsèquement résistantes au traitement, par exemple grâce à l'expression des enzymes de réparation de l'ADN qui diminuent la toxicité de la chimiothérapie, ou encore grâce à l'expression des enzymes de réparation de l'ADN ou encore à un défaut intrinsèque du programme apoptotique [213]. Ces deux mécanismes ne s'excluent pas et peuvent être complémentaires [214].

1.4.3) Causes d'échappement au système immunitaire

1.4.3.1) Le système immunitaire : grands principes

Le système immunitaire représente les moyens de défense de l'organisme envers les organismes pathogènes du non-soi, mais aussi envers les cellules du soi présentant un dysfonctionnement.

Ce système est séparé en deux parties : l'immunité innée, qui grâce à sa réponse rapide est la première ligne de défense de l'organisme, et l'immunité adaptative, dont la réponse nécessite la présentation des antigènes pour activer les cellules immunitaires et induire une réponse (Figure 34).

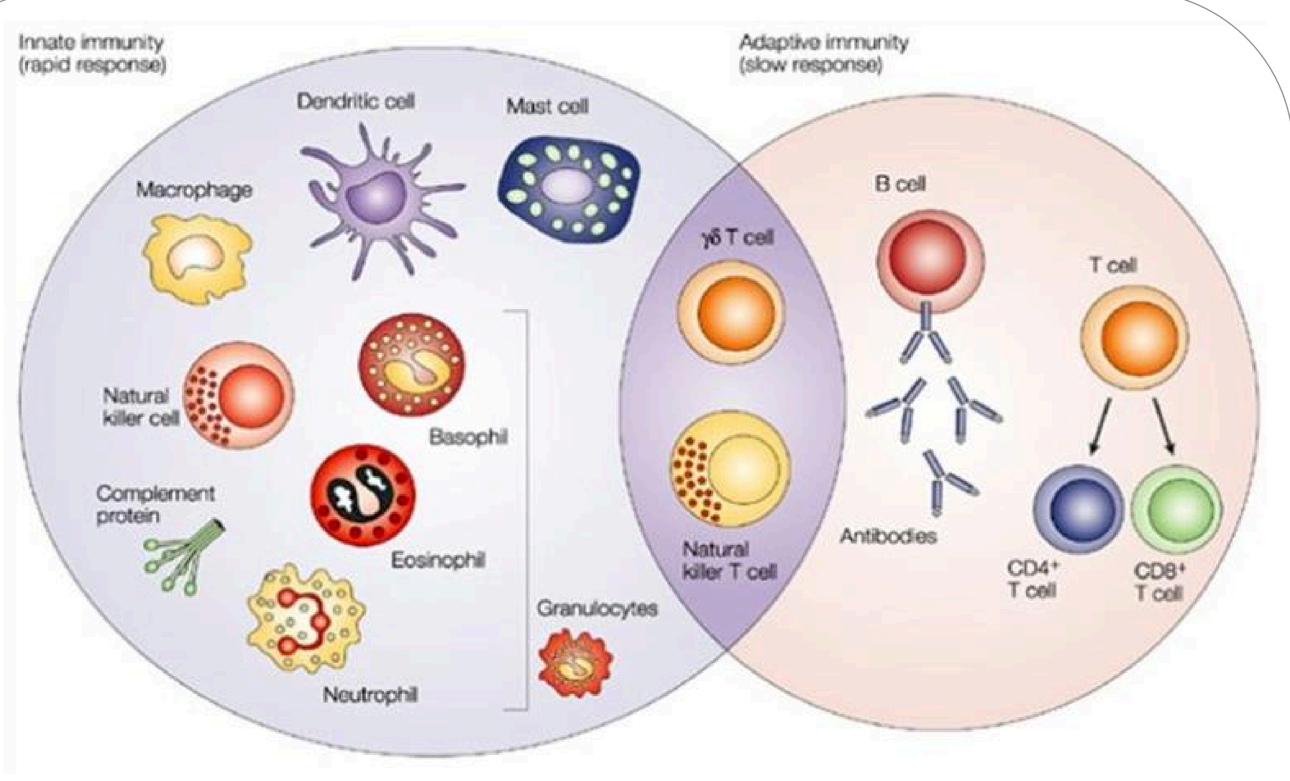

Figure 34 : Vue globale du système immunitaire

Source : [215]

1.4.3.2) Système immunitaire et cancer

Depuis 2000 et la caractérisation des « hallmarks cancer » par Hanahan et Weinberg, l'échappement au système immunitaire des cellules cancéreuses est reconnu comme une caractéristique intrinsèque des cellules tumorales [216]. Si les cellules tumorales sont capables d'échapper au système immunitaire, cela sous-entend que celui-ci a une fonction d'immunosurveillance anti-tumorale en détectant et détruisant les cellules cancéreuses.

Dans l'immuno-editing des tumeurs, qui correspond à la modification de l'immunogénicité des cellules tumorales, trois phases d'interaction entre cellules cancéreuses et immunitaires sont décrites [217, 218] :

- l'élimination, durant laquelle le système immunitaire est opérationnel : il détecte et élimine les cellules cancéreuses

- l'équilibre, où le système immunitaire ne parvient plus à éliminer toutes les cellules tumorales mais où elle ne sont pas encore en nombre suffisant pour induire des symptômes cliniques
- l'échappement, au cours duquel l'immunosurveillance est déficiente et où les cellules tumorales prennent le pas sur le système immunitaire.

Deux processus majeurs d'échappement tumoral ont été décrits : l'immunosubversion, où les cellules du système immunitaire subissent des modifications conduisant à l'inhibition de leurs propriétés anti-tumorales ; et l'immunosélection, dans laquelle les cellules tumorales présentent et/ou acquièrent des modifications empêchant leur reconnaissance par les cellules du système immunitaire. [219]

Sur la base des orientations des travaux de recherche prises durant ma thèse, seules les cellules Natural Killer (NK) et les cellules dendritiques plasmacytoïdes (pDC) seront présentées ici.

1.4.3.3) Les cellules Natural Killer

1.4.3.3.1) Généralités

Les NK sont des cellules appartenant à l'immunité innée qui peuvent tuer des cellules tumorales ou infectées. De plus, elles stimulent la réponse des lymphocytes T (LT) et des lymphocytes B (LB). L'activité cytotoxique des NK est médiée par le granzyme B qui est une protéase induisant l'apoptose de la cellule cible, et la perforine qui forme un canal au travers de la membrane plasmique de la cellule cible.

Dans le cancer, les cellules tumorales ont développé des stratégies pour échapper au système immunitaire, que ce soit en empêchant ce dernier de les reconnaître ou bien en diminuant directement sa cytotoxicité. Par exemple, une forte concentration en TGFβ dans le microenvironnement tumoral inhibe l'action des NK [220].

1.4.3.3.2) Régulation épigénétique de l'activité cytotoxique anti-tumorale des cellules NK

Plusieurs études montrent que l'activité cytotoxique anti-tumorale des cellules NK est épigénétiquement régulée. Les travaux de Shi *et al.* (2016) montrent que l'expression de

NKG2D (natural-killer group 2, member D) est régulée par le niveau (i) de méthylation de l'ADN et (ii) celui de H3K9me2 au niveau du promoteur du gène, et que cela influence l'activité cytotoxique anti-tumorale des cellules NK [221]. Dans ce cas, on peut donc parler de perte d'immunosurveillance par immunosubversion épigénétique.

Les travaux de Wiencke *et al.* montrent que le méthylome des NK varie en fonction du degré d'activation de ces cellules, dans le sens d'une diminution du nombre de CpG méthylés [222]. De plus, cette même étude a mis en évidence l'idée que la déméthylation du gène codant pour le facteur de transcription BHLHE40 (Basic Helix-Loop-Helix Family Member E40) pourrait être utilisée comme biomarqueur de l'activation des NK.

1.4.3.3.1) Régulation de l'activité cytotoxique anti-tumorale des cellules NK par les microARN

Les travaux de Su *et al.* montrent l'existence d'une corrélation positive entre le niveau de cytotoxicité des cellules NK et le niveau d'expression du miR-506 dans le cadre des carcinomes hépatiques [223]. Un autre article montre que l'expression du miR-150 minimise la cytotoxicité des cellules NK en limitant l'expression de la Perforin-1 [224]. De plus, le miR-146a régule négativement la fonctionnalité des cellules NK [225]. Ici, on pourrait parler de perte d'immunosurveillance par immunosubvertion médiée par les miR.

Le miR-20a quant à lui diminue l'expression de MICA (Major Histocompatibility Complex Class I Chain-Related Protein A) au sein de cellules colorectales tumorales, ce qui entraîne l'absence de leur reconnaissance par les NK [226]. Dans ce dernier cas, on parle donc de perte d'immunosurveillance par immunosélection épigénétique.

1.4.3.4) Les cellules plasmacytoïdes dendritiques

1.4.3.4.1) Généralités

Les pDC sont des cellules issues d'un progéniteur de cellule dendritique commun avec les cellules dendritiques myéloïdes et proviennent de la moelle osseuse [227]. Elles représentent moins de 0,4% des cellules mononucléées périphériques du sang (PBMC) présentes dans le sang et les organes lymphoïdes périphériques, et sont capables de sécréter de l'interféron de type 1 [228]. Souvent décrites comme le lien entre l'immunité adaptative et l'immunité innée, elles sont capables d'activer d'autres cellules immunitaires

de par leur aptitude à présenter des antigènes [229]. Dans le cancer, lorsqu'elles n'induisent plus la mort des cellules tumorales elles peuvent promouvoir l'établissement d'un microenvironnement immunosuppressif [230].

Même si elles ne sont pas dans la première ligne de la destruction des cellules tumorales, les pDC restent des acteurs importants du système immunitaire à ne pas négliger, notamment de par leur rôle de « couteau suisse » du système immunitaire. En effet, ces cellules ont un rôle dans la présentation d'antigènes, la production de cytokines, la tolérance et la cytotoxicité [231].

1.4.3.4.2) Régulation épigénétique et miR-médiée de l'activité des pDC

Ma *et al.* montrent que CXXC5 (CXXC-Type Zinc Finger Protein 5) interagit avec TET2 pour réguler les niveau de méthylation et d'expression d'IRF7 (Interferon Regulatory Factor 7) qui va ensuite réguler le niveau d'expression d'interféron (IFN) [232]. Ici aussi, on peut parler de perte d'immunosurveillance par immunosubversion épigénétique.

De plus, le miR-146a est décrit comme régulant la survie et la maturation des pDC [233]. Les travaux de Rossato et al (2017) montrent que la surexpression du miR-618 limite la formation des pDC à partir de cellules hématopoïétiques progénitrices CD34+ [234]. Ici, c'est le phénomène d'immunosélection épigénétique qui est impliqué.

1.5) Inégaux face aux risques : les prédispositions au cancer

S'il est urgent de trouver de nouvelles manières de traiter les patients atteints de cancers, la première étape réside dans la prévention et consiste tout de même à limiter le nombre de patients atteints, ou tout du moins à faciliter un diagnostic précoce en identifiant des facteurs de risque. En effet, en sachant quels patients sont dits "à risque" car ayant été exposés à une quelconque substance environnementale ou possédant une caractéristique spécifique (comme une mutation par exemple), leur surveillance accrue sera facilitée et ils pourront certainement, au vu de l'importance de la précocité de détection de la pathologie, bénéficier d'une meilleure survie.

1.5.1) Le paysage génétique

Dans différents types de cancers, certaines mutations ont été décrites comme impliquées dans la tumorogénèse, leur présence augmente donc le risque de développer un cancer. Les plus connues sont BRCA1 et BRCA2 dans le cancer du sein et de l'ovaire [235–237]. A été également décrit l'implication des mutations d'une des enzymes du système de réparation de l'ADN MisMatch Repair (MMR) ou de l'epithelial cell adhesion molecule (EPCAM) dans le syndrome Hereditary Non Polyposis Colorectal Cancer (HNPCC, ou syndrome de Lynch) [238, 239]. Toujours dans les cancers colorectaux, une séquence «adénome-carcinome » a été décrite et correspond à l'enchainement d'évènements conduisant au développement d'un cancer colorectal. Dans ce cas-là, des mutations successives d'APC (Adenomatous Polyposis Coli Protein), KRAS (Kirsten rat sarcoma viral proto-oncogene) et p53, couplées à des mutations de INK4A (CDKI2A, Cyclin Dependent Kinase Inhibitor 2A), SMAD4 (Mothers against decapentaplegic homolog 4), E-cadhérine, MLH1 (DNA Mismatch Repair Protein Mlh1) et MSH2 (DNA Mismatch Repair Protein Msh2) vont finalement induire le développement d'un adénocarcinome colorectal [240]. De nombreuses autres prédispositions génétiques ont été décrites, telles que RB1 (retinoblastoma 1) dans le rétinoblastome [241].

La détection précoce de ces facteurs permet d'anticiper la survenue d'un cancer, notamment par des examens cliniques réguliers tels que la mammographie ou la coloscopie.

1.5.2) Utilisation de modifications épigénétiques comme biomarqueurs de risques

Plusieurs facteurs épigénétiques ont été décrits comme impliqués dans le cancer, mais permettent-ils de prévoir et/ou de détecter précocement le développement d'une tumeur ? En effet, il semble techniquement compliqué de monitorer la méthylation globale de l'ADN dans l'ensemble des organes, et même si cela était possible la détection de l'hypométhylation serait trop tardive. Il est donc nécessaire de trouver de nouveaux biomarqueurs épigénétiques détectables à partir de biopsies liquides, moins invasives.

Dans le GBM, nous avons démontré que l'exposition à un herbicide, le diuron, entraîne la déméthylation simultanée de PD-L1 (programmed death ligand 1), Bcl-w (Bcl-2-like protein 2) et LLT1 (CLEC2D, C-type lectin domain family 2 member D), la modification épigénétique de ces trois gène pourrait représenter une signature moléculaire et être facilement suivie dans le sang, permettant (i) une prise en charge rapide et (ii) une meilleure évaluation du pronostic (article 1).

Dans le cancer du sein cette fois, nous avons démontré chez des patientes que l'exposition à un autre pesticide, le glyphosate, couplée à la surexpression du miR-182-5p était de mauvais pronostic et augmentait le risque d'apparition d'un cancer du sein [242]. L'idée générale ici est de mettre au point des tests simples permettant, à partir du sang, d'anticiper l'apparition d'un cancer du sein et donc une meilleure prise en charge de par sa précocité : cela correspond à l'étude de biomarqueurs circulants.

Le profil épigénétique étant largement influencée par l'environnement, nous nous sommes intéressés aux modifications épigénétiques induites par l'environnement, comme l'exposition à des polluants.

1.6) Stratification des patients : du pronostic de la survie à la médecine personnalisée

1.6.1) Facteurs pronostiques initiaux liés à la pathologie

1.6.1.1) Caractéristiques anatomopathologiques

Une fois le cancer développé, et afin d'améliorer la lutte, il est important de bien identifier la tumeur, afin de personnaliser au mieux le traitement pour maximiser son effet chez le patient. Le cancer étant une maladie multifactorielle, chaque patient a ses propres spécificités, même si la pathologie est commune. Le principal facteur pronostic est le stade de la tumeur : quatre stades existent, avec des sous classes dans ces stades. La stratification des patients par stade se fait par la classification clinique dite TNM, qui évalue la tumeur primitive (T), le nombre de ganglions lymphatiques atteints (N) et les métastases à distance (M). Dans les tumeurs du système nerveux central, la classification est différente : en effet, les tumeurs nerveuses ne métastasent quasiment pas aux autres organes et il n'y a pas de

ganglions lymphatiques dans le cerveau. La classification se fait tout de même en quatre grades, selon la capacité proliférative des cellules et la délimitation de la tumeur.

1.6.1.2) Mutations génétiques

Les mutations des gènes de la famille RAS sont retrouvées couramment dans les cancers colorectaux [243, 244]. Au-delà de leur implication dans le développement tumoral, certaines mutations sont connues pour être liées à un mauvais pronostic pour la survie du patient. C'est le cas de SMAD4 dans le cancer du pancréas [245], ou bien de RB1 dans le cancer de la prostate [246]. La mutation activatrice d'EGFR (epidermal growth factor receptor) dans le cancer du poumon est quant à elle de bon pronostic, car les inhibiteurs de tyrosine kinases sont plus efficaces lorsque EGFR est muté [247]. Certaines mutations sont à l'interface entre la génétique et l'épigénétique : c'est le cas des mutations de gènes codant pour des protéines directement impliquées dans les régulations épigénétiques au sens large. Par exemple, la mutation de TET2, impliquée dans la déméthylation de l'ADN, est bien décrite dans la leucémie aigüe myéloïde [248, 249] mais aussi dans d'autres types de cancers myéloïdes [208]. De la même manière, KMT2D, une histone méthyltransférase, a été décrite comme étant de mauvais pronostic dans le cancer du poumon non à petites cellules [250]. Ces mutations d'enzymes épigénétiques ont un impact sur leur activité et/ou leur régulation, jouant un rôle dans la progression tumorale.

1.6.1.3) Altérations épigénétiques

Dans le GBM, un lien a été montré entre diminution globale de la méthylation et augmentation de l’agressivité [190].

Comme décrit précédemment, toujours dans le GBM, la méthylation de MGMT est un facteur de bon pronostic, puisque la méthylation du promoteur est corrélée à la non-expression de la protéine et donc à une incapacité à réparer l'ADN, permettant l'induction de l'apoptose suite aux dommages causés par le TMZ [251].

1.6.2) Facteurs pronostiques initiaux liés au malade

D'autres facteurs pronostiques liés directement au malade sont aussi à prendre en compte.

Premièrement, l'âge du patient : pour le GBM, plus le patient est âgé, plus le pronostic de réponse au traitement, et *in fine* de rémission, seront faibles.

Directement lié à son âge, son état de santé général entre aussi en jeu. En effet, un patient en bon état général supportera mieux les traitements. L'état général inclus également le mode de vie du patient : est-il tabagique ou non, a-t-il une activité physique régulière…

Pour évaluer la survie d'un patient, il faut donc tenir compte de ces différents types de facteurs. Cependant, il est important de trouver des méthodes de stratification plus précises et plus efficaces, pour mieux prévoir la survie et adapter le traitement.

1.7) Épimarques circulantes

1.7.1) Épimarques circulantes libres : revue « Cell-free circulating epimarks in cancer monitoring: a systematic review »

Afin de définir les épimarques circulantes (niveau de méthylation de l'ADN, nucléosomes et ARN non codants) et de décrire leur implication dans le cancer, l'introduction de ce chapitre sera basée sur une revue écrite en collaboration avec Manon Duforestel, doctorante au sein de l'équipe, intitulée « Cell-free circulating epimarks in cancer monitoring: a systematic review » dans laquelle nous nous sommes intéressés à un type de biomarqueurs, les épimarques circulantes.

Une tumeur évolue au cours du traitement et des phénomènes de résistance apparaissent, induisant une rechute chez le patient. Cependant, il n'est pas possible de réaliser une biopsie après ou au cours de chaque cure de traitement. Les biomarqueurs circulants sont donc une bonne alternative à la biopsie de la tumeur. En effet, avec une simple prise de sang, il est possible de suivre la réponse au traitement et ainsi de l'adapter au cours du temps.

Cell-free circulating epimarks in cancer monitoring: a systematic review

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Running title: Epimarkers as new cancer biomarkers.

Keywords: epigenetics, biomarkers, cancer, cfc-DNA, cfc-nucleosomes, cfc-ncRNA

ABSTRACT

Cancer numbers increasing, cases heterogeneity and the drug resistance emergence have pushed scientists to search for innovative solutions for patients and epimutation can be a lead. Methylated DNA, modified nucleosomes and non-coding RNAs are found in all cells, including tumor cells. They have an intracellular role, but also an intercellular communication role, being released in extracellular environment and in different body fluids. We reviewed current literature on the use of these blood circulating epimarks in cancer monitoring. They must be considered as "real time" images of the tumor, and can be isolated without invasive methods. In the future, the real challenge lies in the development of specific, sensitive, fast and clinically applicable detection and analysis methods of epimarkers.

I- INTRODUCTION

According to the WHO (World Human Organization), cancer was responsible for an estimated 9.6 million deaths in 2018. It means that about 1 in 6 deaths is due to cancer despite progress in detection and treatment. Cancer is a complex disease, and most of the time, multifactorial. Complexity and heterogeneity of tumors cannot be accounted for by genetic mutations alone, it results from the accumulation of genetic and epigenetic disorders causing neoplasia. Even more, epigenetic alterations tend to be more frequent events than genetic mutations. Epigenetic mechanisms are heritable and reversible, and the sum of the alterations to the chromatin template collectively establish and propagate different patterns of gene expression and silencing [1]. Interest in cancer epigenetics is growing and recent findings in understanding the underlying mechanisms of carcinogenesis are promising. Currently, three types of epigenetic mechanisms are known to be involved in cancer: DNA methylation, histone marks modification and non-coding RNAs expression [2]. For the last few years, these three modifications have been widely studied for their potential as a biomarker. Biomarkers were defined in 2001 by Biomarkers Definitions Working Group as a molecule "that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." In this context the identification of circulating epimarkers in prediction, early diagnosis, prognosis and management of cancer therapy response is a real challenge.

Here, we reviewed the current literature on the use, interest and efficiency of three types of cell-free circulating epimarks in cancer monitoring: cytosine-methylated circulating tumor DNA (ct-DNA-5mC), circulating tumor nucleosome modification (ct-nucleosomes), and cell free circulating non-coding RNA (ct-ncRNA) (**Figure 1**).

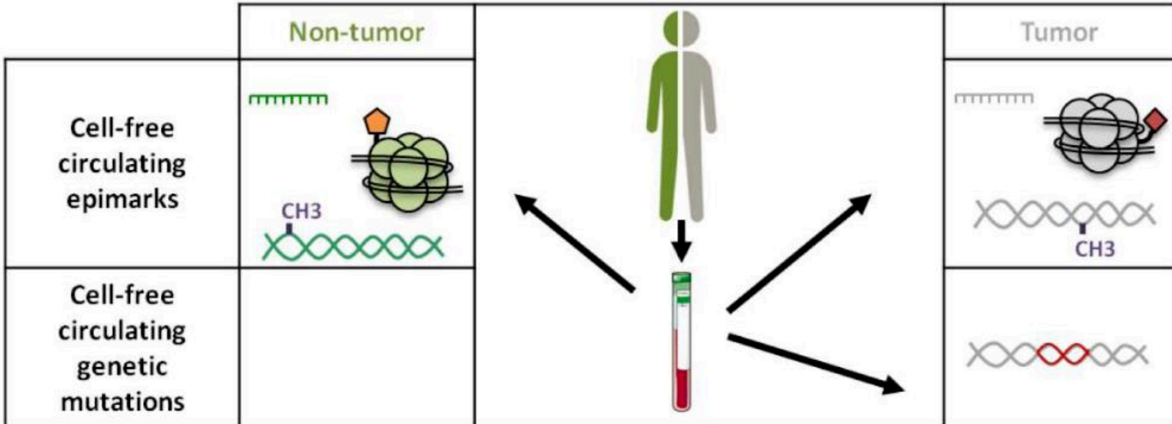

Figure 1: Cell free circulating epimarks and genetic mutations in a physiological and tumoral context.

Cell free circulating epimarks correspond to cfc-DNA, cfc-nucleosomes and cfc-ncRNA. In cancer context, these epimarks are modified and called "circulating tumoral", and ct-DNA can carry mutations.

II- CYTOSINE-METHYLATED CIRCULATING TUMOR DNA

Cytosine-methylated circulating tumor DNA (ct-DNA-5mC) is here defined as a subgroup of cell-free circulating DNA and characterized as being a cytosine-methylated DNA fragment that comes from cancerous cells.

1- Cell-free circulating DNA: the basics

The first demonstration of cell-free circulating nucleic acid in human plasma dates back to 1948, an observation made by Mandel and Metais [3]. In 1968, Barnett EV demonstrated the presence of DNA in sera and synovial fluids in normal and pathologic humans [4] and a few years later in 1973, Davis and Davis detected and quantified this cell-free DNA in the plasma of 418 individuals by counterimmunoelectrophoresis [5]. After this highlight and occasional articles, it was only in the 90's that scientists realized the potential clinical implication of cell-free circulating DNA. Since, researchers' interest in cfc-DNA is growing and nowadays many papers are in favor of its use as a diagnostic and prognostic tool in several diseases [6].

In humans, cfc-DNA is found in several circulating fluids such as blood or lymphatic fluid and other body fluids like synovial fluids, pleural fluid, saliva, urine, milk, etc... [7]. In healthy person, it intuitively seems that cfc-DNA can be originated from all living cells of organism by spontaneous release and by cell death process associated to the cellular homeostasis. Thus, hematopoietic cells are the largest contributors to cfc-DNA during their cell death [8], lymphocytes also seem to be responsible for regulated excretion of DNA [9,10]. Han et al. reports that cfc-DNA is mainly characterized by a size of 70-200 bp with a blood concentration of 0-100 ng/mL [11].

Many articles report that both physiological or physiopathological and (micro)environmental conditions can influence the amount of cfc-DNA such as pregnancy [12], traumatic brain injury [13] or systemic inflammation [14]. Very recently, Hummel and collaborators showed that psychosocial stress exposure leads to increased cfc-DNA as well as fragments size and their methylation pattern [15]. In cancer context, the pool of “physiological” cfc-DNA is enriched by cfc-DNA that originates from tumor cells, and which we here call circulating tumor DNA (ct-DNA).

2- Characteristics of circulating tumor DNA

Ct-DNA can represents 0.01-90% of total cfc-DNA [11,16]. Of course, several parameters influence these percentages such as size, localization, therapy sensitivity, metastasis and vascularity of the tumors [17]. Currently, it is believed that the ct-DNA originates from cancer cells eliminated by cell death processes such as apoptosis and necrosis. However, the hypothesis that living cancer cells release actively DNA is not to be excluded. Abolhassani et al. [18], Stroun et al [19] and Bronkhorst at al. [20] report the existing of a spontaneously extracellular release of DNA from two human cancer cell lines (leukemia and osteosarcoma). Despite clear evidences, circulating tumor cells could also increase the pool of ct-DNA.

Even if the release mechanism is still unclear and remains to be investigated, it is admitted that double-stranded DNA fragments in circulation are mainly small, generally 180 bp [21] or

sized in multiples of the nucleosomal DNA [22]. The size distribution is not completely homogeneous because necrosis is responsible for fragments of several kbp [20]. DNA released from cells is not “naked”, but can circulate as nucleosome or chromatosome [23]. Some studies even suggest that a non-negligible portion of ct-DNA is bound to the cell surface rather than circulating freely in the blood [24].

For about twenty years now, studies about ct-DNA have multiplied and it appears that in this tumor context the quantity of ct-DNA is higher than in physiological condition, 180 ng/ml on average [9,21,25]. Of course, the amount of ct-DNA in blood varies from one individual to another, and can be up to 1000 ng/ml. The half-life of ct-DNA is low since ranged between 16 minutes and 2.5 hours between their biogenesis and their clearance or degradation [26]. Therefore, their turnover is constant. In their study on patients with colorectal tumors, Diehl et al. estimated that for a patient with a tumor of $3x10^{10}$ neoplastic cells (ie 100g), 3.3% of the tumor DNA is daily fed into the circulation [27].

3- Tumor circulating DNA as biomarker

With a rapid and constant/continue turnover and an origin from all cancer cell clones, ct-DNA appears as an ideal “real time biomarker” informing on the heterogeneity, state [28] and size [29] of the tumor within an individual patient. However, distinguish ct-DNA derived from tumor cells and cfc-DNA derived from healthy cells has proven challenging. Differences in size of ct-DNA and cfc-DNA are defines [30]. Though, the size difference between ct-DNA and cfc-DNA can be low: 165 bp for ct-DNA derived from BRAF V600E mutant allele in melanoma patients vs. 132-145 bp for cfc-DNA derived from this wild-type allele in healthy persons [31]. The two main differentiating elements between ct-DNA and cfc-DNA are the presence in ct-DNA of a large number of cancer hallmarks including genetic mutations (such as single-base substitutions, insertions, deletions, or translocations) [28] and epigenetic modifications (such as cytosine DNA methylation).

In 2016, the first liquid biopsy test (based on the monitoring of EGFR mutation in ct-DNA) was approved by the U.S. Food and Drug Administration (FDA), allowing to identify patients with non-small cell lung cancer suitable for EGFR tyrosine kinase inhibitors (TKIs) therapy by analysis of ct-DNA [32]. Liquid biopsy represents a real alternative to the traditional biopsy which is invasive, with long lead time and not always feasible, depending on the location of the tumor and the patient state. This authorization and the emergence of studies [33–37] open concerns on management of all cancers and the use of ct-DNA detection as a prognostic, predictive and post-operative tool [38]. Moreover, a genome-wide sequencing cell-free DNA has been setted up by Cristiano et al, and they found that cancer patients has fragmented profile [39].

4- Cytosine methylated circulating tumor DNA: the new insight for cancer management

In addition to genetic alterations, ct-DNA can harbor tumoral epigenetic modifications such as aberrant cytosine methylation. Cytosine DNA methylation corresponds mainly to an addition

of a methyl group (CH3) on CpG dinucleotide cytosine, which becomes a 5-methylcytosine (5mC). Main actors of DNA methylation are DNA methyltransferases (DNMTs). Global hypomethylation, hypomethylation of DNA repeat elements (such as LINE-1), oncogene hypomethylation and hypermethylation of some gene promoter CpG islands are signatures characterizing the DNA of almost all types of cancer and that can be observed in ct-DNA [40]. These subtypes of ct-DNA is called cytosine methylated circulating tumor DNA (ct-DNA-5mC). Due to its rapid writing or erasing in response to environmental condition changes, cytosine DNA methylation of ct-DNA appears as a real-time reflect of cytosine DNA methylation changes occurring in tumors cells.

In January 2019, Gai and Sun have reported 15 studies less than 6 years old using DNA methylation-based cancer liquid biopsy [41]. The interest in circulating methylome is growing, indeed, the major benefit of large-scale epigenetic modifications is their tissue specificity and therefore their potential to inform about cancer specificities. Several teams have succeeded in identifying the origin of ct-DNA by analyzing methylation patterns [42–44]. Among them, Kang et al. (2017) developed CancerLocator, a probabilistic approach, based on CpG cluster features and Moss et al. [45] worked out a comprehensive human cell-type methylation atlas to decipher the cellular identity of ct-DNA.

A good example of the usefulness of circulating methylome as diagnostic tool is SEPT9 (septin 9) gene in colorectal cancer (CRC). The 5'-end regulatory regions of *SEPT9* gene contains a CpG island, which when is methylated can silence SEPT9 expression and may enhance cell proliferation, cell migration, angiogenesis and so, neoplastic progression [46]. SEPT9 seems to be methylated in over 90% of CRC tissues [47]. After the validation by many papers [48], in 2016 the only FDA-approved molecular blood test for colorectal cancer [49] was commercialized under the name Epi ProColon. This test is based on a real-time PCR assay to detect the hypermethylated promoter region of SEPT9 DNA in plasma [50]. More recently, studies have favorably evaluated SEPT9 as a prognostic factor and significant post-operative indicator for CRC recurrences [51,52]. Over the last years, many studies have been conducted on the CRC to identify the regions in which analysis of the changes in methylation provides the highest clinical value. These studies of ct-DNA-5mC to evaluate the tumor occurrence, progression and recurrence have spread to other types of cancers, such as hepatocellular carcinoma [53], ovarian cancer [54], breast cancer [55], prostate cancer [56], etc... But research work remains to be done to develop new molecular blood tests.

III- CIRCULATING NUCLEOSOMES AND THEIR POST-TRANSLATIONAL MODIFICATIONS

1- Histones and Nucleosomes: the basics

The nucleosome was first described in 1974 by Kornberg [57]. It represents a repetitive unit of chromatin structure and is essential for facilitating the very tight compaction of DNA into a chromosome. Concretely, DNA is complexed with histones to form nucleosomes, the first level of DNA compression [58]. The core of the nucleosome is an octamer of eight histone proteins,

with 145 base pairs (bp) of DNA wrapped around [59]. This octamer is composed of two copies of each histone: H2A, H2B, H3 and H4 to which is added the histone linker H1 to strongly maintain the 2 full turns of DNA around the octamer, this structure is called chromatosome [60]. Histones are small basic proteins containing a globular domain and a N-terminal tail rich in basic amino acid residues. These core histone tails can protube from their own nucleosome and interact with adjacent nucleosomes and other proteins. Thanks to these interactions, the architecture in "string-of-beads" will undergo another shortening and assembling into higher order three-dimensional structure to finally fit into the nucleus. The amino acids residing in histone tails may be subject to post-translational modifications (PTMs). PTMs refers to chemical events including acetylation (Ac), methylation (Me), phosphorylation (P), ubiquitylation (Ub) and sumoylation (Sumo), the most common [61]. It turns out that PTMs can occur at different residues (e.g arginine, serine, threonine, tyrosine) but mainly on lysines and, to several degrees (e.g mono-, di-, tri-) at a single residue.

The combination of all marks in a nucleosome determines outcomes. Till today, more than one hundred distinct modifications have been described [62] and are related to gene activation or silencing. As example, acetylation may have direct effects on nucleosomal architecture and influence chromatin accessibility to transcriptional machinery [63] by affecting histone-histone and histone-DNA interactions [64] while histone methylation regulates gene expression through recruiting histone-tail binding proteins [65]. Those chromatin PTMs are established from effector, also named writer proteins. The underlying DNA methylation template functions as platforms for the recruitments of readers of histone language able to entail downstream effects such as recruiting other chromatin remodeling complexes [66]. Eraser proteins have the role to remove those modifications. Histone modifications are essential for physiological process such as cellular differentiation, DNA repair [67] and probably other cellular mechanisms remaining further exploration, as maintenance of telomeric and sub-telomeric chromatin [68].

2- Histone modifications in cancer

The global function of histones PTMs is to regulate access to DNA and consequently genes expression. Thereby, deregulation of histones modification mechanisms are often involved in carcinogenesis. Upregulation, downregulation or mutation of writers proteins including histone acetyltransferases (HATs), methyltransferases (HMTs) or erasers (e.g deacetylases, demethylases) can lead to an overall alteration of the histone patterns [69]. For example, in 2005, Fraga et al. showed that a loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 are hallmarks often associated with cancer occurrence [70]. Sometimes, hypoacetylated and hypermethylated histones are associated with hypermethylated CpGs islands, an another characteristic of transformed cells [71]. Kondo and his team have reported that G9a, a well described PTMs writer, is higher expressed in hepatocellular carcinomas (HCC) than in non-cancerous liver tissues. G9a is an HMTs responsible for H3K9me1, H3K9me2, H3K27me1 and H3K27me2. It seems that the histone H3K9 methylation combined with promoter methylation on CpG is related with the silencing of P16, a tumor suppressor [72].

Gene repression mediated by G9a overexpression have also been reported in breast cancer [73], immune escape and chemoresistance of colon carcinoma [74], etc... Phosphorylation is also a crucial, but less studied, post-translational modification. Histone phosphorylation appears to play a role in DNA repair [75], transcription and chromosome segregation during mitosis [76]. Obviously, a change in the histone phosphorylation pattern may promotes tumorigenesis [77–79]. Example are numerous, and quite logically, cancer therapy is turning more and more to the use of epigenetic drugs, and the PTMs players are excellent targets. Among the privileged targets of the epidrugs are the writers and the erasers, but it can be counterproductive to inhibit a whole class of major transcription regulatior proteins.

For instance, studies of HDAC inhibitors (HDACi) have exploded this last years and many are already approved and used clinically in various pathologies [80]. HDACi are relatively new anti-cancer drugs but are the most used epidrugs targeting PTMs players. Restoration or inhibition of HMT activity also are of great importance for tumor therapy. Different HMT inhibitors (HMTi) are the subject of clinical trials at different stages such as Tazemetostat, an EZH2 inhibitor [81].

Now that the relationship between histone modifications and cancer is establish and the lack of effective therapies is therefore not a hindrance, the use of nucleosomes and their specific PTMs as cancer biomarkers shows a real potential. As for ct-DNA-5mC, to take advantage of PTMs as biomarkers and avoid the inconveniences associated with invasive solid biopsy, the alternative of liquid biopsy is strongly considered [82].

3- Circulating nucleosomes as cancer biomarkers

As cfc-DNA and ct-DNA, circulating nucleosomes are released into biologic fluid, including blood, following cell death such as necrosis and apoptosis, and many circulating nucleosomes can be captured and digested by macrophages. In plasma, nucleosomes have an initial half life of 4 minutes before being degraded by circulating endonucleases [83]. Like ct-DNA-5mC, these circulating nucleosomes can be isolated with specific antibodies [84]. Nucleosomes can also be secreted in blood by active mechanisms [85]. Either they joined bloodstream by both damaged and activated cells, the role of nucleosomes is not yet completely clear but it seems that they exhibit an inflammatory state and mediate cell death [86,87]. As with cfc-DNA, multiple biological conditions, both physiological and pathological, cause an increase in the amount of cell-free nucleosomes [88,89], including cancer [90].

The utility of cfc-nucleosomes as biomarkers has already been explored in two studies in 2017 and 2018. The two papers discussed the interest of cfc-nucleosomes in early diagnosis of colorectal cancer [91,92]. Both performed ELISA tests on a cocktail of circulating nucleosome epitopes, some of which epimodified as H4K20me3, H3K9Me3 and H3K9Ac. Cfc-nucleosomes carrying epigenetic modifications are present from tumor initiation to advanced stages. In spite of that, neither of the two studies is really conclusive. Designated biomarker models (e.g cfc-nucleosomes combination) are promising for early detection but the link between cfc-nucleosomes levels and CRC diagnosis or prognosis is still unknown. Finally, these articles

suggest to deepen the research, with larger numbers of patients for example, or considering the association of biomarkers of different types, such as proteins. Circulating nucleosomes also appear to be interesting biomarkers for early predicting the efficacy of a treatment as it has been demonstrated in lung [93], colon [94] and breast cancers [95].

In many cases, histone PTMs in the initial tumor, such as those described in the previous section, are used in prognosis. But studies of these biomarkers in the blood are scarce while the therapeutic arsenal is full of possibilities. A concrete example is glioblastoma, an aggressive brain tumor that becomes rapidly resistant to standard temozolomide and irradiation treatment [96]. Therapeutic failures with glioblastoma drives scientists to wonder if it is not an epigenetic disease [97]. In 2015, Pacaud et al. demonstrated that three different phosphorylated signatures of histone H3 are associated with a poor prognosis in patient with the reference treatment [98]. They highlight that the use of kinase inhibitors abrogated the high level of phosphorylated histones residues. Nowadays, plethora of kinase inhibitors are available, but the limiting step is still in sample collection, extremely invasive and not always feasible in the case of glioblastoma. Finally, to benefit from kinase inhibitors in anti-glioblastoma therapy, the alternative of using cfc-nucleosomes as blood biomarkers could once again raises barriers.

IV- CELL-FREE CIRCULATING NON-CODING RNA

1- Non-coding RNA: the basics

For a long time, researchers were only interested by coding DNA, they were focused on RNA transcription and proteins translation. Non-coding DNA, which represents 98% of total DNA was called "junk DNA". Moreover, even if DNA is coding for an RNA, it does not always end with the production of a protein: it is non-coding RNA (ncRNA). Since a few years, researchers started to focus on ncRNAs. In humans, only 2% of the genome code for proteins [99], whereas 80% are transcripted [17], which means that most of the transcripted sequences are ncRNAs. There are two main families of ncRNAs : long non coding RNA (lncRNA) (>200nt) and short ncRNA (19nt<size<200nt).

The lncRNA family is very heterogeneous and not well classified. Among the lncRNA family, we here distinguish the large intergenic non-coding RNAs (lincRNAs) and circular RNA (cirRNA). LincRNAs represents the half of lncRNA and are defined as autonomously transcribed non-coding RNAs longer than 200 nucleotides, that do not overlap annotated coding genes [100]. They exercise functions such as remodeling chromatin and genome architecture, RNA stabilization and transcription regulation, including enhancer-associated activity. CircRNA are defined as endogenous RNAs that can forms between a downstream 3′ splice site and an upstream 5′ splice site in a linear precursor mRNA and act as miRNA sponges, competitor of RNA, sequestrator of proteins and splicing modulator [101]. The short ncRNA family there can be divided in several subtypes : microRNA (miRNA) which target mRNA and induce their degradation, piwiRNA (piRNA) implicated in transposons silencing, small nuclear RNA (snRNA) with a role in splicing, small nucleolar RNA (snoRNA) which participate to

ribosomal RNA maturation [102], but also ribosomal RNA (rRNA), transfer RNA (tRNA) and its derived fragments.

2- Characteristics of cfc-ncRNA

Multiple mechanisms participate to the release of ncRNA in body fluids from cells, including necrosis and apoptosis [103]. Certain ncRNA can also be released and transported in body fluids in association with proteins such as miRNA-AGO1/2 [104]. In theory, all ncRNA can be detected in body fluids and particularly in blood. A study performed from 477 cancer patients illustrates this point by reporting that the core of cfc-ncRNA notably includes 258 miRNA, 441 piRNA, 411 tRNA, 125 snRNA and 24 snoRNA [105].

The half-life of cfc-ncRNA in blood is distinct between the different ncRNA subtypes. In theory, the half-life of all ncRNA in the plasma is affected by circulating RNAses. An interesting study reports that half-life of miRNA in blood could be of 14 days since the expression level of tumor-associated miRNA decreased to basal levels two weeks after the tumor resection [106]. Butova et al. (2019) reports that 6% of lncRNAs show a half-life over 12h, and only 29% of lncRNAs may be considered unstable with a 2h half-life [107]. The average half-life of circRNAs in plasma exceed 48h [108].

3- cfc-ncRNA as cancer biomarkers

Being easy to isolate and to study by RT-qPCR, ncRNA could be very good biomarkers in cancer diagnosis and therapy. This is why they are pretty much studied in several cancer types. In this part, we will focus only on cell-free-circulating ncRNA, since they do not give same informations about tumors than ncRNA in extracellular vesicles that could be the source of intercellular communication.

Many cfc-ncRNAs have been described as deregulated in several cancer types and can be used as biomarkers, like cfc-lncRNA in non small cell lung cancer [109], cfc-miRNA in colorectal cancer [110], cfc-cirRNA in breast cancer [111], to cite only a few.

More than focusing on unique biomarker, some papers tried to identify a panel of ncRNA as biomarker to increase the diagnosis precision, the prediction of response to anticancer therapy and or to anticipate toxicity. For example, Peng et al. [112] found 4 miRNA and lncRNA to be used in early diagnosis of non-small cell lung cancer (NSCLC). A circulating non coding RNA panel has also been identified in hepatocellular carcinoma [113]. A panel of serum ncRNAs, including let-7a, miR-155, miR-574-5p, and MALAT1, was shown to be present in patients with breast cancer [114]. Even if some ncRNA are not pertinent in diagnosis, they can be used in therapy, like the miR-370-3p in glioblastoma for example, which is not prognostic but able to reduce tumor volume in mice [115].

VI- CONCLUSIONS, CHALLENGES AND FUTURE DIRECTIONS

Cancer monitoring is a topic at the heart of scientific research. In parallel with the studies for innovative treatments or to get around the phenomena of resistance, the early detection of the malignancy is the Holy Grail of cancer research. Accumulating evidence indicates that epigenetic modifications happens prematurely in oncogenesis [116,117]. Therefore, as we reviewed, circulating epimarks, whatever their nature, are allies in cancer diagnosis. A tool to assist with the detection of circulating epimarks can also be useful to stratify the tumors and to emit a prognosis. In cancer therapy, epimarkers may have a role in treatment efficiency and toxicity monitoring. Thus, cell-free circulating epimarks are real swiss army knives, giving us a real-time image of the tumor and its adaptation to its microenvironment (**Figure 2**).

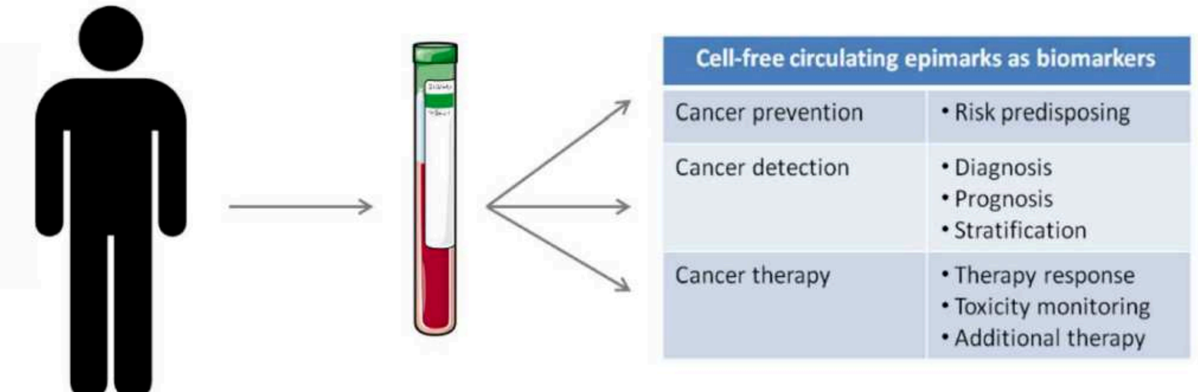

Figure 2: Cell-free circulating epimarks as biomarkers, for cancer detection, prevention and therapy. These epimarks can be very useful to monitor cancer evolution during its development.

Despite the use of plasma methylome, nucleosome and non-coding transcriptome as biomarkers is a promising insight for the development of precision medicine [118] and personalized therapy, the identification of biomarkers with adequate clinical accuracy stays a challenge. The relevance plasma methylome study is no longer to prove considering the specificity of the marker, but the test sensitivity is discussed, not always satisfactory probably due to the noises from the background DNA. Even if false positives should be rarer with cfc-DNA than with protein biomarkers (e.g. antigen dosage), the screening will be no less tedious for a given cancer, the methylation changes would not be systematically known a priori, and a panel of genes would have to be tested. Another need is to find a way to discriminate between ct-DNA-5mC and methylated non-tumor DNA, since the size criterion evidenced by certain papers is not technically applicable. However, as sequencing technologies quickly develop, the eventual use of ct-DNA in clinical seems to be promised. The other challenge that remains is the discovery of appropriate epidrugs, in order to really valorize these discoveries [119]. The existence of drug is not a limiting factor in the case of modified nucleosomes in cancer. However, studies on the use of cfc-nucleosomes in cancer management require further exploration. The few existing studies are inconclusive. However, the isolation of

nucleosomes circulating in the blood could facilitate the monitoring of the hard-to-access tumors known to display histones profile changes, as glioblastoma.

Now that many papers studied circulating epimarks, it seems important to go further and design clinical trials to validate epimarks use in clinic. Indeed, our priority research must remain patients.

LIST OF ABBREVIATIONS

Ac: acetylation

cfc: cell free circulating

cfc-ncRNA: cell free circulating-non coding RNA

cfc-nucleosomes: cell free circulating-nucleosomes

cirRNA: circular RNA

CRC: colorectal cancer

ct: circulating tumor

ct-DNA: circulating tumor DNA

ct-DNA-5mC: cytosine-methylated circulating tumor DNA

ct-DNA-5mC: cytosine-methylated circulating tumor DNA

ct-ncRNA: circulating tumor non coding RNA

ct-nucleosomes: circulating tumor nucleosomes

FDA: Food and Drug Administration

HATs: histone acetyltransferases

HDACi: histone deacetylase inhibitors

HMTi: HMT inhibitors

HMTs: methyltransferases

lincRNAs: large intergenic non-coding RNAs

lncRNA: long non coding RNA

Me: methylation

miRNA: microRNA

ncRNA: non-coding RNA

NSCLC : non-small cell lung cancer

P: phosphorylation

piRNA: piwiRNA

PTMs : post-translational modifications

rRNA: ribosomal RNA

snoRNA: small nucleolar RNA

snRNA: small nuclear RNA

Sumo: sumoylation

tRNA: transfer RNA

Ub: ubiquitylation

World Human Organization: WHO

DECLARATIONS

Ethics approval and consent to participate

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Concent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

All authors were responsible for the draft of the work or critical revision for important intellectual content. All authors read and approved the final manuscript.

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1.7.2) ÉPIMARQUES CIRCULANTES DANS DES VÉSICULES EXTRACELLULAIRES

En plus de circuler librement dans le sang, les épimarques circulantes (ADN méthylé, nucléosomes, miRNA) peuvent être exportées dans les vésicules extracellulaires et donc utilisées comme biomarqueurs. Il existe plusieurs types de ces vésicules, généralement discriminées par leur taille, alors que leur biogénèse est différente [135]. Même si toutes ne possèdent pas le même contenu et ne portent donc pas les mêmes informations (notamment les corps apoptotiques), les microvésicules et les exosomes ont tous deux un rôle de communication intercellulaire. Dans le cadre du cancer, il nous semblait intéressant de nous concentrer sur cet aspect de communication plutôt que sur la nature des vésicules extracellulaires.

Comme décrit dans la revue, l'ADN méthylé est une des épimarques circulantes existantes. Mais contrairement aux ARN, sa présence dans les vésicules extracellulaires a peu été étudiée, sûrement dû à un problème de localisation cellulaire : l'ADN est situé dans le noyau, alors que les exosomes se forment dans l'endosome donc au niveau du cytoplasme. Cependant, quelques articles décrivent la présence d'ADN double brin dans les exosomes [252, 253], mais peu ont étudié la méthylation de cet ADN [254].

La question qui se pose aussi à ce stade est : quel est le rôle de cet ADN (méthylé ou non) dans les vésicules extracellulaires ? Participe-t-il à un transfert de gènes ? Un article montre que l'ADN transféré par des vésicules extracellulaires et comprenant KRAS muté induit une prolifération plus importante chez des cellules normales [255], mais d'autres études restent à mener à ce sujet.

Les microARN sont couramment retrouvés dans les vésicules extracellulaires. De par leur fonction intrinsèque, et sous réserve que la cible soit exprimée, leur effet sera le même quelle que soit la cellule. En d'autres termes, ils peuvent jouer leur rôle de répresseur traductionnel quelle que soit la cellule dans laquelle ils sont. C'est pourquoi ce sont des acteurs majeurs de la communication intercellulaire.

Ces microARN peuvent être exportés dans les exosomes et autres vésicules extracellulaires par des RNA binding proteins telles que SYNCRIP [256] ou HNRNPA2B1 [257]. Ces dernières

se fixent à des séquences consensus appelées exomotifs : GGGCA/UG/C pour SYNCRIP [149, 150] et GGAG pour HNRNPA2B1 [151].

Les deux types d'épimarques (circulantes ou dans des vésicules extracellulaires) donnent des informations pertinentes pour l'évolution du traitement et le suivi du patient : efficacité du traitement, rechute, etc…

Dans le glioblastome spécifiquement, une signature de miRNA circulants a été décrite comme étant caractéristiques de la pathologie [258, 259].

Plutôt dans le cadre de la communication intercellulaire, Yin *et al.* ont démontré que le miR-1238 était capable de conférer aux cellules du microenvironnement une résistance au témozolomide [260].

1.8) Les épithérapies, nouvel outil de la médecine moderne

1.8.1.1) Stratégies innovantes

Afin de réussir à traiter les nombreux types de cancers existants, plusieurs nouvelles stratégies sont actuellement en cours de développement.

Premièrement, il est possible de combiner différentes thérapies déjà existantes en ciblant au mieux les caractéristiques du patient : c'est la médecine personnalisée. Par exemple, cet article de Flashner-Abramson *et al.* est parti d'une base de données contenant les informations du réseau protéique de 3500 tumeurs pour déterminer quelle(s) voie(s) de signalisation cibler pour chaque tumeur [261]. Cette méthode est efficace car très personnalisée, cependant, l'analyse protéique des tumeurs reste coûteuse et techniquement compliquée. Avec l'amélioration constante des techniques de séquençage, d'autres études s'intéressent à l'utilisation du Next Generation Sequencing (NGS) pour étudier en profondeur les tumeurs et stratifier les patients [262]. L'inconvénient de ces deux stratégies est qu'elles permettent de bien cerner la tumeur initiale au moment du diagnostic, mais ne permettent pas un suivi du patient au cours du traitement.

Deuxièmement, depuis quelques années, une des stratégies consiste à utiliser le système immunitaire afin de tuer les cellules tumorales. Les CAR-T cells en sont un exemple : ces lymphocytes T prélevés au patient puis modifiés de manière à leur faire exprimer un récepteur chimérique capable de reconnaitre spécifiquement les cellules tumorales ont démontré leur efficacité dans un premier essai clinique en 2013 [263]. Une autre approche consiste à limiter l'échappement au système immunitaire des cellules tumorales, notamment en inhibant PD-L1 sur la cellule tumorale ou PD-1 sur les LT grâce à des anticorps bloquants : atezolizumab pour PD-L1 et nivolumab pour PD-1 par exemple [264]. Il peut cependant être risqué de bloquer complètement PD-1 (programmed cell death protein 1) et/ou PD-L1, ce mécanisme étant impliqué dans l'immunotolérance et empêche donc la destruction des cellules du soi et l'apparition de maladies auto-immunes [265]. Diverses méthodes ciblant des points de contrôle immunitaires autres que l'axe PD-1/PD-L1 sont également utilisées, comme des anticorps anti-CTLA4 (cytotoxic T-lymphocyte associated protein 4), cette protéine étant elle aussi un inhibiteur de l'action des LT via l'axe CTLA4/B7-1 et B7-2 (ipilimumab).

Le troisième axe à améliorer est la méthode d'administration : les chimiothérapies et radiothérapies restent toxiques pour l'ensemble de l'organisme. Malgré les progrès de la radiothérapie qui permet de cibler de mieux en mieux la tumeur, des tissus sains sont malgré tout systématiquement atteints. Idem pour la chimiothérapie, administrée le plus souvent de manière systémique, soit directement dans la circulation sanguine soit par voie orale. Ces thérapies peuvent gravement détériorer l'état de santé du patient, et la radiothérapie peut même induire le développement d'un autre cancer à l'issu du premier [266]. De plus, l'irradiation ainsi que certains traitements anti-cancéreux tels que le Tamoxifène ou l'Etoposide sont classés comme carcinogéniques par l'IARC [267, 268]. Il est donc crucial de développer de nouvelles méthodes d'administration de chimiothérapie et de radiothérapie, afin de limiter la toxicité globale. L'idée principale est que ces thérapies doivent atteindre uniquement les cellules tumorales, et non pas les cellules saines. Pour la radiothérapie, l'utilisation d'anticorps radiomarqués permet une meilleure spécificité [269]. Pour la chimiothérapie, certains traitements tels que l'ABT-737 (un inhibiteur de BCL2, BCL-XL et BCL-W permettant d'augmenter l'apoptose des cellules) ou certaines

méthodes d'administration comme les injections intrathécales (directement dans le liquide cérébrospinal), intrapéritonéales ou intravésicales (dans la vessie) sont actuellement utilisées, mais même si elles limitent la toxicité systémique de la chimiothérapie, elles n'empêchent pas un impact sur les cellules saines de l'organe traité. Une méthode à l'étude actuellement consiste à modifier la chimiothérapie pour diminuer sa cytotoxicité. Dans cet article d'Ibsen *et al.*, pour que la doxorubicine joue son rôle uniquement dans la tumeur, elle sera clivée spécifiquement par photoactivation [270]. Cependant ces deux méthodes ne sont pas utilisables dans tous les organes, notamment le cerveau. Il est aussi possible d'encapsuler la chimiothérapie, et d'induire le largage de ses composants par des ultrasons [271], un pH spécifique (l'environnement tumoral est légèrement acide) [272], ou des phospholipases (plus présentes dans les cellules tumorales) [273].

Enfin, développer des thérapies novatrices reste un axe important de la lutte contre le cancer, et cibler l'épigénétique est une piste prometteuse.

1.8.1.2) Épithérapies : manipuler les interrupteurs épigénétiques

Des thérapies ciblant l'épigénétique, ou épithérapies, sont déjà utilisées chez des patients. Il en existe deux types principaux : les HDACi, inhibiteurs des histones déacétylases, et les DNMTi, inhibiteurs des méthylases de l'ADN. Plusieurs HDACi sont approuvés par la FDA dans le traitement du lymphome cutané à cellules T, mais aussi dans le myélome multiple [274] : le SAHA (suberoylanilide hydroxamic acid) et le panabiostat respectivement. Ces HDACi ciblent les classes cinq classes d'HDAC, mais des molécules plus spécifiques d'une seule classe sont à l'étude, telles que le tacedinaline qui ne cible que les HDAC de classe I (essai clinique NCT00005624). De plus, d'autres essais cliniques sont en cours pour différents types de cancers, tels que les ovaires, le col de l'utérus ou encore le cancer de la prostate [274]. Pour les DNMTi, deux sont utilisés dans le cadre du traitement du syndrome myélodysplasique : l'azacitidine et la décitabine, deux analogues de la cytidine ne pouvant être méthylés.

Les HDACi et les DNMTi ciblent directement des enzymes épigénétiques, mais il est possible d'agir sur le profil épigénétique de manière moins directe. Par exemple, à ce jour il n'existe

pas de molécules inhibitrices des TET, et encore moins spécifique d'une seule protéine de cette famille, mais des stratégies détournées ont tout de même été mises au point. Par exemple, le dimethyloxallyl glycine (DMOG) inhibe toutes les enzymes dépendantes du 2-oxoglutarate, impliquées dans de nombreux rôles physiologiques dans la cellule (réparation d'acides nucléiques, régulation de la biosynthèse de transcrits et de protéines, métabolisme lipidique, détection de l'hypoxie, biosynthèse du collagène et des protéines associées…) [275] et notamment la déméthylation active de l'ADN, puisque les TET font partie de cette classe de protéines. Il existe aussi des inhibiteurs d'IDH, une classe d'enzymes impliquées dans la production d'α-cétoglutarate, cofacteur des TET [276].

Cependant, comme pour beaucoup de chimiothérapies, ces inhibiteurs manquent de spécificité et peuvent induire une toxicité importante. Mais au-delà d'un manque de précision en terme d'accès à la cellule, ces épithérapies souffrent d'un manque de spécificité au sein même d'une cellule. En effet, les inhibiteurs de DNMT vont diminuer la méthylation globale de l'ADN, mais que se passe-t-il si c'est un oncogène qui est déméthylé ? Idem pour les HDACi, pouvant induire la réexpression d'oncogènes. Les autres types d'épithérapies "indirectes" présentées vont en plus induire des changements en dehors des modifications épigénétiques, notamment au niveau du métabolisme.

À ce jour, il semble donc important de trouver des méthodes affinant la précision de ces épithérapies.

Une première stratégie consiste à changer les modifications épigénétiques sur une séquence précise de l'ADN. Pour cela, il est nécessaire d'identifier des complexes enzyme épigénétique/facteur de transcription de mauvais pronostic afin de spécifiquement rompre ces complexes. C'est le cas d'un peptide capable de casser l'interaction DNMT3A/ISGF3γ dans le glioblastome multiforme [195]. La même stratégie pourrait être utilisée avec des enzymes de la famille TET, puisqu'il a été démontré que ces protéines pouvaient interagir avec des facteurs de transcription tels que WT1 (Wilms tumor 1) ou PU.1 (hematopoietic transcription factor PU.1) [277, 278]. La méthode de genome editing qui consiste à modifier le génome pour changer l'information génétique peut être adaptée à l'épigénome. En effet, la conjugaison d'une enzyme épigénétique (DNMT ou TET notamment) à une Cas9 (CRISPR

associated protein 9) désactivée pourrait permettre, après ajout d'un sgRNA la modification épigénétique d'une séquence précise de l'ADN [279, 280]. Même si cette méthode est difficilement applicable en thérapie, elle pourrait être utilisée pour screener les séquences susceptibles d'être impliquées dans la tumorogénèse.

La seconde consiste à utiliser des miRNA ou des anti-miR, selon si le miR cible est de bon ou de mauvais pronostic. En effet, de nombreux miRNA ont été identifiés comme suppresseurs de tumeurs : le miR-29b dans l'AML [281], miR-27a-3p dans le cancer du poumon [282] ou le miR-551a dans le cancer gastrique [283]. Au contraire, le miR-494 a été décrit comme étant oncogénique dans le cancer du poumon [284], et le miR-23b dans le cancer du sein [285]. Le principe est de traiter les patients avec un miR mimétique des miR suppresseurs de tumeurs, et/ou avec un anti-miR capable d'empêcher l'effet répresseur de transcription d'un miR oncogène. Cependant, à ce jour les méthodes d'administration ne sont pas optimales, comme abordé dans la discussion.

2. Objectifs

Avec un nombre de cancers en constante augmentation dans le monde, le traitement de cette pathologie est un véritable enjeu de santé publique. En effet, les chiffres de prévalence ne cessent d'augmenter, d'une part à cause de l'augmentation des nouveaux cas, mais de façon plus positive grâce à une survie prolongée due à une meilleure prise en charge [286]. Il est donc urgent de trouver des solutions pour diminuer la mortalité induite par cette pathologie. C'est une maladie des plus complexes, avec de nombreux niveaux de régulation et une hétérogénéité importante.

C'est pourquoi plusieurs axes sont explorés à ce jour : la découverte de nouveaux traitements, l'optimisation de traitements déjà existants, l'augmentation de l'efficacité du système immunitaire mais aussi l'identification de facteurs de risques pour améliorer la prévention, ou encore la recherche de facteurs pronostiques et le suivi de leur évolution au cours du temps, permettant l'adaptation du traitement selon la réponse du patient.

Dans ce dernier axe d'étude, le but est de personnaliser le traitement en fonction du patient afin d'induire une meilleure réponse. En effet, il a été démontré que certains traitements, même s'ils se montrent efficaces dans un premier temps, peuvent induire des phénomènes de résistance et donc à long terme empêcher la mort des cellules tumorales. Suivre l'évolution des pathologies au cours du temps permettrait d'éviter ces phénomènes de résistance en changeant plus régulièrement le traitement.

Dans ce travail de thèse, nous nous sommes intéressés à différents aspects de la lutte contre le cancer, en se concentrant sur le prisme de l'épigénétique. Dans ce cadre, nous avons décrit plusieurs implications épigénétiques dans le cancer, notamment dans le glioblastome multiforme (

Figure 35).

Dans un premier temps, nous avons démontré que l'hypométhylation de l'ADN est associée à la résistance au traitement et à l'échappement tumoral. En effet, la déméthylation de l'ADN joue un rôle dans la glioblastomagenèse en modifiant notamment l'immunosélection (article 1), mais aussi dans la rechute du glioblastome via la surexpression de TET2 (article 3), et dans l'immunosubversion (articles 2 et 4).

Toujours à propos de la méthylation de l'ADN, nous avons prouvé qu'elle pouvait être utilisée comme outil pronostique de risque (article 1).

Ensuite, nous avons décrit les miR comme étant des marqueurs de l'acquisition de la résistance, que ce soit avec l'exomiR-378a-3p dans les NK ou avec l'adénosine méthylation du miR-200b-3p (articles 4 et 6).

Enfin, nous avons identifié le miR-370-3p comme outil thérapeutique potentiel dans le traitement du glioblastome multiforme (article 5).

Ces différents travaux corrélés mettent en évidence l'importance de la prise en compte de l'épigénétique à chaque stade de la pathologie, de la prévention au développement de nouvelles thérapies.

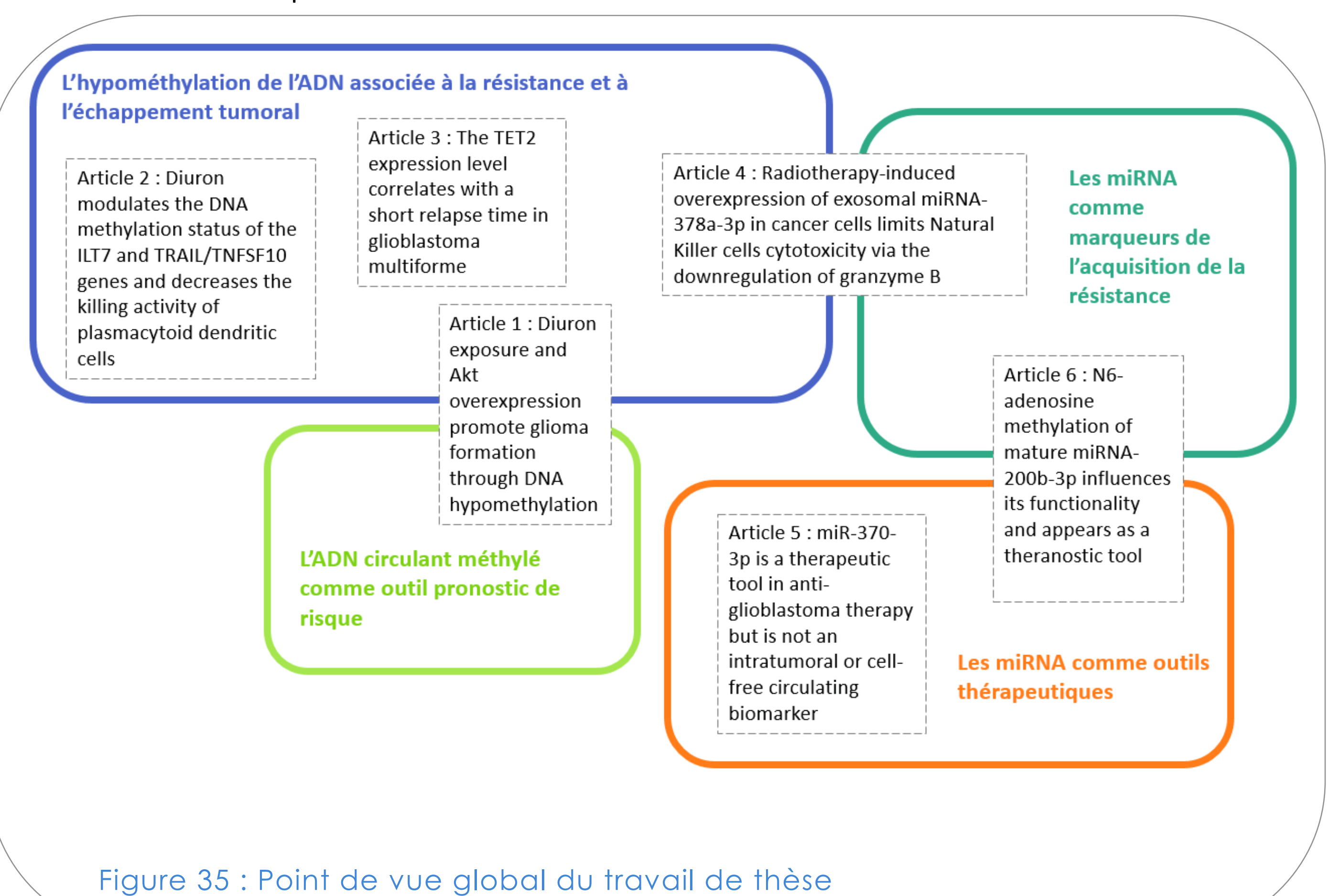

Figure 35 : Point de vue global du travail de thèse

3. RÉSULTATS

Article 1 -
Diuron exposure and Akt overexpression promote glioma formation through DNA hypomethylation

Dans cet article, nous nous sommes intéressés à l'impact du diuron, un herbicide, sur la gliomagenèse. Cet herbicide a été démontré comme étant carcinogène dans plusieurs organes (vessie, glande mammaire, rein…) [287–291] et l'exposition aux pesticides a été reconnue comme augmentant le risque de tumeurs cérébrales [292–295] . Les questions étaient multiples, mais la principale était : le diuron est-il capable d'induire la transformation de cellules astrocytaires normales en cellules tumorales ? Et si oui, existe-t-il des mécanismes épigénétiques impliqués ?

Nous avons démontré que le diuron seul ne permettait pas d'induire la transformation d'une cellule normale en cellule tumorale, mais lorsque l'exposition est couplée à la surexpression d'Akt, la gliomagenèse a lieu dans 60% des cas. Akt est une protéine kinase impliquée dans la survie cellulaire en inhibant l'apoptose. Sa surexpression est directement liée à la capacité d'invasion des cellules de glioblastome [296], notamment via la phosphorylation de DNMT1 et la rupture du complexe majeur de la méthylation DNMT1/UHRF1/PCNA [59].

Cette transformation a lieu via un mécanisme de déméthylation globale de l'ADN médiée par APOBEC3γ et par la perte du complexe DNMT1/UHRF1/PCNA, complexe majeur du maintien de la méthylation de l'ADN.

Diuron exposure and Akt overexpression promote glioma formation through DNA hypomethylation

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Running title: Diuron promotes glioma?

Keywords: DNA methylation, diuron, gliomagenesis, apoptosis, PD-L1

ABSTRACT

Background: Diuron is an environmental component listed as a likely human carcinogen. Several other studies report that diuron can be oncogenic for bladder, urothelial, skin and mammary cells. None study mentions the putative effect of diuron on the glioma occurrence.

Objectives: We here wanted to investigate the effects of diuron exposure on the glioma occurrence while wishing to incriminate a putative implication of DNA methylation modulation in this process.

Methods: In *in vivo* model of glioma, diuron exposure was firstly compared or combined with oncogenic overexpressions already known to promote gliomagenesis. ELISA quantifying the 5-methylcytosine level on DNA was performed to examine the global DNA methylation level. Quantitative real-time polymerase chain reaction and Proximity Ligation In situ Assay were performed to identify the molecular causes of the diuron-induced changes of DNA methylation. The signatures diuron-induced changes of DNA methylation were analyzed in a cohort of 23 GBM patients.

Results: Diuron exposure is not sufficient to promote glioma, such as the oncogenic overexpression of Akt or Ras. However, the combination of diuron exposure and Akt overexpression promotes glioma. We observed that the diuron/Akt-induced glioma is characterized by three phenotypic signatures characterizing cancer cells: a global DNA hypomethylation, a loss of sensitivity to cell death induction, and a gain of signals of immune escape. Our data associated these phenotypes with three aberrant DNA methylation signatures: the *LLT1, PD-L1* and *Bcl-w* hypomethylations. Strikingly, we observed that these three concomitant hypomethylations were only observed in GBM patients having a potential exposure to diuron via their professional activity.

Conclusions: As single player, diuron is not an oncogenic of glioma, but it can participate to the glioma formation in association with other events (also devoid of oncogenic property as single player) such as Akt overexpression.

BACKGROUND

The European Cancer Observatory (ECO) estimates the number of brain tumor at 30 715 in Europe (http://eco.iarc.fr) and the Central Brain Tumor Registry of the United States (CBTRUS) estimates at 26 070 the number of brain primary malignant tumor in 2017 (http://www.cbtrus.org). In 2016, the World health Organization (WHO) classification of central nervous system (CNS) tumors was revised to integrate molecular signatures tohistological parameters in order to enhance the robustness of the classification of CNS tumors.

During the last decades, different molecular oncogenic events have been described to induce primary tumor brain formation. Thus, oncogenic overexpression and induction of global DNA hypomethylation are described to promote the gliomagenesis[1-2-3-4]. In addition, several risk factors have been described as potential contributors/inducers of glioma risk. These environmental risk factors include allergies, exposure to ionizing and non-ionizing (cellular phones) radiations, exposure to chemicals, solvents and pesticides[5-6-7].

A study performed in southwestern of France reports that a high level of occupational exposure to pesticides might be associated with an excess risk of brain tumors[8]. Meta-analysis of brain cancer and farming indicated that pesticides exposure commonly experienced by farmers may contribute to the increased risk of brain cancer[9]. A study performed in Nebraska significantly associated some specific agricultural pesticide exposures and the risk of glioma among male farmers[10]. However, these studies need to be counterbalanced by study reporting the absence of association between pesticide exposure and risk of glioma[7].

Diuron is an herbicide and an antifoulant listed as a likely human carcinogen by US Environmental Protection Agency (USEPA) in 1997. Due to this dual and extensive utilization, diuron contamination is frequently observed[11]. Diuron and its metabolites appear as the third pesticide detected in surface water in Italy with a concentration of 3.10^4ng/L [12]. Diuron is detected in Great Barrier Reef lagoon at a concentration exceeding the Australian and New Zealand guideline trigger value of 0.2 µg/L [13]. The CEREMA study reports that diuron is detected in air with a peak at 0.31 ng/m^3. This study also indicated that diuron is detected in approximately 30% of groundwater samples with a maximum concentration of 0.279 µg/L. The CEREMA study also reports that diuron was detected in 70% of samples with a maximum concentration of 0.864 µg/L and the mean concentration of 0.041 µg/L in european river waters. Diuron is also detected in food and vegetables. European Food Safety Authority mentions an ADI (Acceptable daily intake) of 0.007 mg/kg bw/d and an ARfD (Acute Reference Dose) of 0.016 mg/kg bw/d. In a document edited in 2011, EFSA reports that the highest chronic exposure represented 1.8 % of the ADI (German child) and the highest acute exposure amounted to 6.1 % of the ARfD (apple). Thus, in addition to professionals using diuron in their activity, diuron exposure is multiple for Human.

Despite its classification as a likely human carcinogen, little is known about the combination effect of oncogenic overexpression and the pesticide exposure on the glioma occurrence. More generally little is known about the diuron-induced tumor molecular mechanism even if diuron has been already reported to promote the bladder[14-15], urothelial[16], skin [17-18] and mammary[15-19] carcinogenesis.

In this article, we asked the question to determine whether the diuron exposure of glial cells could promote glioma formation and if that is the case, we will analyzed the putative involvement of DNA methylation modifications in the diuron-induced glioma.

Here, we show that the unique diuron exposure is not oncogenic for glial cells. However, the combination of diuron exposure and Akt overexpression promote glioma. In terms of molecular mechanisms, our data indicate that the diuron/Akt-induced glioma is characterized by active and passive processes of DNA demethylation that promote an epigenetic reprogramming of certain apoptosis and immune system actors. Thus, this supports the escape of cell death and immune system.

METHODS

Cell culture and diuron treatment

Ntv-a cell line used was obtained from Holland[20] team and corresponds to newborn tv-a transgenic mice brain cells infected with viruses containing RCAS vectors with LacZ, Ras and/or Akt. These cells are cultivated with DMEM with 10% fetal calf serum, 1% glutamine, 1% penicillin/streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO_2-air. Cells were treated with 100µM of diuron dissolved in DMSO each 2 days during 14 days, control cells received DMSO only.

Tumorigenicity assay

After diuron exposure Ntv-a cells were harvested by trypsinization, washed and resuspended in saline buffer. Cell suspensions were injected s.c. (subcutaneous) as 2.10^6 cells in 0.05 ml of PBS with equal volume of matrigel matrix (Becton Dickinson, France) in the flank of 7 groups of 5 7/8-weeks-old Nude NMRI-nu female mice (Janvier, France). Tumor volumes based on caliper measurements were calculated by the modified ellipsoidal formula (*Tumor volume* = 1/2(*length* × *width*^{2})).

The experimental procedures using animals were in accordance with the guidelines of Institutional Animal Care and the French National Committee of Ethics. In addition, all experiments were conducted according to the Regulations for Animal Experimentation at the « Plate-forme Animalerie » of « Institut de Recherche en Santé de l'Université de Nantes (IRS-UN) » and approved by the French National Committee of Ethics (Agreement number: B44278).

Measure of global DNA methylation

DNA was extracted by using the QiaAmp DNA mini Kit (Qiagen, France). Next, global DNA methylation was estimated by quantifying the presence of 5-methylcytosine using 5-mC DNA ELISA Kit (Zymo Research, France) according to the manufacturer's instructions.

RT-qPCR analysis

RNA extract is performed using RNeasy Mini QIAcube Kit and QIAcube (Qiagen, France). RT-qPCRs are performed using QuantiTect Reverse Transcription Kit, Rotor-Gene SYBR Green PCR Kit, QuantiTect Primer Assays and Rotor-Gene Q as real-time thermocycler (Qiagen, France). Reference gene RPLP0 was used, with the $2^{-\Delta\Delta Ct}$ relative quantification method.

Proximity Ligation In Situ Assay (P-LISA)

Cells were cultured for 24h on cover slip. Cells were then fixed with 4% paraformaldehyde in PBS pH 7.4 for 15min at room temperature. Permeabilization is performed with PBS containing 0.5% Triton X-100 for 20min at room temperature. Blocking, staining, hybridization, ligation, amplification and detection steps were realized according to manufacturer's instructions (Olink Bioscience, Sweden). All incubations were performed in a humidity chamber. Amplification and detection steps were performed in dark room. Fluorescence was visualized by using the Axiovert 200M microscopy system (Zeiss, Le Pecq, France) with ApoTome module (X63 and numerial aperture 1.4). Preparations were mounted

by using ProLong® Gold antifade reagent with DAPI (Life Technologies, France). Pictures acquisition was realized in structured illumination microscopy. After decovolving (3.5 Huygens Essential software (SVI)), 3D view was obtained by using Amira.4.1.1 program. Finally, images were analyzed by using the freeware "BlobFinder" available for download from www.cb.uu.se/~amin/BlobFinder. Thus, we obtained either number of signals per nuclei since nuclei can be automatically identified. DNMT1 and UHRF1 were detected with anti-DNMT1 (Santa Cruz, sc10221, France) and anti-UHRF1 (Santa Cruz, sc98817, France).

Measure of genes methylation by quantitative methylation-sensitive restriction enzyme digestion (qMSRE).

qMSRE combine the use of methylation sensitive restriction enzyme and real-time PCR.

For qMSRE, 500 ng of purified genomic DNA (QIAamp DNA Mini QIAcube Kit and QIAcube (Qiagen, France)) is subject to digestion with adequate methylation sensitives enzymes. For each assay, these enzymes can be changed, but generally, we used enzymes that can digest unmethylated CG sites but not methylated CG sites. A parallel "mock" reaction containing all reaction components except enzyme (replaced with glycerol) is included for each sample. DNA from the digested or mock reaction is then amplified by real-time PCR with specific primers. Thus, 5µL of digested or mock solutions were used to perform qPCR using the Rotor-Gene SYBR Green PCR Kit and Rotor-Gene Q as real-time thermocycler (Qiagen, France). DNA was digested by AciI, BspEI and HpaII (4h at 37°C) for the *Bcl-w*-qMSRE, AciI and BsrBI (2 digestions: 4h at 37°C and 4h at 65°C) for the *LLT1*-qMSRE or by Hpa I, HhaI and AvaI (4h at 37°C) for the *PD-L1*-qMSRE. qPCR was performed with the and the following primers: Bcl-w: CTCTGCTTTTCCTAGGCACGCAA and AGGGCTGTTCAGAGGCCATAGT, LLT1 : primers: ACCATCTGGCCTGGATCACA and GGAGAGTTACCCATTTGGCCCATT and PD-L1: AAATGCAGTGATGGCCCATTTC and GATCCACATAGGTTGCCTTCCTCT. The methylation level for any amplified region can be determined using the following equation Percent Methylation = 100 x $2^{-\Delta Ct}$ where ΔCt = the average Ct value from the digested reaction minus the average Ct values from the reference/undigested reaction.

Sequences of the considered promoters were obtained from the Eucaryotic Promoter Database (https://epd.vital-it.ch).

Measure of Cell Death

Percentages of cell death were evaluated by using a Trypan Blue Stain 0.4%, and the Countess® Automated Cell Counter (ThermoFisher, France). Cell death was induced using temozolomide (25µM, Torcis, France). Two Gy irradiation was performed by using X-Rad 225cx (Precision X-Ray inc., North Bradford, CT, USA).

siRNA transfection protocol

In a six-well culture plate, 2.10^5 cells were incubated for 24 h at 37° C in a CO_2 incubator. Then, 60 pmol of siRNA was added and the cells incubated for 7 h at 37° C in a CO_2 incubator. Without removing the siRNA, 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration was added and cells incubated for 24 h.

Then, cells were cultured for 48 h in normal culture medium. Thus, analyses were realized 72 h after the siRNA transfection. The following siRNA were used: siRNA-A (control) (sc-37007, Santa Cruz), siRNA-Bcl-w (sc-37294, Santa Cruz) and siRNA-APOBEC3 (sc-60091, Santa Cruz).

Measure of Akt and APOBEC3γ expression

The Akt and APOBEC3γ expression level were estimated by ELISA method and the use of the PathScan® Total Akt1 Sandwich ELISA Kit (Cell Signaling, France) and APOBEC3γ ELISA kit (MyBioSource, USA) according to the manufacturer's instructions.

Patient samples

Patient samples were collected from GBM patients treated at the "Institut de Cancérologie de l'Ouest" (ICO, http://www.ico-cancer.fr). All patients recruited gave signed, informed consent. All the samples collected and the associated clinical information were registered in the database (N° DC-2018-3321) validated by the French research ministry. Biological resources were stored at the "Centre de Ressources Biologiques-Tumorothèque" (Institut de Cancérologie de l'Ouest, Saint-Herblain, F44800, France).

Statistical analysis

All experiments were done at least in triplicates. Significance of the differences in means ± standard deviations was calculated using Student-t test. Significance of correlation between two parameters was calculated using Pearson's test.

RESULTS

The combination of diuron exposure with Akt overexpression induces glioma, while neither Diuron nor Akt alone is sufficient to induce glioma formation

The RCAS/tv-a model has been a very useful and productive tool for studying the gliomagenesis[21]. In this model, PDGF-B overexpression promotes oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes; and the combination of activated Ras and Akt induces high-grade gliomas[1], while neither activated Ras nor Akt alone is sufficient to induce GBM formation[2].

We first have asked the question to know whether the diuron exposure on Ntv-A cells overexpressing LacZ, Ras or Akt had the ability to promote the gliomagenesis such as the Ras+Akt combination. For this purpose, Ntv-a/LacZ, Ntv-a/Akt and Ntv-a/Ras cells were exposed to 100µM diuron each 2 days during 14 days (Figure 1) to generate Ntv-a/LacZ+Diuron, Ntv-a/Akt+Diuron and Ntv-a/Ras+Diuron cells. Five independent exposures were performed for each cell types. The diuron exposure dose (100µM or 23mg/L) was determined as being i) a dose devoid of cytotoxicity (Figure S1), and ii) a dose inferior to the one seen in human blood (that is 100mg/L [22]). Tumorigenicity assays were performed via the injection of diuron-exposed cells. Five mice were used for the Ntv-a/LacZ, Ntv-a/Akt, Ntv-a/Ras and Ntv-a/Ras+Akt cells. Each independent diuron exposure was subcutaneously injected in one mice. As expected, our experiments confirmed that the Ras+Akt combination acts as oncogenic event for the glioma formation whereas neither Ras nor Akt alone is sufficient to induce GBM formation (Figure 1). We next noted that diuron exposure is not sufficient to induce glioma formation, while its combination with Akt promotes the glioma formation in 60% of our experiments (3/5).

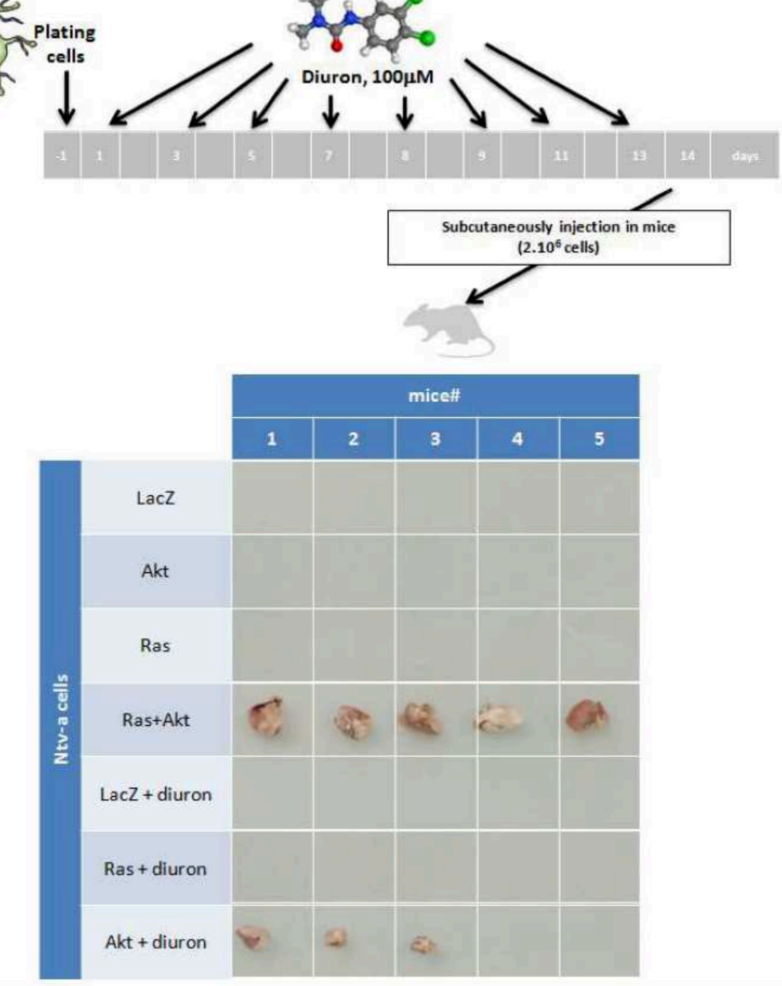


Figure 1: Diuron exposure is not sufficient to induce gliomagenesis, but its combination with Akt overexpression promotes gliomagenesis.

Experimental procedures were here summarized. Five mice per condition were xenografted subcutaneously with indicated cells. 60 days after cells injection, mice were euthanized and tumors were collected. Pictures illustrate the resected tumors.

Next, we have compared the tumor forming potential of Ras+Akt cells and Akt+diuron cells by comparing the tumor growth monitored by caliper measurements. Kinetic of tumor growth and the measure of tumor volume indicated that the volume of tumors induced by the Ras+Akt combination and the tumor growth of these tumors were superior than the one induced by the Akt+diuron combination (Figure 2A and Figure 2B).

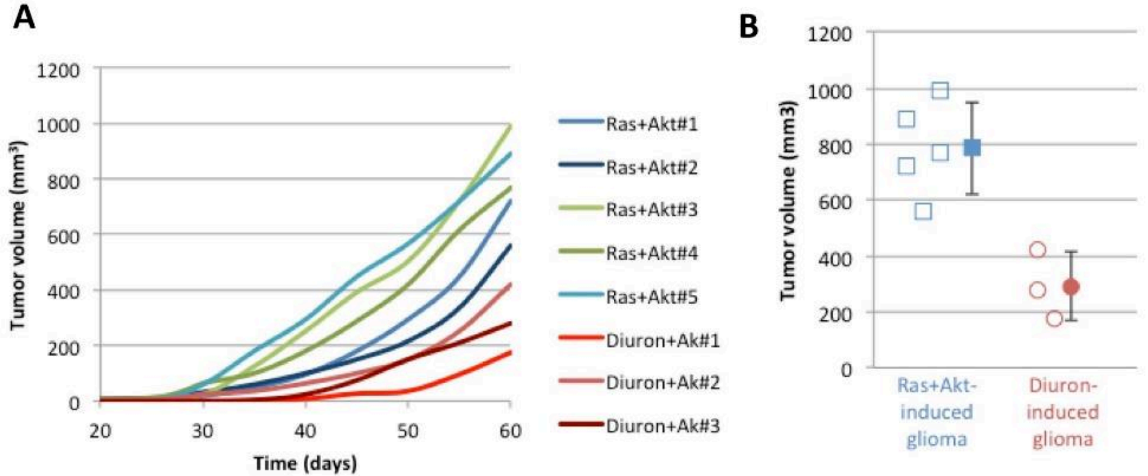


Figure 2: The growth of gliomas induced by the Akt+diuron condition is less aggressive than the one induced by Ras+Akt overexpression.

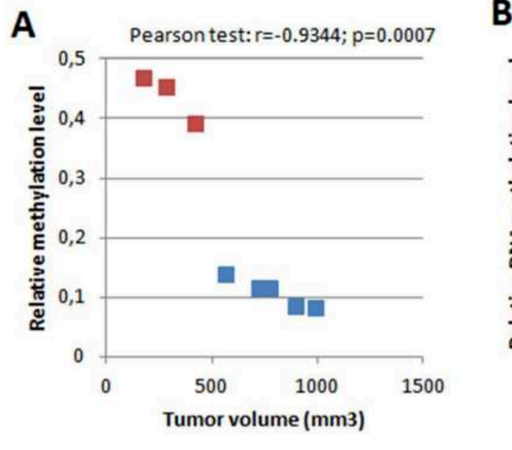
A. Curves illustrate, for each mice, the tumor growth of Ras+Akt- and Akt+diuron-induced glioma.

B. Graph illustrates and compares, sixty days after the cells injection, the volume of all Ras+Akt-induced glioma (n=5, blue squares) with the volume of all Akt+diuron-induced glioma (n=3, red circles).

Cells promoting Akt+diuron-induced tumors are globally hypomethylated via active and passive DNA demethylation processes

In human glioma, the global level of DNA methylation is associated with the tumor grade and with the aggressiveness of tumors[23-3-24]. Our results correlated the relative methylation level of Ras+Akt-induced and Akt+diuron-induced tumors with the tumor volume (Figure 3A).

To characterize the 5mC methylation level in cells at the origin of the Akt+diuron-induced glioma (Ntv-a/Akt+diuron cells), we next compared the methylation level of DNA extracted from Ntv-a/LacZ (control, Ctrl), Ntv-a/Ras+Akt and Ntv-a/Ras+Akt+Folate cells. Folate treatment of Ntv-a/Ras+Akt cells (40µg/ml, 72h) was here used as a treatment induced a gain of 5mC. 5mC ELISA reveals that Ntv-a/Akt+diuron cells were hypomethylation in comparison with Ntv-a/LacZ cells (control cells) (Figure 3B). In addition, we noted that Ntv-a/Ras+Akt cells were more hypomethylated than Ntv-a/Akt+diuron. We also noted that Akt+diuron induced a strong global DNA hypomethylation resulting from synergic effect between the Akt overexpression and the Diuron exposure since Akt+diuron DNA demethylation percentage is higher than the theoric addition of diuron effect only and Akt overexpression alone (Figure 3B).



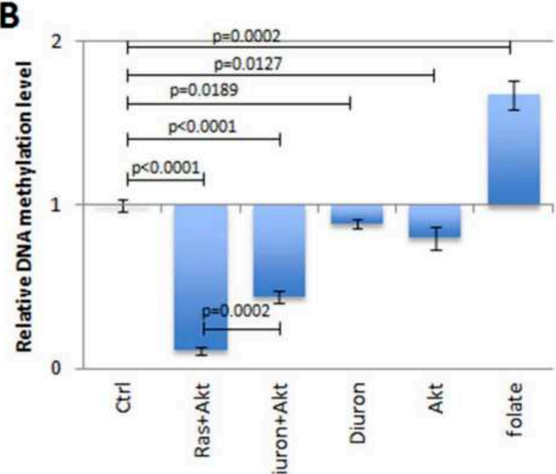


Figure 3: Global DNA hypomethylation occurs in Akt+diuron-induced glioma.

A. Correlation between the relative level of DNA methylation and the tumor volume. Blue squares represent the Ras+Akt-induced glioma (n=5) and red squares represent the Akt+diuron-induced glioma (n=3).

B. Graph illustrates the relative DNA methylation level (mean•SD) seen in Ntv-a/Ras+Akt- and Ntv-a/diuron+Ak cells (n=3). "Folate" represents the relative DNA methylation level in cells treated with folate i.e. as a positive control of gain of DNA methylation. "Ctrl" represented the methylation level of Ntv-a/lacZ cells.

In order to determine a molecular cause at the origin of the global DNA hypomethylation seen in Ntv-a/Akt+diuron cells, we first analyzed the expression level of several actors of the DNA methylation and DNA demethylation machineries. RT-qPCR analyses indicated that *APOBEC3*γ and *TDG* were overexpressed in Ntv-a/Akt+diuron cells compared to Ntv-a/Akt cells (Figure 3C).

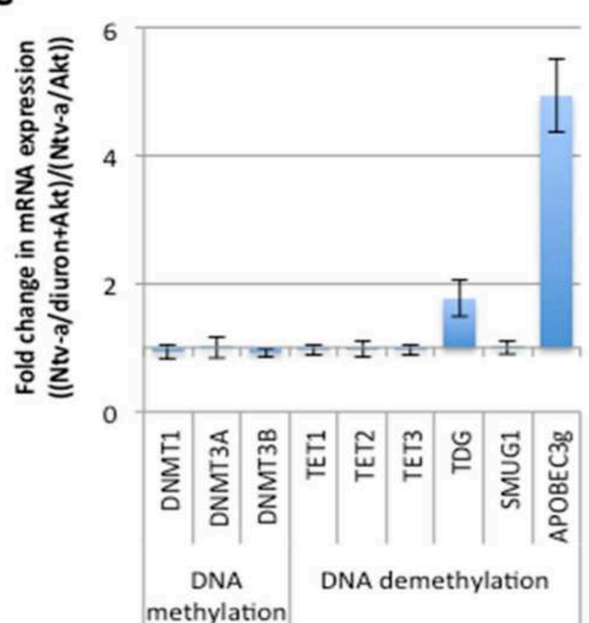


Figure 3: Global DNA hypomethylation occurs in Akt+diuron-induced glioma.

C. Graph represents the fold change expression in mRNA encoding for DNA methylation players (DNMT1, 3A and 3B) and DNA demethylation players (TET1, 2 and 3, TDG, SMUG1 and APOBEC3•).

In addition, we also studied the integrity of the DNMT1/UHRF1/PCNA complex through the integrity of the DNMT1/UHRF1 interaction. This focus is due to the fact that the loss of DNMT1/UHRF1/PCNA integrity promotes global DNA hypomethylation and inducestumorigenesis[3-4]. Our study reports that the DNMT1/UHRF1 signals decrease in Ntv-a/Akt+diuron cells in comparison with Ntv-a/Akt cells (Figure 3D). Thus, we conclude that the loss of DNMT1/UHRF1/PCNA and the APOBEC3γ overexpression are the two leading molecular causes of the global DNA hypomethylation seen in cells at the origin of Akt+diuron-induced glioma.

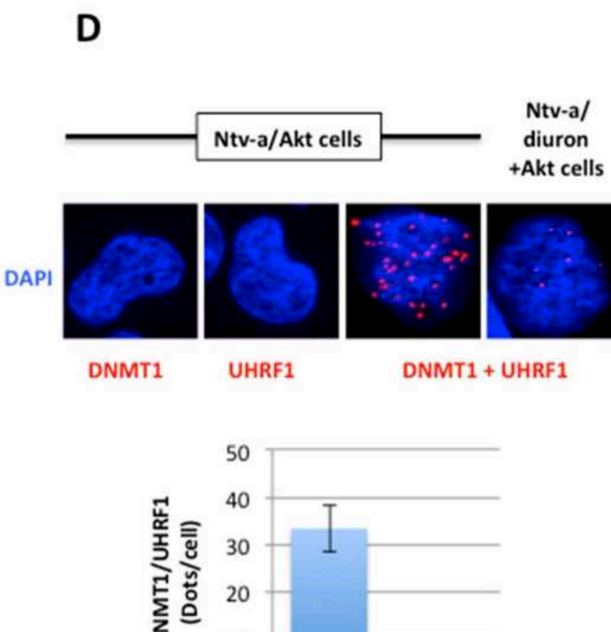


Figure 3: Global DNA hypomethylation occurs in Akt+diuron-induced glioma.

D. ApoTome view of the DNMT1/UHRF1 dots in indicated cells. Red dots symbolize the DNMT1/UHRF1 interactions. Nucleus/DNA are stained in blue via the use of DAPI. Graph (mean ± SEM) illustrating the number of DNMT1/UHRF1 dots per cells in indicated cells. The number of DNMT1/UHRF1 dots is calculated from the analysis of, at least, 50 nucleus in three independent experimentations. The absence of dots in presence of the unique use of DNMT1 or UHRF1 antibodies underlines the specificity of signal obtained in presence of the DNMT1+UHRF1 antibodies.

The above data support the hypothesis that Akt induces the global DNA hypomethylation through the disruption of the DNMT1/PCNA/UHRF1 complex via the DNMT1 phosphorylation, such as previously described[3]. To address this point, we analyzed the impact of an Akt inhibitor on the level of pDNMT1, the level of DNMT1/UHRF1 interactions and 5mC. Our data confirmed that Akt overexpression promotes the DNMT1 phosphorylation, the decrease of DNMT1/UHRF1 interaction, and the decrease of 5mC in DNA studied (Figure 3E). We also observed that Perifosine, an Akt inhibitor, limited the DNMT1 phosphorylation and restored the DNMT1/UHRF1 interactions but is ineffective to restore the global level of DNA methylation (Figure 3E).

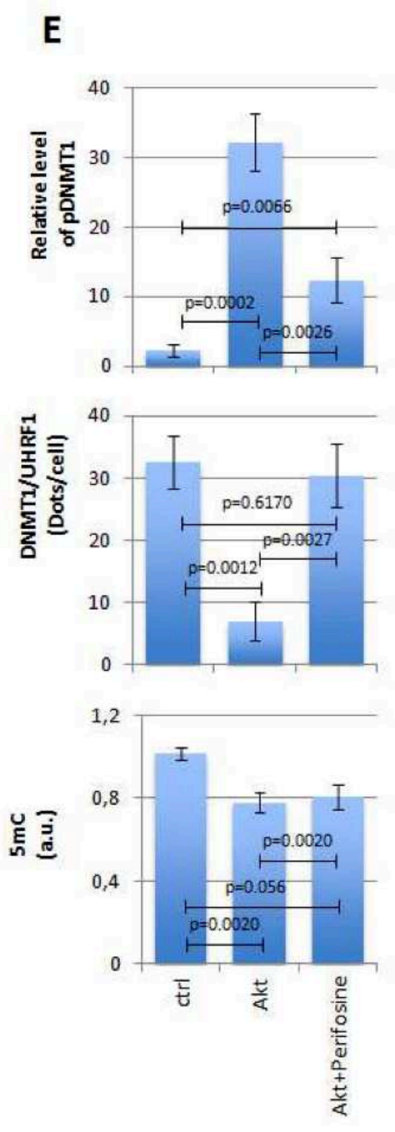


Figure 3: Global DNA hypomethylation occurs in Akt+diuron-induced glioma.

E. Graphs illustrate the effect of Akt overexpression and/or inhibition (via the use of Perifosine) on the relative level of phospho-DNMT1 (pDNMT1) (top), the DNMT1/UHRF1 interactions (middle) and on the global level of 5-methylcytosine (5mC, bottom). P-LISA estimates the number of DNMT1/UHRF1 interactions. In cell ELISA performed with anti-pDNMT1S127 was used to analyze the DNMT1 phosphorylation such as previously described3. ELISA-5mC (Zymo Research, France) was used to quantify the 5methylcytosine (5mC) i.e. to study the global level of DNA methylation.

Concerning the diuron-mediated global DNA hypomethylation, our data suggest a mechanism mainly involving the APOBEC3γ overexpression. To investigate this point, we analyzed the impact of a siRNA-mediated APOBEC3γ down-regulation on the global level of 5mC. Our results indicated that siRNA-APOBEC3γ abrogated the diuron-mediated global DNA hypomethylation, while siRNA-A (control) has no effect on the diuron-mediated global DNA hypomethylation (Figure 3F).

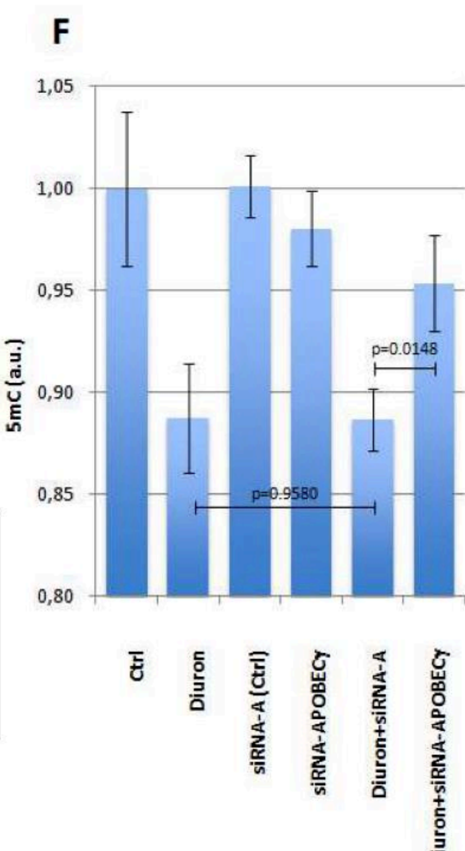


Figure 3: Global DNA hypomethylation occurs in Akt+diuron-induced glioma.

F. Graph illustrates the modification of 5-methylcystosine seen in cells treated with diuron and/or siRNA directed against APOBEC3•. ELISA-5mC (Zymo Research, France) was used to quantify the 5methylcytosine (5mC) i.e. to study the global level of DNA methylation.

Cells promoting Akt+diuron-induced gliomas are resistant to the temozolomide/irradiation-induced cell death through the Bcl-w overexpression.

The standard anti-GBM therapy includes a surgical resection followed by radiation and chemotherapy using temozolomide. Thus, we first studied whether Ntv-a/Akt+diuron cells are sensitive to the temozolomide/radiation therapy. For this purpose, we have calculated the percentage of cell death in Ntv-a/LacZ (Ctrl), Ntv-a/Ras+Akt and Ntv-a/Akt+diuron. For this purpose, cells were treated with 25µM of temozolomide (TMZ) for 72h and irradiated (2 Gy) (Figure 4A). Thus, we noted that Ntv-a/Ras+Akt and Ntv-a/Akt+diuron cells were strongly resistant to the temozolomide/radiation-induced cell death since this treatment promoted the death cell in a small percentage of cells (5% and 15%, respectively) (Figure 4B). In order to determine a molecular cause at the origin of this resistance, we analyzed the expression level of 11 apoptotic players. RT-qPCR experiments indicated that mRNA encoding for two anti-apoptotic proteins were overexpressed in Ntv-a/Akt+diuron cells in comparison with Ntv-a/Akt cells: 3 fold for *Bcl-xl* and near to 10 fold for *Bcl-w* (Figure 4C).

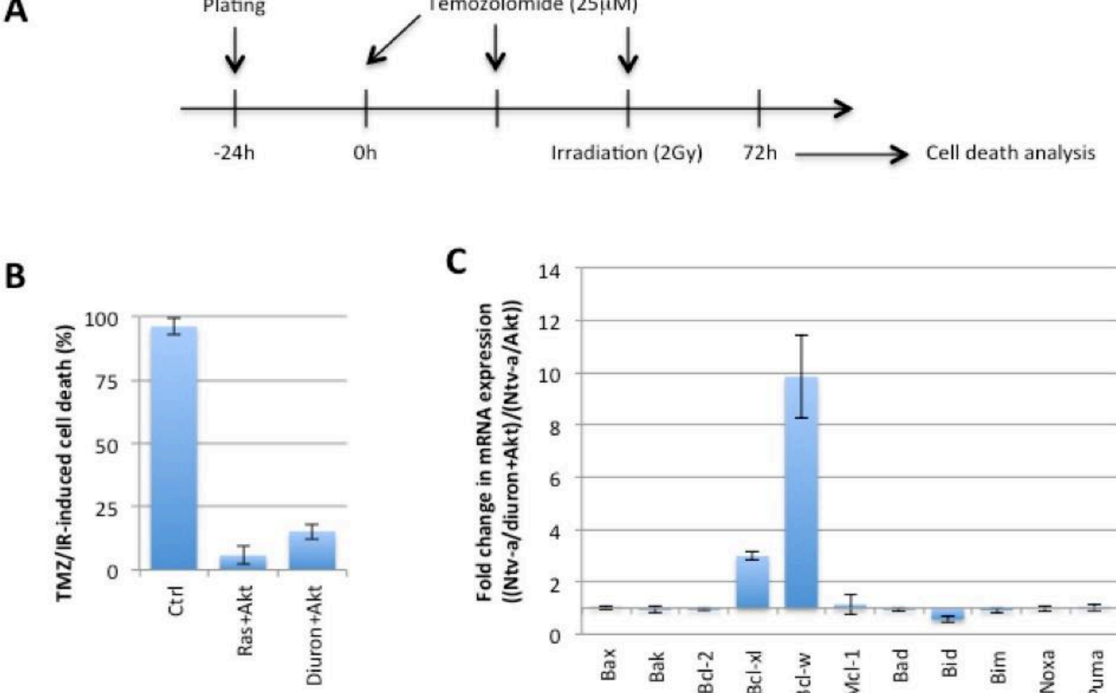
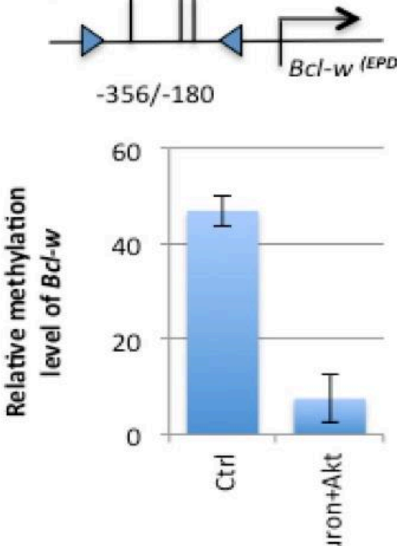


Figure 4: The Akt+diuron-induced gliomas are resistant to the temozolomide/irradiation-induced cell death.

A. Schematic representation of the temozolomide/irradiation-induced cell death.

B. Graph compares the percentage of Temozolomide/Irradiation-induced cell death in indicated cells according to Trypan blue method.

C. Graph represents the fold change expression in mRNA encoding for 11 proteins of the BCL2 family.



qMSRE analyzing the methylation level of the *Bcl-w* promoter indicated that the promoter region of *Bcl-w* was hypomethylated in Ntv-a/Akt+diuron cells in comparison with Ntv-a/Akt cells (Figure 4D).

Figure 4: The Akt+diuron-induced gliomas are resistant to the temozolomide/irradiation-induced cell death.

D. qMSRE estimates the relative level of DNA methylation of Bcl-w promoter.

In order to determine whether the diuron-induced Bcl-w overexpression played a crucial role on the temozolomide/radiation resistance, we then measured the percentage of temozolomide/radiation-induced cell death in Akt+diuron cells presenting a Bcl-w down-regulation induced through the use of siRNA directed against Bcl-w (Figure S2). This experiment indicated that siRNA-Bcl-w induced gain of 26% of TMZ/IR-induced cell death (Figure 4E). This finding confirms that the Bcl-w overexpression plays a role in the TMZ/IR-resistance phenotype seen in cells treated with Akt+diuron.

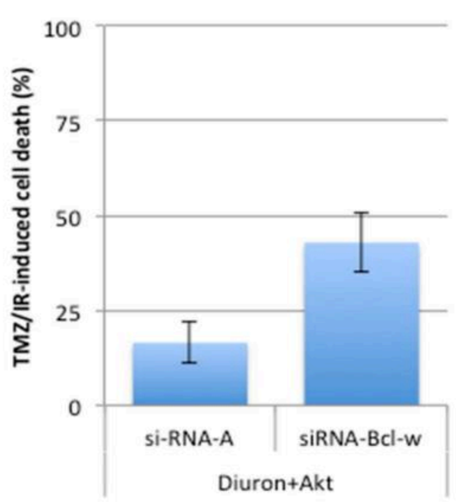


Figure 4: The Akt+diuron-induced gliomas are resistant to the temozolomide/irradiation-induced cell death.

E. Graph compares the percentage of Temozolomide/Irradiation-induced cell death in indicated cells according to Trypan blue method.

Cells promoting Akt+diuron-induced gliomas are characterized by the DNA hypomethylation-mediated PD-L1 and LLT1 overexpression.

Among the various mechanisms of resistance acquired by tumor cells during tumorigenesis, there is the immune system evasion. A pleiotropic number of molecular signatures can be associated to the immune escape of glioma. Here, we have decided to focus our study on 4 main actors of the immune escape of glioma cells: PD-L1 (since PD-L1 is upregulated in glioma[25]), Fas (a crucial player of the immune system-induced apoptosis[26]), DcR3 (a negative regulator of Fas[27]) and LLT1 (since LLT1 acts as a mediator of immune escape[28]). Our data indicated that Akt+diuron cells overexpressed PD-L1 and LLT1 (Figure 5A).

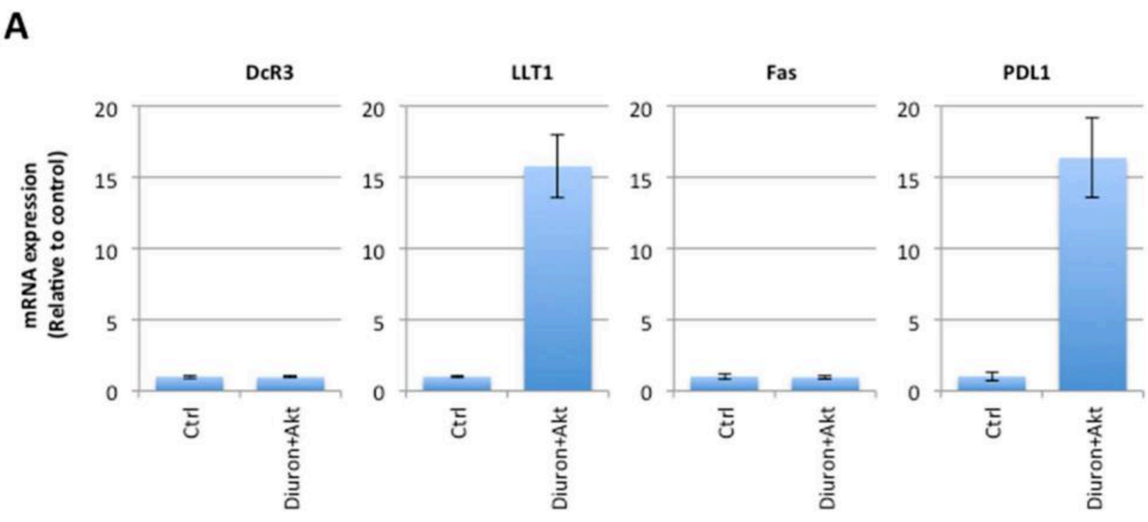


Figure 5: Akt+diuron-induced gliomas are characterized by the DNA hypomethylation-mediated PD-L1 and LLT1 overexpression.

A. Graphs compare the relative mRNA expression encoding for immune players (DcR3, LLT1, and PD-L1 for immunotolerance, and Fas for immunostimulation) in control (Ctrl) and diuron+Akt cells.

qMSRE analyses indicated that LLT1 and PD-L1 promoter were hypomethylated in Akt+diuron cells (Figure 5B and Figure 5C).

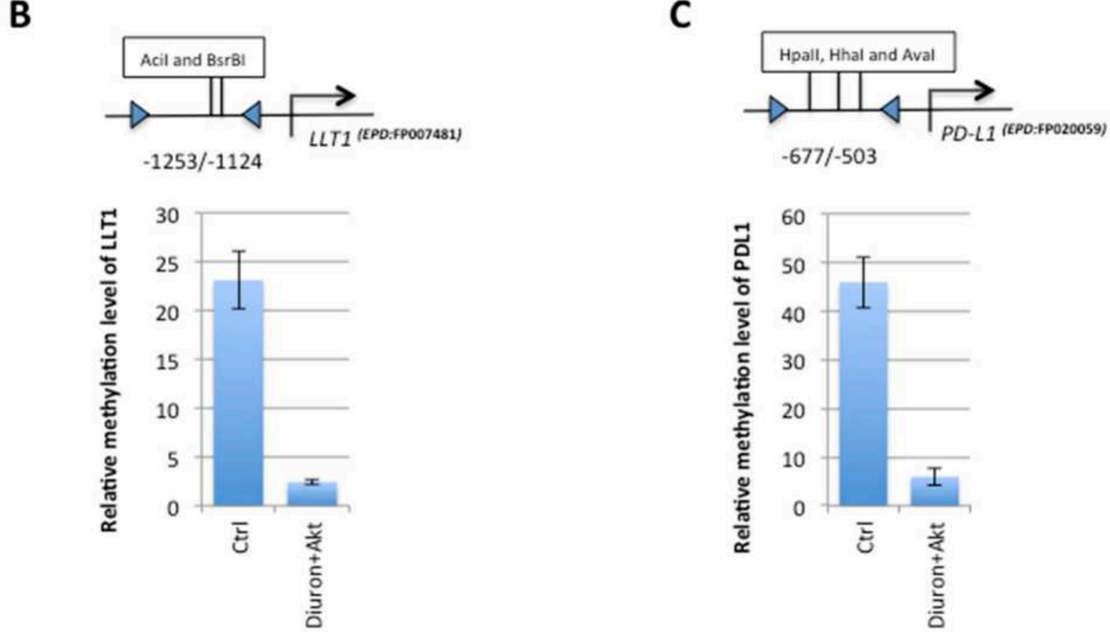


Figure 5: Akt+diuron-induced gliomas are characterized by the DNA hypomethylation-mediated PD-L1 and LLT1 overexpression.

B. qMSRE estimates the relative level of DNA methylation of LLT1 promoter

C. qMSRE estimates the relative level of DNA methylation of PD-L1 promoter.

Hypomethylation of *Bcl-w* and *PD-L1* promoters is associated with the APOBEC3γ overexpression, while hypomethylation of *LLT1* promoter is associated with the DNMT1/PCNA/UHRF1 disruption.

Akt+diuron cells presenting an APOBEC3γ overexpression and a loss of DNMT1/UHRF1/PCNA, we hypothesized that these 2 causes of DNA hypomethylation could be involved in the hypomethylation of *Bcl-w, PD-L1* and *LLT1* promoters. To test this hypothesis, APOBEC3γ was overexpressed by transfecting a plasmid coding for this protein (Figure S3), while the loss of DNMT1/UHRF1/PCNA complex was obtained through the use of a plasmid encoding for a peptide disrupting this complex (named UP, such as previously described[3-4]). qMSRE analyses indicated that the APOBEC3γ overexpression promoted the *Bcl-w* and *PD-L1* hypomethylation, while the UP-induced loss of DNMT1/UHRF1/PCNA complex induced the LLT1 hypomethylation (Figure 6).

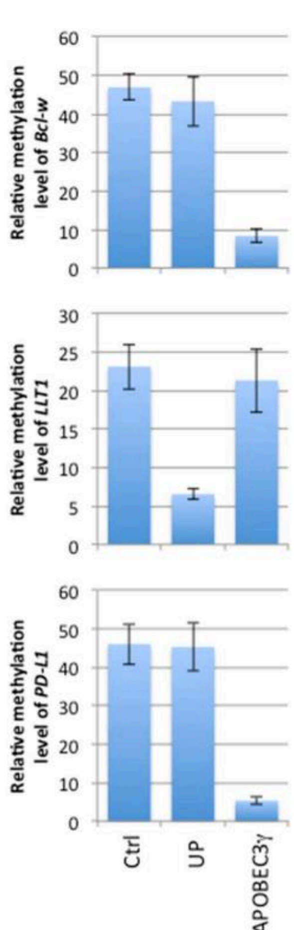


Figure 6: APOBEC3 promotes the PD-L1 and Bcl-w demethylation, while the DNMT1/PCNA/UHRF1 disruption promotes the LLT1 demethylation.

qMSRE (as previously described) compare the relative methylation level of Bcl-w, LLT1 and PD-L1 genes in cells transiently transfected with the pcDNA.3.1 (Ctrl), pcDNA.3.1-UP plasmid and with pcDNA.3.1-APOBEC3· plasmids. pcDNA.3.1-UP is a plasmid encoding for a chimeric protein having the ability to selectively disrupt the DNMT1/UHRF1 interaction3-4.

Presence of concomitant *LLT1, PD-L1* and *Bcl-w* hypomethylation in GBM patients.

Our data indicate that the gliomagenesis induced by the Akt overexpression and Diuron exposure is associated with the hypomethylation of the *LLT1, PD-L1* and *Bcl-w* genes. In other terms, our data indicate that the *LLT1, PD-L1* and *Bcl-w* hypomethylation are associated with the Diuron-induced modifications of methylome. Based on this finding, we hypothesized that GBM patients having had a potential exposure to Diuron via their professional activity could have a tumors characterized by the concomitant *LLT1, PD-L1* and *Bcl-w* hypomethylations. To investigate this idea, a GBM sample cohort of 23 patients was used (Supplementary table T1). Interestingly, two of these patients have had a potential exposure to Diuron via their professional activity (group#A), while all other patients have a supposed absence of Diuron contact (group#B). qMSRE indicated that the concomitant *LLT1, PD-L1* and *Bcl-w* hypomethylation (defined by a relative methylation level inferior to 5) was only observed in group#A and not in group#B (Figure 7). We also noted that the two GBM patients included in group#A have high levels of APOBEC3γ and Akt expressions (Figure 7).

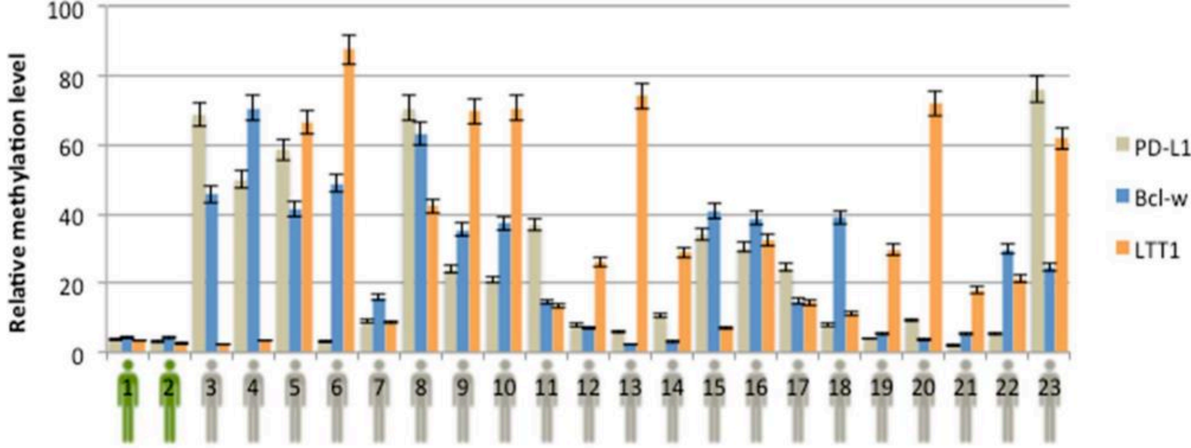


Figure 7: Methylation status of PD-L1, Bcl-w and LLT1 genes in GBM patients putatively exposed to diuron via their professional activity (green, n=2) or not (grey, n=21).

qMSRE (as previously described) compare the relative methylation level of Bcl-w, LLT1 and PD-L1 genes. APOBEC3· and AKT expressions were estimated by ELISA method. GBM patients potentially exposed to diuron via their professional activity (farmer, gardener, ...) are in green (n=2), and GBM patients potentially unexposed to diuron via their professional activity are in grey (n=21).

DISCUSSION

During the last decades, several works define the gliomagenesis as a tumor development generated by multistep and/or chromothripsis (a phenomenon characterized by thousands of chromosomal rearrangements) processes that involve various genetic and/or epigenetic alterations. Thus, during tumor initiation step, genetic and/or epigenetic alterations can promote the acquisition of cancer hallmarks such as the apoptosis and immune evasion. In previous articles, we and others have begun to unveil the mechanisms through which DNA hypomethylation or the Akt/Ras overexpression can promote gliomagenesis and the acquisition of apoptosis and immune evasion phenotypes [3-4-29-30]. However, in these papers, DNA hypomethylation was induced by a chimeric peptide having the ability to disrupt the integrity of the main complex responsible for the DNA inheritance, the DNMT1/PCNA/UHRF1 complex.

In the present article, we report that Akt overexpression and diuron exposure promote the glioma formation from neural progenitor cells via the induction of DNA hypomethylation mediated by two distinct pathways: the loss of DNMT1/PCNA/UHRF1 interactions and the APOBEC3γ overexpression. The identification of these 2 molecular causes of hypomethylation reinforce the idea that DNMT1/PCNA/UHRF1 play a crucial role in the maintenance of DNA methylation level, such as already described in several articles[31-32-3-4]. This last point provides a new evidence for the involvement of APOBEC3γ in DNA demethylation mechanisms[33-34], despite the discussion surrounding the role of APOBEC3γ on these mechanisms (a discussion mainly associated with the publication of Wijesinghe and Bhagwat[35]).

Our work is the first to report the evidence that the diuron exposure can promote to the gliomagenesis. However, in gliomagenesis, the oncogenic effect is not due to itself since diuron needs to be associated to Akt overexpression to promote the gliomagenesis. The involvement of diuron in carcinogenesis is not new since diuron is already reported for the bladder[14-15], urothelial[16], skin[17-18] and mammary[15-19] carcinogenesis. Nevertheless, none of these publications mentioned an impact of diuron on the global level of DNA methylation and on the methylation level of certain gene promoters.

The fact that diuron needs to be associated with another oncogenic hit (Akt overexpression) to promote gliomagenesis reinforces the idea that tumorigenesis can results from the accumulation of several hits such as mentioned in the Knudson hypothesis, also known as the two-hit hypothesis[36-37] .

To our knowledge, the impact of diuron on methylome is to date only reported in oyster [38-39-40]. More generally, several publications report the fact that pesticide exposure affects the methylome. Recently, Rusiecki et al. (2017) reports that pesticide exposure has been associated with acute and chronic adverse health effects and that DNA methylation may mediate these effects [41]. Besides in this article, the authors report that pesticide exposure could modulate the *MGMT* methylation. In our study, we noted that the methylation of

MGMT methylation was unchanged (Figure S4). We also observed that the two GBM patients potentially exposed to diuron via their professional activity are "MGMT unmethylated". Zhang et al. (2012) report that diazinon-treated cells exhibited increased DNA methylation levels in certain genes defined as tumor suppressor genes such as RASSF1A and PTEN. A finding supporting the idea that the diazinon-induced hypermethylated could play a pathological role in cancer development [42]. On contrary to this study, our work shows that the Akt+diuron-induced tumors are characterized by both global and local DNA hypomethylations. Our data clearly indicated that the diruon+Akt-induced tumorigenesis is associated with the hypomethylation and the overexpression of genes that responsible for apoptosis (Bcl-w) and immune evasion (PD-L1 and LLT1). Indeed, *bcl-w/bcl2l2* (bcl-2-like protein 2) gene encodes an anti-apoptotic protein which expression is regulated by DNA methylation in glioma [43-44]. In addition, Bcl-w expression contributed to the aggressiveness of glioma [45]. Lectin-like transcript-1 (LLT1) is a newly identified ligand for the inhibitory natural killer (NK) cell receptor CD161 which the expression acts as a mediator of immune escape and contributes to the immunosuppressive properties of glioma cells [28]. PD-L1 is a protein encoded by the CD274 gene, which plays a major role in suppressing the immune response. The high PD-L1 expression is associated with a worse outcome of patients suffering from GBM according to certain study [46], while others studies contradict this finding [47]. Be that as it may on this point, our observation associating the Akt+diuron-induced demethylation/overexpression of Bcl-w, PD-L1 and LLT1 is consistent with the Akt+diuron-induced gliomagenesis. In other terms, it appears that diuron promotes the reprogramming of expression of certain apoptotic and immune actors. Other pesticides have the same effect. Thus, we recently observed that a pesticides mixture modulates the Mcl1 expression in MSC cells[48].

Finally, we made the dual observing that 1) the gliomagenesis induced by the Akt overexpression and diuron exposure is associated with the hypomethylation of the *LLT1, PD-L1* and *Bcl-w* genes and 2) the concomitant *Bcl-w, PD-L1* and *LLT1* hypomethylation occurs in 2/2 tumors of patients having had a potential exposure to diuron via their professional activity. We did not observe this situation in 21 tumors of patients devoid of potential exposure to diuron via their professional activity. These observations are troubling and tend to support the idea that diuron exposure can play a role of one oncogenic hit on the road to gliomagenesis.

CONCLUSION

Since several years, a large number of studies analyzed the pesticide exposure and the carcinogenesis initiation. Our data strongly identify a causal link between diuron exposure and cancer development. However, our data underline that diuron is not oncogenic by itself since diuron promotes gliomagenesis only when its exposure is associated with the Akt overexpression (another non-oncogenic event of gliomagenesis by itself) in our model. Mechanistically, we determined that diuron promotes gliomagenesis through the induction of DNA methylation modification. Thus, a better understanding of the diuron-induced alterations of DNA methylation mechanisms could provide rational biomarkers for detecting diuron-induced glioma and to design efficient therapeutic strategies against these types of tumors. In addition to our finding, several reports mention the idea that the exposure of environmental chemicals can modulate the epigenome to potentialize or directly induce the carcinogenesis[49-50-51]. Thus, epigenetic reprogramming by environmental chemical could represent a novel mechanism to explain their carcinogen impact. Thus, without being oncogenic alone, exposure to an environmental chemical such as diuron could be the first step on the carcinogenesis road. And this step needs to "be complete" by other(s) step(s) (such as exposure to other(s) environmental chemical(s) or the genes overexpression, mutation, or deletion, etc...) in order to arrive at its term i.e. the tumor formation (Figure 8).

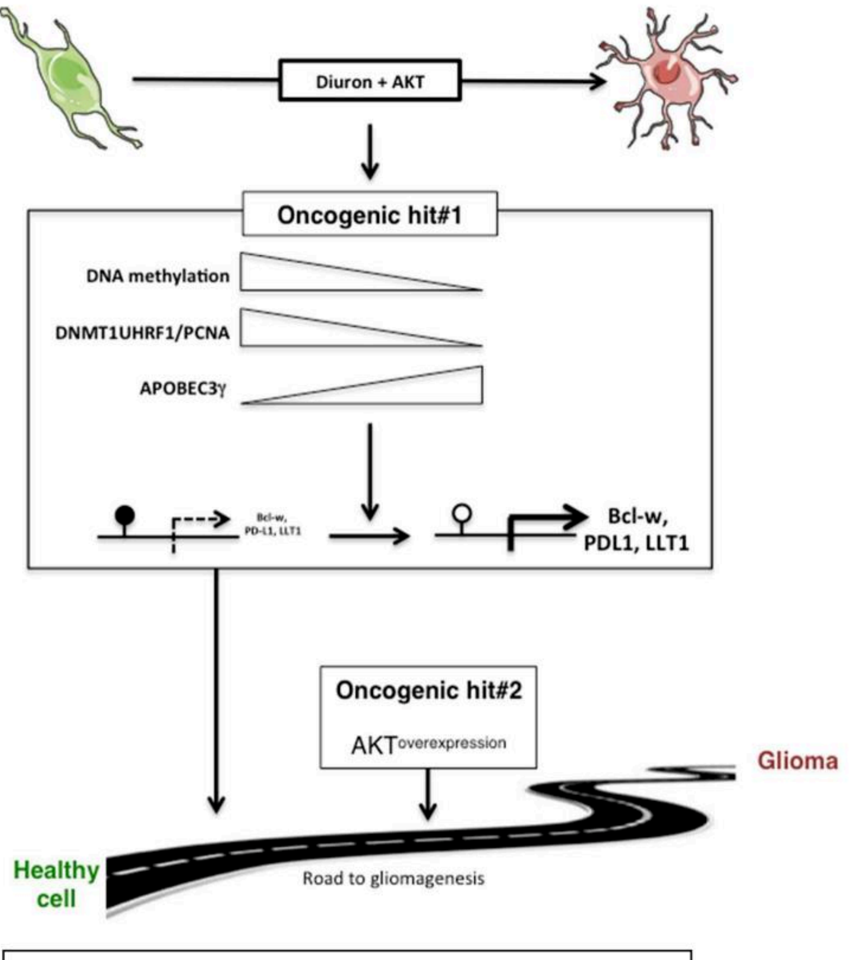


Figure 8: Signaling pathway of the Akt+diuron-induced

ABBREVIATIONS

P-LISA: Proximity ligation In situ Assay. 5mC : 5methylcystosine.

qMSRE: quantitative methylation-sensitive restriction enzyme digestion.

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the Ethics Committee of LaBCT and was performed according to the Helsinki Declaration.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. All other datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Conflicting interests

The authors declare that they have no conflicting interests.

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Authors' contributions

PFC designed and coordinated the project.

JB, AN, GBC, and PFC performed all experiments.

JB, GCB, CB, FMV and PFC interpreted and discussed the data.

PFC wrote the first version of the manuscript and all authors reviewed and approved it.

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SUPPLEMENTARY FIGURES

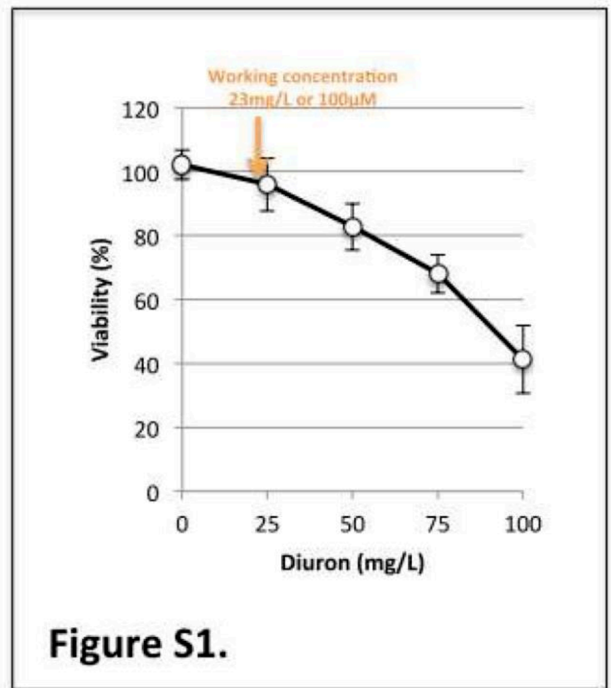


Figure S1.

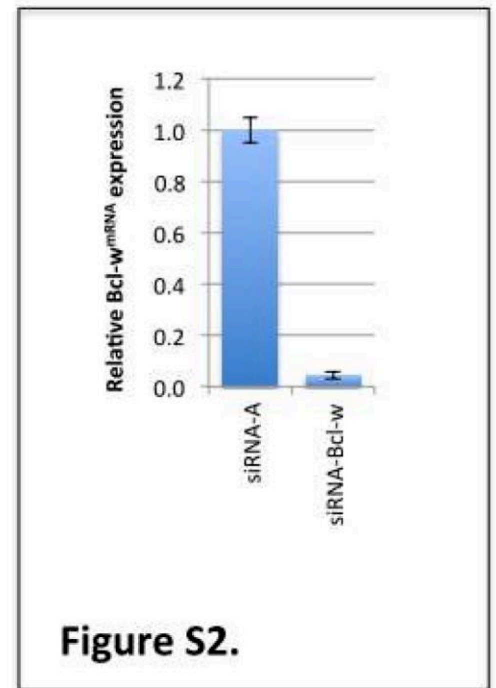


Figure S2.

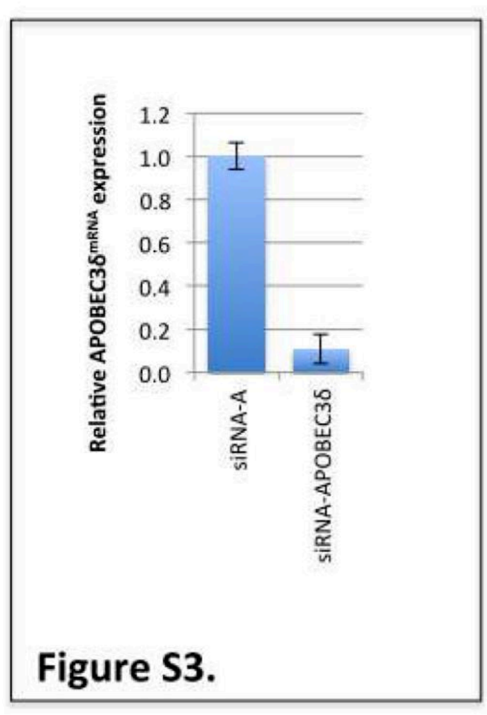


Figure S3.

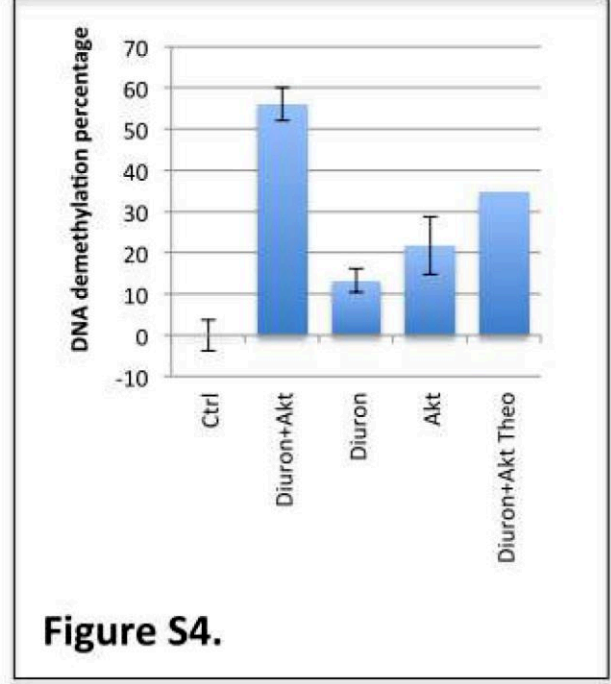


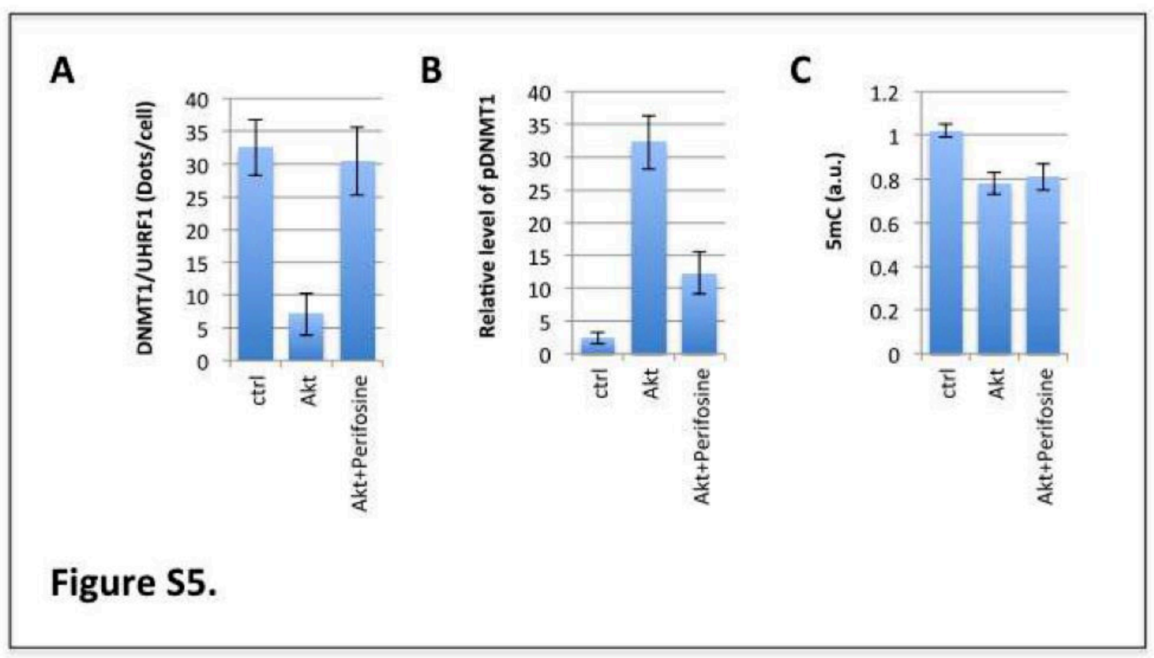
Figure S4.

Figure S1. Cell viability of Ntv-a/lacZ cells exposed to diuron.

Different doses of diuron were incubated on Ntv-a/lacZ cells. XTT Cell Viability Kit (Ozyme, France) was used to calculate the percentage of cell viability after 48h of diuron. The values are means±SD from three independent experiments performed in duplicate.

Figures S2 and S3. qRT-PCR were done to validated the siRNA downexpression. Down-regulation of Bcl-w and APOBEC3• were performed via cells transient transfection with siRNA-Bcl-w (Santa-Cruz, sc-37294, France) and siRNA-APOBEC3• (Santa-Cruz, sc-60091, France). siRNA-A is a control (Santa-Cruz, sc-37007, France).

Figure S4. Graph illustrates the percentage of DNA demethylation seen in indicated cells. This percentage is calculated from data obtained with 5-methylcytosine ELISA (Zymo Research,, France). “Diuron+Akt Theo (theoric)” represents the addition of values obtained from Akt and Diuron.



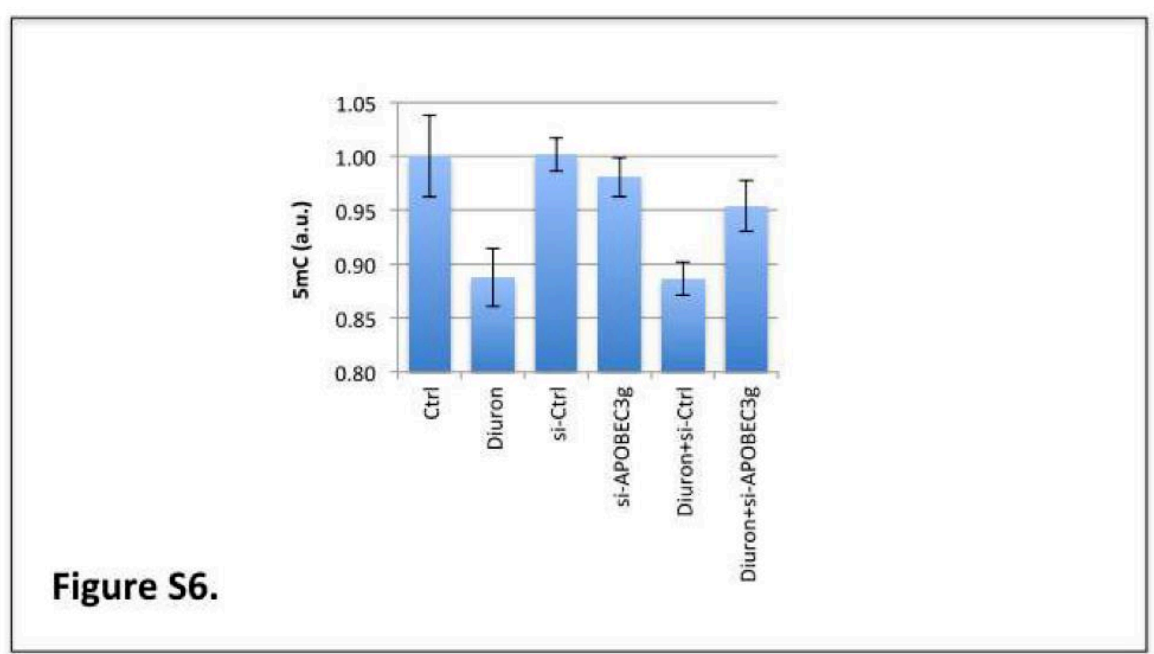


Figure S5. Graphs illustrate the effect of Akt overexpression on the DNMT1/UHRF1 interactions (A), the relative level of phospho-DNMT1 (pDNMT1) (B) and on the global level of 5-methylcytosine (5mC) (C). P-LISA estimates the number of DNMT1/UHRF1 interactions. ELISA performed with anti-pDNMT1S127 was used to analyze the DNMT1 phosphorylation such as previously described3. ELISA-5mC (Zymo Research,, France) was used to quantify the 5methylcytosine (5mC) i.e. to study the global level of DNA methylation.

Figure S6. Graph illustrates the modification of 5-methylcystine seen in cells treated with Diuron and/or siRNA directed against APOBEC3•. ELISA-5mC (Zymo Research, France) was used to quantify the 5methylcytosine (5mC) i.e. to study the global level of DNA methylation.

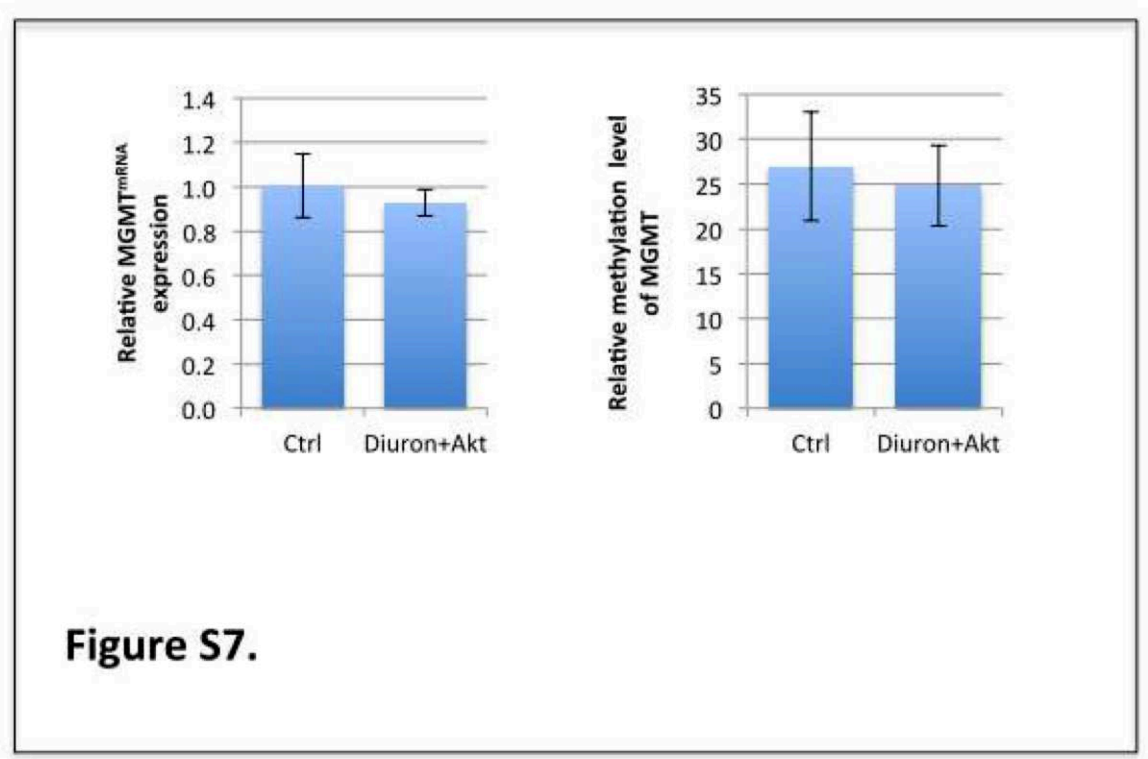


Figure S7. Graphs illustrate the effect of Diuron+Akt on the MGMTmRNA expression (A) and the MGMT methylation level (B). RT-qPCR estimates the MGMTmRNA expression. qMSRE (OneStep qMethyl™ Kit - Zymo Research, France) estimates the MGMT methylation level.

Article 2 - Diuron modulates the DNA methylation status of the ILT7 and TRAIL/TNFSF10 genes and decreases the killing activity of plasmacytoid dendritic cells

Le diuron étant retrouvé dans le sang, nous nous sommes intéressés à son impact sur le système immunitaire [297]. Bien qu'un article ai déjà montré un impact du diuron sur le système immunitaire, cette étude a eu lieu chez le rat, et contrairement à notre article ne s'intéresse pas au volet épigénétique [298].

Nous avons démontré que le diuron était capable de diminuer la cytotoxicité des pDC envers les cellules tumorales, en induisant une déméthylation des promoteurs de TRAIL et ILT7. TRAIL est impliqué dans l'immunostimulation, au contraire d'ILT7 qui joue un rôle dans l'immunotolérance. Les promoteurs de ces deux gènes sont déméthylés, pourtant les effets sont opposés : la transcription d'ILT7 augmente, alors que celle de TRAIL diminue. Dans le premier cas, la réponse correspond au dogme disant qu'un gène avec un promoteur déméthylé sera transcrit, mais dans le cas de TRAIL ce dogme n'est pas respecté. En effet, la déméthylation de TRAIL induit une diminution de la transcription. Ce phénomène est dû au recrutement de CTCF, un répresseur transcriptionnel, sur ce promoteur.

Les mécanismes épigénétiques sont donc d'importants régulateurs du système immunitaire et permettent une meilleure compréhension de l'impact du diuron sur les pDC.

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Diuron modulates the DNA methylation status of the ILT7 and TRAIL/TNFSF10 genes and decreases the killing activity of plasmacytoid dendritic cells

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Abstract

Background: Plasmacytoid dendritic cell (pDC) is described as the Swiss knife of immune system. Thus, the understanding of aberrant epigenetic reprogramming of genes governing the pDC functionality by pollutants appears such as an attractive research point.

Results: Our study has investigated the effect of Diuron (an herbicide) on the pDC-killing activity towards cancer cells. Thus, we observed that the Diuron exposure of pDC promotes a context of global DNA hypomethylation, which is associated with a phenotype of decrease of the killing activity of pDC towards cancer cells. At molecular level, our data associated the Diuron-induced global DNA hypomethylation with the elevated expression of TET2, an epigenetic player involved in DNA demethylation processes, and the decrease of the pDC-killing activity with the decrease of TRAIL expression and the increase of ILT7 expression.

Conclusions: Thus, our article reports that a pollutant (Diuron) induces an epigenetic reprogramming of a subtype of immune cell (pDC), which decreases its killing activity towards tumors cells. In some context, this mechanism might be conducive to the initiation of pathologies.

Keywords: DNA methylation, Plasmacytoid dendritic cells, TET2, Diuron

Background

Originally described in human lymph nodes in the 1950s plasmacytoid dendritic cells (pDCs) are a rare type of immune cells since these cells constitute < 0.4% of peripheral blood mononuclear cells (PBMC) [1, 2]. Despite this relative rarity, pDC plays a crucial role in immune response since they link the innate and adaptive immune systems [3–5]. Thus, pDC has potential multifaceted roles in the pathogenesis of autoimmune diseases, allergy, cancer and human immunodeficiency virus (HIV) infection [6]. Consequently, the understanding of aberrant epigenetic reprogramming of certain genes governing functionality of pDC by pesticides appears such as an attractive research point. Besides literature reports that certain genes involved in pDC functionality can be epigenetically regulated by DNA methylation process. As example, pDCs can act as tolerogenic cells when expressing the programmed death 1 ligand (PD-L1) [7], and the PD-L1 expression can be regulated by the DNA methylation status of its promoter [8]. pDC-killing activity can be mediated by TRAIL [9] and Granzyme-B, which expression can be epigenetically regulated [10–12]. The ILT7 expression, in which the interaction with BST2 assures an appropriate TLR response by pDCs during viral infection and participates in pDC-tumor crosstalk, can be also epigenetically regulated by DNA methylation [13, 14].

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Diuron is a substituted urea compound used as herbicide and antifouling. Consequently, the Diuron toxicity can affect all the aquatic ecosystem (fish, algae, oyster, etc.) but also human health. Thus, literature reports that Diuron modulates the methylome of oysters [15–17], and affects the multixenobiotic resistance activity in Zebrafish [18]. Reports also mention a health risk for population exposed to Diuron [19–23].

The current study analyzed the putative effect of a Diuron exposure on the pDC-killing activity via the investigation of the DNA methylation status of genes regulating this activity.

Methods

Cell culture and exposure

The plasmacytoid dendritic cell line CAL-1 (Dr. T Maeda, Nagasaki University, Japan) has been derived from a blastic plasmacytoid dendritic cell neoplasm (BPDCN) patient [24]. These cells were cultured with RPMI 1640 supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO_2-air. Cells were exposed to Diuron (Santa Cruz, France) dissolved in dimethyl sulfoxide, (DMSO, Sigma). Control cells were exposed to 0.1% DMSO.

DNA extraction, 5mC ELISA and qMSRE

DNA extract is performed using QIAamp DNA Mini QIAcube Kit and QIAcube (Qiagen, France).

The quantification of 5-methylcytosine is performed using 5mC DNA ELISA kit (Zymo, Ozyme, France) according to the manufacturer's instruction.

qMSRE combines the use of methylation-sensitive restriction enzyme and real-time PCR. Briefly, 20 ng of DNA was digested or not by Hpy188III and HpyCH4IV. Then, 5 µL of digested or not mix solutions were used to perform qPCR using the Rotor-Gene SYBR Green PCR Kit and Rotor-Gene Q as real-time thermocycler (Qiagen, France). Primers are: ILT7-S: TGTGAGGACCCT GGACTTCCTTTT, ILT7-AS: CAGCCTGGGCAACAA GAACAAA, TRAIL-S: ATGCCTGTAATCCCAGCA CGTT and TRAIL-AS: GGTTTTACCATGTAGGCC AGGCT. The methylation level for any amplified region can be determined using the following equation: Percent Methylation $= 100 \times 2^{-\Delta Ct}$ where $\Delta Ct =$ the average Ct value from the digested reaction minus the average Ct values from the reference/undigested reaction. If DNA is methylated, Hpy188III and HpyCH4IV will not be able to digest DNA, and the DNA region will be amplified by PCR.

RNA extraction and RT-qPCR

RNA extract is performed using RNeasy Mini QIAcube Kit and QIAcube (Qiagen, France). RT-qPCRs are performed using QuantiTect Reverse Transcription Kit, Rotor-Gene SYBR Green PCR Kit, QuantiTect Primer Assays and Rotor-Gene Q as real-time thermocycler (Qiagen, France). Normalization of gene expression was performed using RPLP0. The $^{-}2^{\Delta\Delta Ct}$ method was used to calculate the fold change of mRNA expression between two conditions.

Protein extraction and TET2 ELISA

Protein extracts were obtained using RIPA Lysis and Extraction Buffer (Thermo Scientific, France) in accordance with the manufacturer's instructions. TET2 ELISAs were performed according to the manufacturer's instructions (MyBiosource, MBS9317739, USA).

siRNA transfection

In a six-well culture plate, 2×10^5 cells were incubated for 24 h at 37 °C in a CO_2 incubator. Then, 60 pmol of siRNA was added with 100 µL of siRNA transfection medium and the cells incubated for 7 h at 37 °C in a CO_2 incubator (Santa Cruz, France). Without removing the siRNA, 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration was added and cells incubated for 24 h. Then, cells were cultured for 48 h in normal culture medium. Thus, analyses were realized ~72 h after the siRNA transfection. The following siRNA were used: siRNA-A (control) (sc-37007, Santa Cruz) and TET2 (sc-88934, Santa Cruz, France).

Cytotoxicity assays

CAL-1 cells (7×10^5) were seeded overnight in a 24-well plate in 2 mL medium devoid of Diuron. A total of 1.1 mL medium was replaced with 100 µL FBS-free RPMI medium containing 12.5 µg/mL CpG B or Ctrl CpG B (Invivogen, France). The ability of pDCs to kill tumor cells was assessed in a classic Europium-TDA release assay (DELFIA; PerkinElmer), according to manufacturer's instructions. Briefly, tumors cells were labeled with the fluorescence-enhancing ligand BATDA, which was released into the supernatant after cytolysis. Tumor cells or PBMC and pDC were then co-cultured for 4 h at indicated E/T ratios. Supernatants were collected and the released Europium-TDA release was counted using a fluorometer. Target cells were treated with 1% NP40 and sonicated as a measure of maximal release. Target cells incubated without effector cells were used to measure spontaneous release. Percent of specific lysis was calculated using the following equation: % specific

lysis = [experimental release − spontaneous release]/[maximum release − spontaneous release] × 100.

Chromatin immunoprecipitation (ChIP) experiments

Briefly, cross-linking step was performed by adding 37% formaldehyde to a final concentration of 1% [12]. Cells were incubated on a rotator for 10 min at room temperature (RT), and formaldehyde was quenched by adding 1.25 M glycine to a final concentration of 125 mM. Cells were rocked for 5 min at RT, washed with cold PBS, and flash-frozen in liquid nitrogen. Cells were lysed in buffer containing 1% SDS, 5 mM EDTA, and 50 mM Tris–HCl (pH 8.0) with freshly added protease inhibitor. The chromatin was fragmented to 200–500 bp with a Bioruptor plus sonicator (Diagenode, France) at 4 °C. ChIP experiments were performed using ChIP-IT kit (Active Motif, France). The ChIP-grade Anti-CTCF (Abcam#Ab70303, France) and IgG (Abcam#AB2410, France) were used. DNAs eluted from ChIP were applied to real-time qPCR analysis using a QuantiFast SYBR green qPCR (Qiagen, France) on the Rotor-Gene Q (Qiagen, France). ChIP-quantitative PCR enrichment of target loci was normalized to input DNA.

Statistical analysis

All experiments were done at least in triplicates. Significance of the differences in means was calculated using Student *t* test. The probability level for statistical significance was $p < 0.05$ throughout the study.

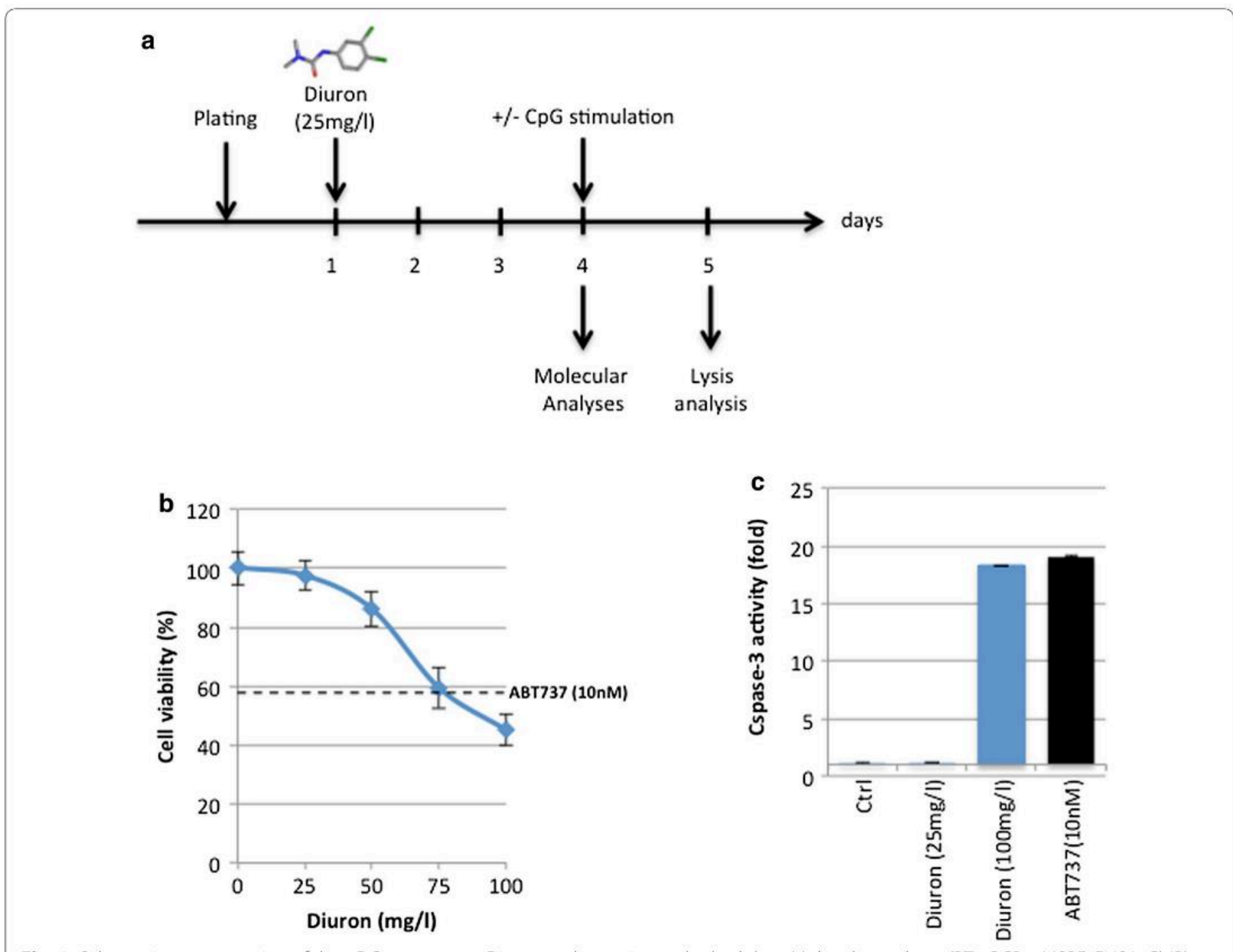

Fig. 1 Schematic representation of the pDC exposure to Diuron and experimental schedule. **a** Molecular analyses (RT-qPCR, qMSRE, ELISA, ChIP) were performed 72 h after the Diuron exposure (25 mg/L). Lysis assay was performed 96 h after the Diuron exposure (25 mg/L). **b** The impact of Diuron on the viability of pDC was investigated using the XTT Cell Viability Kit (Cell Signaling, France). ABT737, a BH3 mimetic drug, was used as positive control for apoptosis induction. **c** Caspase-3 activity (Caspase-3 assay kit, Abcam, France) was measured to determine whether the pDC exposure to Diuron or ABT737 promoted apoptosis. Ctrl represents control exposure performed with DMSO

Results

Diuron exposure promotes the TET2-mediated DNA hypomethylation of pDC

Literature reports that Diuron affects the global DNA methylation level [15–17]; we first investigated whether Diuron exposure could affect the global DNA methylation level of the pDC-like cell line CAL-1. For this purpose, CAL-1 cells were expose to 25 mg/L of Diuron for 72 h since this herbicide and four of its metabolites had a total concentration as high as 100 mg/L in plasma and urine [25] (Fig. 1a). The dose of 25 mg/L of Diuron was also chosen since it had no effect on the cell viability, on contrary to ABT737, a BH3 mimetic drug-inducing apoptosis [26, 27] (Fig. 1b). We also observed that the dose of 25 mg/L of Diuron was unable to promote Caspase-3 activation, on contrary to ABT737 (Fig. 1c). The dose of 25 mg/L of Diuron being devoid of toxicity/apoptogenicity and "compatible" with a dose observed in human blood, we decided to continue our study with this dose.

The global DNA methylation level was here estimated by ELISA quantifying the presence of 5-methylcytosine (5mC). Thus, we noted that Diuron exposure decreased the percentage of 5mC (Fig. 2a). In other terms, Diuron promotes the global DNA hypomethylation of pDC.

RT-qPCR performed to quantify the mRNA encoding for the major DNA methylation players shown that the Diuron exposure promoted the $TET2^{mRNA}$ overexpression (Fig. 2b). Our study also indicated that the Diuron exposure promoted the TET2 protein overexpression (Fig. 2c).

Diuron exposure of pDC decreases the killing activity of these cells

To analyze the impact of the Diuron-induced DNA hypomethylation on the pDC-killing activity, we have compared the cell lysis activity of CpG-stimulated pDC previously exposed or not to Diuron (Fig. 1). The calculation of the cell lysis percentage indicated that the Diuron exposure decreased the killing activity of these cells toward several tumor cells such as glioblastoma cells (Fig. 3a), breast cancer cells (Fig. 3b), and ovarian cancer cells (Fig. 3c), but not toward PBMC (Fig. 3d).

The Diuron-mediated decreased killing activity of pDC is associated with the modulation of the methylation status of ILT7 and TRAIL

Several molecular actors play a crucial role in the killing activity of pDC. In our study, we have focused our analysis on PD-L1, ILT7, TRAIL and Granzyme-B, i.e., on genes whose expression can be epigenetically regulated. The RT-qPCR analyses comparing the CpG-stimulated pDC pre-exposed or not to Diuron indicated that Diuron exposure increased the ILT7 ^{mRNA} expression and decreased the $TRAIL^{mRNA}$ expression. No expression changes were observed for $PD\text{-}L1^{mRNA}$ and $Granzyme\text{-}B^{mRNA}$ (Fig. 4a). ELISA experiments

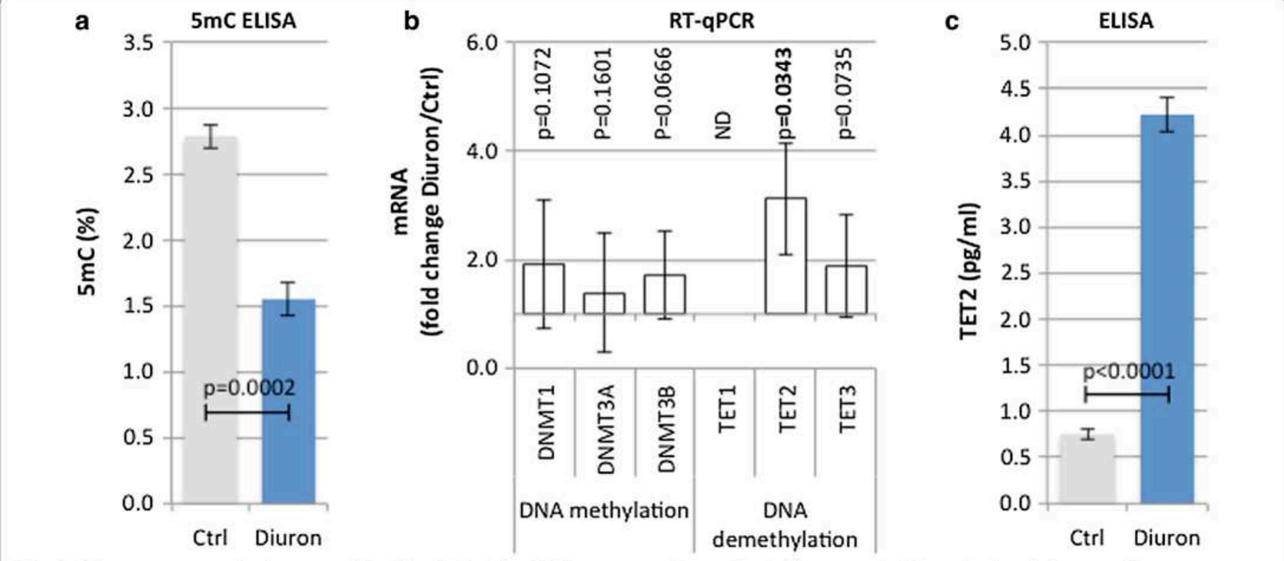

Fig. 2 Diuron promotes the decrease of 5mC in pDC via the TET2 overexpression. **a** Graph illustrates the Diuron-induced decease of 5-methylcytosine (5mC) in pDC exposed to Diuron. Ctrl represents control exposure performed with DMSO. ND: not detected. **b** Graph illustrates the fold change expression of mRNA encoding for the major DNA methylation players that are DNMT1, DNMT3A, DNMT3B, TET1, TET2 and TET3 compared to control. Ctrl represents control exposure performed with DMSO. **c** Graph from ELISA analysis illustrates the Diuron-induced increase of TET2 in pDC exposed to Diuron. Ctrl represents control exposure performed with DMSO

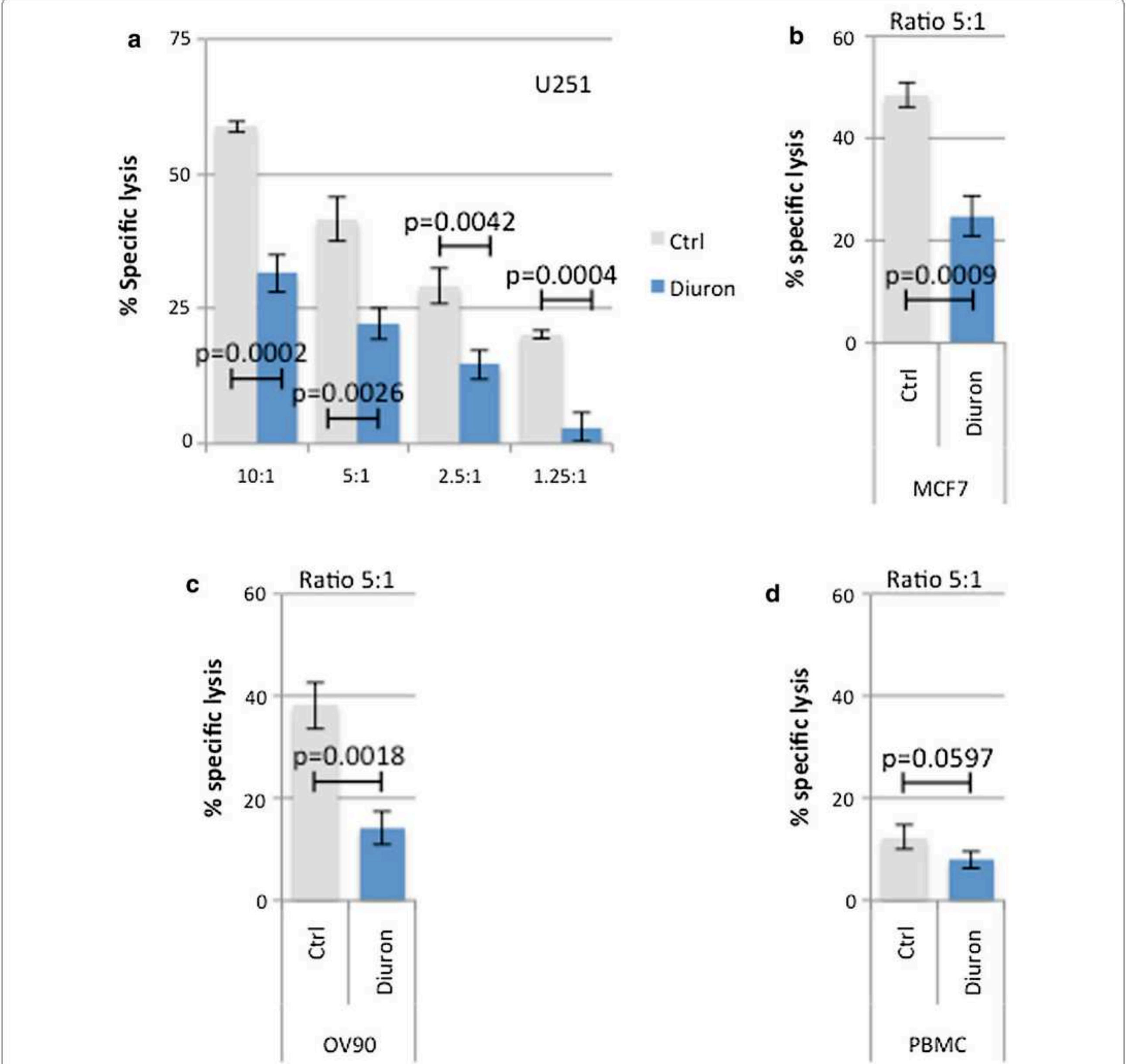

Fig. 3 Diuron exposure of pDC decreases the killing activity of these cells. After a pre-exposure to Diuron or DMSO (Ctrl), CAL-1 cells were stimulated by CpG for 36 h, and were co-cultured with U251 (**a**), MCF (**b**) OV90 (**c**) cells and PBMC (**d**) for another 4 h. The percentage of specific lysis was determined by cytotoxicity assay. Specific lysis (%) = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100

indicated that Diuron exposure increased the ILT7 expression and decreased the TRAIL expression (Fig. 4b). qMSRE investigating the methylation level of the TRAIL and ILT7 promoters shows an hypomethylation in pDC pre-exposed to Diuron (Fig. 4c). Thus, our data associated with the DNA hypomethylation of the ILT7 promoter with a gain of mRNA expression in pDC pre-exposed to Diuron, which agrees closely with the dogma: "DNA demethylation promotes a gain of transcription". Paradoxically, our data indicated that the DNA hypomethylation of the TRAIL promoter is associated with a loss of mRNA expression in pDC pre-exposed to Diuron. By analyzing the hypomethylated region of TRAIL promoter with the CTCFBS

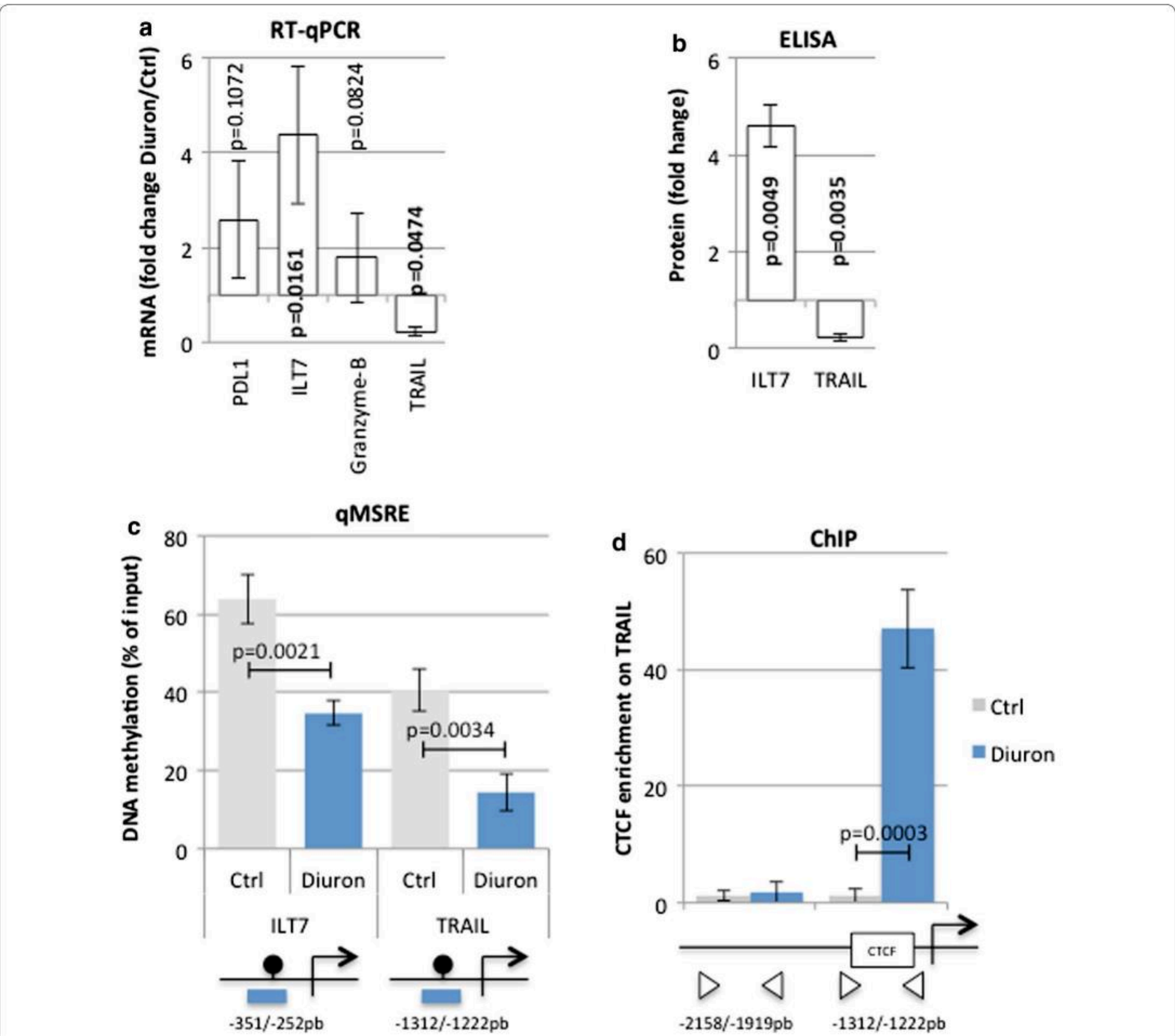

Fig. 4 The Diuron-mediated decreased killing activity of pDC is associated with the modulation of the methylation status of ILT7 and TRAIL. **a** Graph illustrates the Diuron-induced fold change of mRNA expression encoding for molecular actors of killing pDC activity and shows an increase of ILT7 and a decrease of TRAIL expression. **b** Graph illustrates the Diuron-induced fold change of ILT7 (In-cell ELISA, Abcam, France) and TRAIL (ELISA, Abcam, France) expression at protein level. The ILT7 signal was normalized by total cell numbers using Janus Green, a whole cell stain. **c** Graph illustrates the percentage of DNA methylation of the ILT7 and TRAIL promoters. Diuron exposure induced a decrease of both. **d** Graph illustrates the Diuron-induced increasing of CTCF enrichment on the TRAIL promoter

prediction tool, we noted that this region included putative binding sites for the transcriptional repressor CTCF. ChIP experiments confirmed that CTCF was recruited on the Diuron-induced hypomethylated region of TRAIL promoter (Fig. 4d). Taken together, these data support the idea that the Diuron-mediated decreased killing activity of pDC is associated with the modulation of the methylation status of ILT7 and TRAIL.

siRNA TET2 abrogates the cascade of events promoting decreased killing activity of pDC

Our data suggest that TET2 plays a crucial role in the Diuron-mediated decreased killing activity of pDC via the modulation of the methylation-mediated expression of ILT7 and TRAIL. Based on this finding, we next analyzed the impact of the siRNA-induced TET2 down-regulation on the TET2 expression (ELISA), the global level of 5-methylcytosine (5mC, ELISA), the methylation level of ILT7 and TRAIL promoters (qMSRE), the

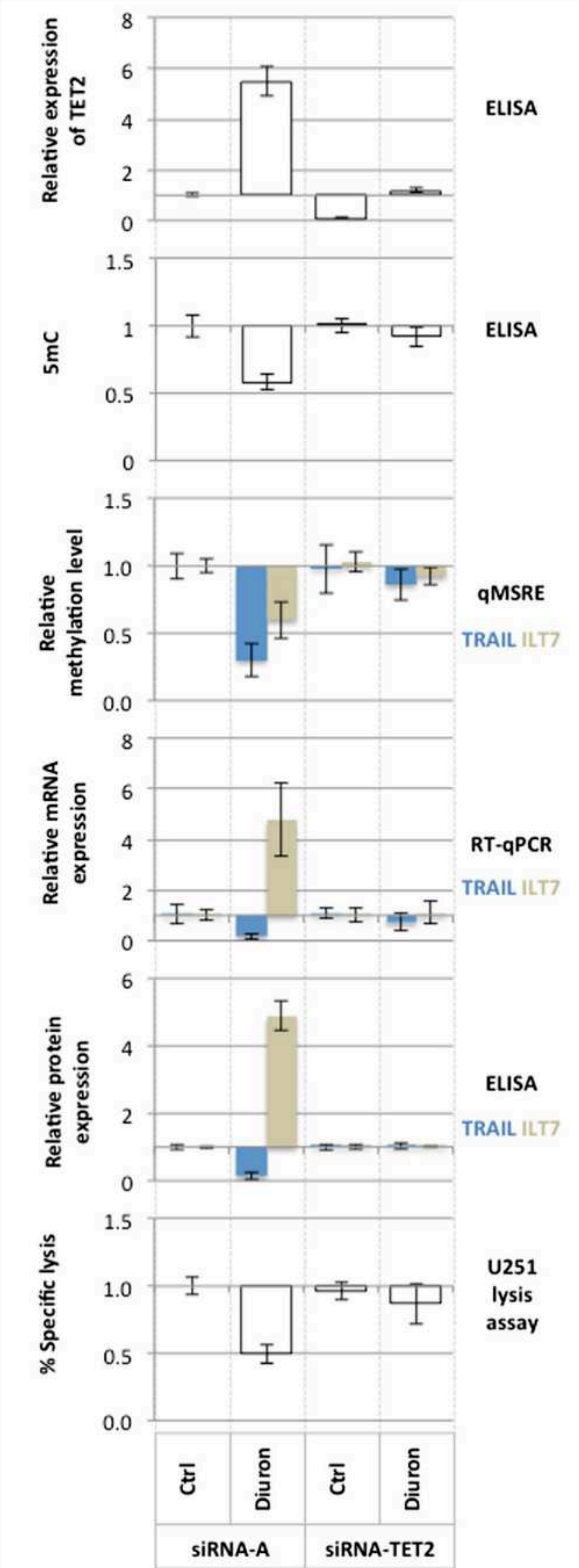

Fig. 5 Impact of the siRNA-mediated TET2 down-regulation on the cascade of events promoting decreased killing activity of pDC. Each parameter was calculated relatively to the siRNA-A/Ctrl condition

expression of TRAIL and ILT7 at mRNA (RT-qPCR) and protein (ELISA) levels and on the lysis toward U251 cells. As expected, we noted that the siRNA-induced TET2 down-regulation abrogated the decreased killing activity of pDC by abolishing the ILT7 overexpression and the TRAIL down-regulation occurring in a context of local and global DNA hypomethylation (Fig. 5).

Discussion

Due to its role of Swiss knife of immune system (such as described by Karrich et al. [28]), the understanding of aberrant epigenetic reprogramming of genes governing functionality of pDC by pesticides appears such as an attractive research point. Our data indicated that the Diuron exposure decreased the killing activity of pDC towards three different cancer cell types (glioblastoma, breast cancer, and ovarian cancer) but not toward PBMC. Thus, it appears, in some way, that the Diuron exposure impoverishes the immunosurveillance concept by acting on one effective unit killing pre-cancerous and/or cancerous cells: the pDC. To date, few other publications report that Diuron affects the immune system via a process of immunotoxicity [29]. Domingues et al. report that rat exposure to Diuron presented a decrease in macrophages spreading, but no change was observed regarding the phagocytosis index [30].

Our data also identified a molecular pathway associated with the decrease of pDC-killing activity since the Diuron exposure promotes the TET2 overexpression, which promotes the TRAIL down-expression and the ILT7 overexpression in a context of local and global DNA hypomethylation. Besides, the dual observation of the loss of TRAIL expression (a cytokine-promoting cell death [31]) and the overexpression of ILT7 (whose interaction with BST2 at the surface of tumor cells promotes the immune tolerance [13]) is consistent with the idea that the Diuron exposure decreased the killing activity of pDC.

By incriminating a TET2-mediated mechanism in the Diuron-mediated reduction of the pDC-killing activity, our data are one of the first to show that an environmental pollutant can affect the immunity via an epigenetic mechanism. Indeed, literature reports that environmental pollutants can affect the immunity, but no reports (at our knowledge) clearly incriminate an epigenetic player in this process. Taylor et al. report that Ziram (a broad-spectrum fungicide) activates mitogen-activated protein kinases and decreases cytolytic protein levels in human natural killer cells [32]. Lepeule et al. reports that the subchronic exposure to traffic-related pollutants was associated with significantly reduced lung function in the elderly and that epigenetic mechanisms related to inflammation and immunity may influence these associations

[33]. However, the authors of this article did not clearly identify the involved molecular epigenetic mechanism in their observation.

Classically, the promoter DNA hypomethylation is associated with an increase of gene expression [34, 35]. Of course, certain individual cases refute this dogma. Our study illustrates one of these individual cases since the TRAIL DNA hypomethylation is associated with its down-regulation. Our experiments support paradoxically situation by showing that the hypomethylated area of TRAIL recruits CTCF, a transcriptional repressor. More general, it is easy and intuitive to think that the DNA methylation status of the different gene areas (promoter, regulator, enhancer, super-enhancer, insulator, and others) could have distinct effects on genes expression.

The health outcomes resulting from environmental exposure(s) are highly varied and remarkably complex. In this article, we report that Diuron, an herbicide, induces an epigenetic reprogramming of a subtype of immune cells, pDC, which decreases its killing activity towards tumors cells. In some context, this mechanism might be conducive to the initiation of pathologies (such as cancer) via a process of "pollutants-induced alteration of immune surveillance". Besides, Nadeau et al. report that increased exposure to AAP (Ambient Air Pollution) is associated with hypermethylation of the Foxp3 locus, impairing Treg-cell function and increasing asthma morbidity [36].

Conclusion

Our study tends to reinforce the converging evidences supporting the fact that Diuron may be a potential tumorigenic substance. This effect was direct such as already reported to the bladder [14, 15], urothelial [16], skin [17, 18] and mammary [15–19] carcinogenesis or indirect via the decrease of immunosurveillance phenomenon. However, in the absence of study in human, our study should not be considered as an absolute proof of the guilt of Diuron in the occurrence of cancer but as a scientific rationale incriminating a potential cellular mechanism that may be at the origin of the initiation of cancer.

Abbreviations

pDC: plasmacytoid dendritic cell; PD-L1: programmed death 1 ligand; TRAIL: TNF-related apoptosis-inducing ligand; TET: ten–eleven translocation; PBMC: peripheral blood mononuclear cells.

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Authors' contributions

PFC and SP designed and coordinated the project. JB, MPJ, AN, and PFC performed all experiments. FMV, GBC, SP and PFC interpreted and discussed the data. PFC wrote the first version of the manuscript and all the authors reviewed and approved it. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets obtained and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicting interests.

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Article 3 -
The TET2 expression level correlates with a short relapse time in glioblastoma multiforme

Dans le glioblastome, l'hypométhylation de l'ADN a été identifiée comme étant un facteur pronostic négatif de la survie [190]. De plus, TET2 est connu comme étant muté dans de nombreuses hémopathies, mais plusieurs altérations ont aussi été décrites dans le GBM [208, 209]. Nous nous sommes donc intéressés à l'expression de TET2 dans le GBM. Dans un premier temps, nous avons étudié son expression au niveau protéique au moment de la première résection, mais elle n'est pas pronostique à ce moment-là. Ensuite nous avons eu l'opportunité d'accéder à des tumeurs plus rares, puisqu'elles proviennent de patients dont la résection tumorale a été effectuée deux fois. Cette configuration est inhabituelle puisque la survie étant courte chez les patients atteints de glioblastome multiforme, il est rare de les opérer deux fois consécutives, bien que cela améliore la survie [299–301].

Nous avons donc établi une cohorte de 10 patients ayant été opérés deux fois, chez lesquels l'expression protéique de TET2 a été analysée et corrélée au temps entre les deux résections. Lorsque l'expression de TET2 augmente entre les deux résections, le temps entre les deux opérations diminue. Ces résultats semblent montrer une implication de TET2 dans l'agressivité tumorale suite à la première résection.

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The TET2 Expression Level Correlates with a Short Relapse Time in Glioblastoma Multiforme

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Abstract

Background: Epigenetic changes play crucial roles in cancer initiation and progression and in tumor recurrence. Glioblastoma multiforme (GBM) is by far the most common and most malignant of the glial tumors, and its recurrence is ineluctable. Thus, anti-GBM therapy need for new treatments for recurrent GBM. We here in focused on identifying whether TET2 (an epigenetic players involving in the DNA demethylation process) as a putative target for the development of anti-GBM therapy.

Results: We observed that the expression level of TET2 at mRNA and protein levels is not associated with a prognosis value of survival in GBM patients. However, in a cohort of 10 GBM patients having received two surgical resections (rGBM), we noted that three in ten rGBM patients have an increase of TET2 expression between resection#1 and #2.

Conclusions: By observing an increase of TET2 expression between two surgical resections an despite the fact that expression level of TET2 is devoid of prognosis value of survival in GBM patients, our data provide a promising starting point for the use of TET2 inhibitors administrable in the subgroup of patients with recurrent GBM.

Keywords: DNA methylation; TET; TET2; Glioblastoma multiforme; TET inhibitors

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Introduction

The Ten-Eleven Translocation-2 (TET2) is a member of the TET family proteins that include 2 other members: TET1 and TET3. These proteins catalyze the steps of the active DNA cytosine-demethylation via the conversion of 5-methylcytoisne (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5CaC) [1-3]. TET2 such as other TET proteins is an α-ketoglutarate- and Fe^{2+}-dependent dioxygenase and the oncometabolite 2-hydroglutarate inhibits its activity [4].

In cancer, the TET2 mutational state is frequently associated with a prognosis value. However, TET2 mutations are more present in liquid tumors than in solid tumors. As example, TET2 mutations are rare in breast, ovarian, lung and brain cancers and observed with a prevalence of 6%-26% in MDS, or in 12-27% in adult AML [5]. In glioblastoma multiforme (GBM), 7 genetic alterations within TET2 (p.V218M, p.G355N, p.P363L, p.L1721W, p.P1723S, p.I1762V, p.H1778R), are described, but the presence of these alterations was not associated with a prognosis value [6]. Investigations performed with human samples and murine models suggest that TET2 deficiency does not promote the tumorigenesis but predisposes to the development of certain tumors such as MPN, MDS and lymphoma [7]. In other terms, these works suggest that TET2 could act as tumor suppressor gene [8-10]. However, TET2 could act as an oncogene by

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participating to the global DNA hypomethylation phenotype i.e. oncogenic phenotype. A recent article suggests that TET2 sustains immunosuppressive function of tumor-infiltrating myeloid cells to promote melanoma progression [11]. Thus, this last article supports the idea that TET2 could be a therapeutic target in certain cancers (other than hematologic cancer, certainly).

The role of TET proteins in glioblastoma multiforme is not fully documented. Two articles report the involvement of TET3 in GBM. Cui et al. suggest that TET3 acts as a tumor suppressor gene in glioblastoma stem cells [12]. Two articles report that TET3 is involved in the dynamic methylation/demethylation mechanism in GBM cells [13,14]. Takai et al. report that TET1-mediated production of 5-hydroxymethylcytosine (5hmC) is required for the tumorigenicity of glioblastoma cells [15]. The nuclear exclusion of TET1 is also reported as a source of DNA methylation and a loss of 5-hydroxymethylcytosine [16]. However, the large majority of articles investigating the role of TET1 in glioma is focused on low-grade glioma. A similar observation can be performed about TET2 [17,18]. Chen et al. reports that the modulation of TET2 expression affects the invasion potential and the proliferation rate of glioma cells [19]. Always in low-grade glioma, Guilhamon et al. report that TET2 interacts with EBF1 to promote a sequence-specific mechanism of DNA demethylation [20]. Thus, there is a lack of investigation on the role of TET2 on GBM. In order to try to overcome this deficiency, our present study investigated the putative association of TET2 with a prognosis value in the context of GBM and its dynamic expression following first resection and standard anti-GBM treatment.

Methods

Tumors samples

Tumors were collected from the "Réseau des tumorothèques du Canceropole Grand-Ouest/réseau Gliome", the "Base clinico-biologique des Glioblastomes (n°BRIF: BB-0033-00093), CHU Angers, France" and the Biological Resource Center of University hospital of Angers. Tumor patient material and records (diagnosis, age, sex, date of death) were used with confidentiality in accordance with French laws and the recommendations of the French National Ethics Committee. In accordance with the regulations, all subjects signed a specific informed consent form for this biocollection, approved by an Ethics Committee, the French State Department for National Education, Higher Education and Research and the CNIL.

Protein analysis: ELISA

Proteins extracts were obtained by using RIPA Lysis and Extraction Buffer (Thermo Scientific, France) in accordance with the manufacturer's instructions. TET2-ELISAs were performed according to the manufacturer's instructions (MyBiosource, MBS9317739, USA).

Statistics

The log-rank test was used to test whether the difference between survival times of two groups is statistically different or not. The Kaplan-Meier survival curves were used to represent the probability of surviving in a given length of time of subgroups of patients. T-test is performed to estimate whether the difference between a parameter characterizing two groups is statistically different or not. Pearson's correlation test is performed to estimate the significant character of a correlation between two parameters.

Results

The TET2mRNA level is not associated with a prognosis value

To investigate whether TET2mRNA can be used as a biomarker associated with a favorable survival, we first analyzed data available on "Betastais/REMBRANDT database" (http://www.betastasis.com/glioma/rembrandt/kaplan_meier_survival_curve/). Thus, we observed that the expression level of TET2mRNA in GBM is not associated with a prognosis value of survival, neither in term of overall survival (OS) **(Figure 1).**

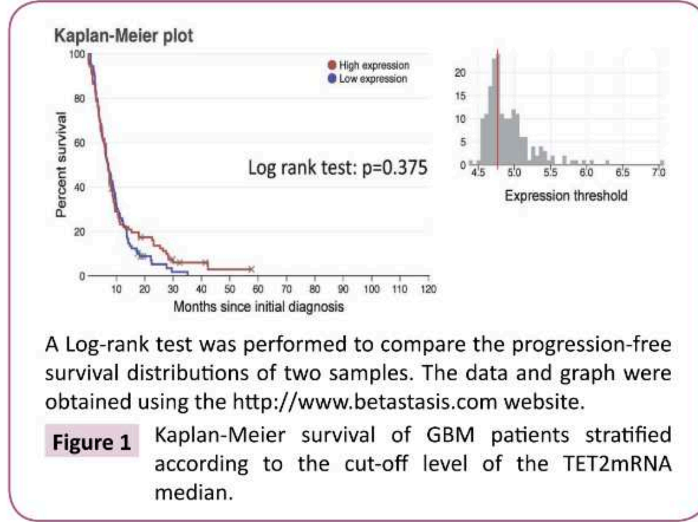
The TET2 expression level is not associated with a prognosis value

In a second approach, we have considered the TET2 protein expression level as a putative biomarker associated with a prognosis survival. TET2 expression level was analyzed in 31 GBM patients. The patients' characteristics of this cohort (cohort#1) are listed in **(Table 1).** ELISA was performed to calculate the TET2 expression level in GBM **(Figure 2A).** Our data indicates significant heterogeneity in the TET2 expression since a 11-fold increase was observed between the minimal value and the maximal value for TET2.

Then, our cohort of 31 GBM samples has been divided in 2 subgroups using the median value as threshold. Survival curves were visualized in Kaplan-Meier plot. A log-rank test indicates a lack of difference between the overall survival of GBM patients having a high level of TET2 and those having a low level of TET2 **(Figure 2B).**

The TET2 expression level correlates with a short relapse time in recurrent GBM

We extended our study by asking if the TET2 expression could be

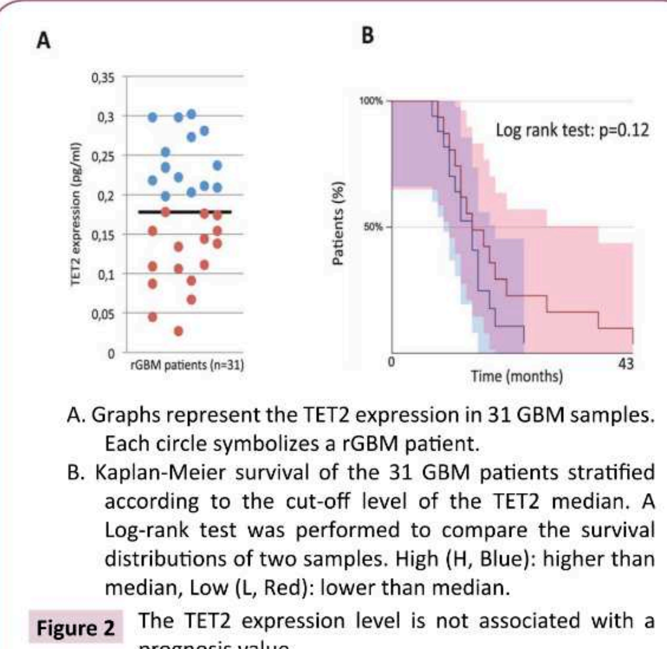


A Log-rank test was performed to compare the progression-free survival distributions of two samples. The data and graph were obtained using the http://www.betastasis.com website.

Figure 1 Kaplan-Meier survival of GBM patients stratified according to the cut-off level of the TET2mRNA median.

Table 1 Patients' characteristics of this cohort (cohort#1).

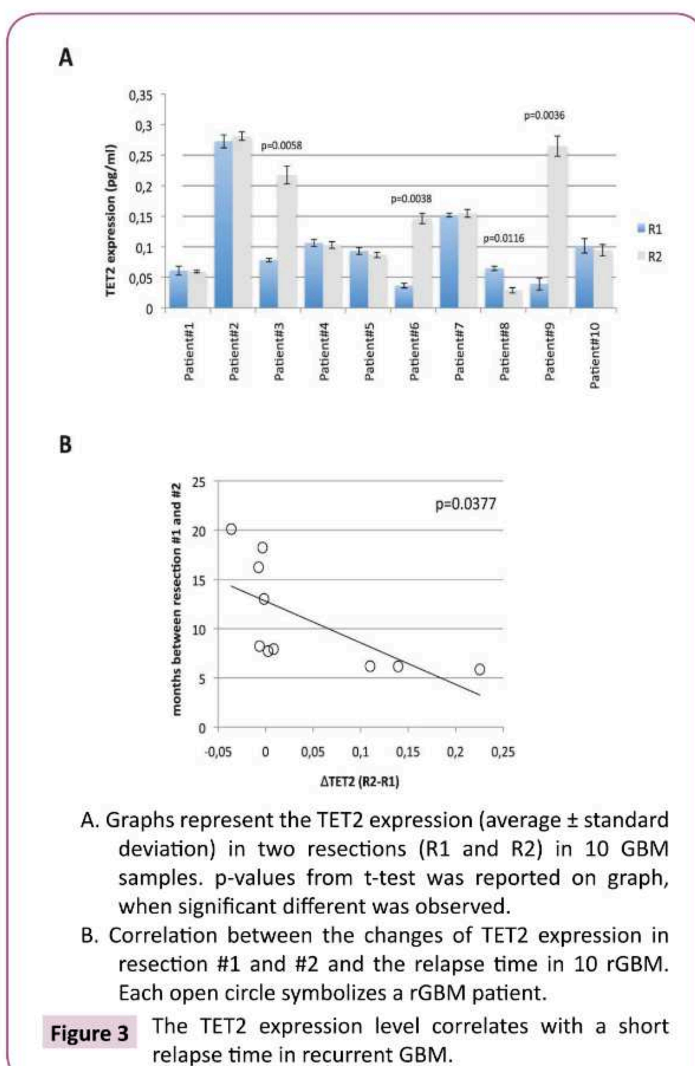
Cohort#1 (n=31)	Characteristics		Patients	Log-rank test
	Age (years) Median (range)		56 [46;69]	p=0.30
	Gender			p=0.29
		Male	20	
		Female	11	
	Survival time (months)			
	Median (range)		14.1 [4.6;42.6]	
	Extent of surgery			
		Biopsie or partial resection	0	
		Complete resection	31	
Cohort#2 (n=10)	**Age (years) Median (range)**		51 [42;58]	np
	Gender			np
		Male	7	
		Female	3	
	Survival time (months)			
	Median (range)		23.6 [11;36.3]	
	Extent of surgery#1 and #2			
		Biopsie or partial resection	0/0	
		Complete resection	10	



A. Graphs represent the TET2 expression in 31 GBM samples. Each circle symbolizes a rGBM patient.
B. Kaplan-Meier survival of the 31 GBM patients stratified according to the cut-off level of the TET2 median. A Log-rank test was performed to compare the survival distributions of two samples. High (H, Blue): higher than median, Low (L, Red): lower than median.

Figure 2 The TET2 expression level is not associated with a prognosis value.

associated with a short relapse time in recurrent GBM (rGBM). To address this question, TET2 expression level was analyzed by ELISA in resection#1 and #2 of 10 GBM patients having received two surgeries. The relapse time is here defined such as the months separating the both resections. The patients' characteristics of this cohort (cohort#2) are listed in **Table 1.** All GBM of this cohort are IDH1 wild-type and are primary GBM (i.e. that the patients are devoid of medical history with low grade glioma). Thus, we noted that three in ten rGBM patients have an increase of TET2



A. Graphs represent the TET2 expression (average ± standard deviation) in two resections (R1 and R2) in 10 GBM samples. p-values from t-test was reported on graph, when significant different was observed.
B. Correlation between the changes of TET2 expression in resection #1 and #2 and the relapse time in 10 rGBM. Each open circle symbolizes a rGBM patient.

Figure 3 The TET2 expression level correlates with a short relapse time in recurrent GBM.

expression between resection#1 and #2 **(Figure 3A).** One in ten rGBM patients harbors a decrease of TET2 expression **(Figure 3A).** We next calculated the changes of TET2 expression between resection #1 and #2 (#TET2=TET2Resection#2-TET2Resection#1). Graph and Pearson's correlation test indicate that the changes of TET2 expression between resection#1 and #2 was correlated with relapse time (p=0.0377) (**Figure 3B**).

Discussion

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain cancer. Despite the gain of survival associated with the use of a standard of care treatment composed by a maximal safe surgery followed by adjuvant chemoradiotherapy and adjuvant chemotherapy, recurrence is an inevitable event. Besides, literature reports that GBM patients underwent repeat resections with a median time between resections of 6-9 months [21,22]. On contrary to the first line of treatment, the treatment of recurrent GBMs (rGBMs) poses a great challenge since no well-defined recommendation of therapy is suggested. This point finds its echo in the multiples clinical trials performed about the rGBM treatment and with a large panel of drugs including: PARP inhibitor (Veliparinb, ClinicalTrials.

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gov Identifier: NCT01026493), vEGF inhibitor (ClinicalTrials.gov Identifier: NCT01474239), or EGFr inhibitor (ClinicalTrials.gov Identifier: NCT01310855). Several epidrugs such as HDAC inhibitor (ClinicalTrials.gov Identifier: NCT01110876) and BET inhibitor (OTX015 ClinicalTrials.gov Identifier: NCT02296476) have also been investigated for the treatment of rGBM [23]. The data included in our study suggests that TET2 expression could be used as a putative biomarker and/or target for the development of precision and personalized medicine for the treatment of rGBM, but not for the initial GBM treatment. Despite the limitation of this initial study (low number of rGBM), our data constitute a promising starting point for the design of TET2 inhibitors administrable at patients with rGBM i.e. for patients having a strong medical need.

The observation of a correlation between an elevated TET2 expression and a short time of relapse also asks the question of the role played by TET2 in the GBM recurrence. Thus, several hypotheses can be formulated from this observation: is the TET2 overexpression an actor of the GBM recurrent?; is the TET2 overexpression a biomarker associated with the acquired resistance of the standard anti-GBM treatment? Is the TET2 overexpression a consequence of the GBM recurrence?....and others. Investigations replying at these questions are ongoing in our lab.

Conclusion

Finally, our data reinforce the need for the development of TET2 inhibitors. On contrary to the other epigenetic players, the epidrug pipeline is, to date, poor in TET inhibitors and more particularly in TET2 inhibitors. Several points can explain this relative lack. Thus, the fact that TET2 protein was later characterized as connected to cancer than others epigenetic players could explain the lack in TET2 inhibitors of the epidrug pipeline [24,25]. The focus on TET2 mutations and not on the TET2 expression level could be another possible explanation. The description of TET2 as both tumor suppressor gene and oncogene also appears be a drag for the development of TET2 inhibitors. Literature reports that DMOG (an antagonist of α-ketoglutarate cofactor and inhibitor for HIF prolylhydroxylase) and 2-hydroxyglutarate can be used to inhibit TET2 [26,4]. However, these molecules having a large spectrum of selectivity, their use such as selective TET2 inhibitors can be discussed.

In conclusion, our data and the current lack of selectivity of TET2 inhibitors support the research for a better understanding of the role played by TET2 in rGBM and for the design/development of highly selective TET2 inhibitors.

Declarations

Acknowledgements

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Authors' Contributions

PFC designed and coordinated the project.

JB, AN and PFC performed all experiments.

FMV and PFC interpreted and discussed the data.

PFC wrote the first version of the manuscript and all authors reviewed and approved it.

Conflicting Interests

The authors declare that they have no conflicting interests.

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Article 4 - Radiotherapy-induced overexpression of exosomal miRNA-378a-3p in cancer cells limits Natural Killer cells cytotoxicity via the downregulation of granzyme B

Dans cet article, nous nous sommes intéressés à l'étude de l'exomiR-378a-3p dans différents cancers traités par irradiation (GBM, cancers de la prostate et du col de l'utérus). L'irradiation de cellules de GBM induit la déméthylation du promoteur du miR-378a-3p par TET2, et l'induction de la transcription par le ATF. Le miR est alors exporté dans les exosomes et va agir sur les cellules NK, où il va induire la dégradation de l'ARNm codant pour le granzyme B et diminuer leur cytotoxicité. Chez les patients, l'expression du miR-378a-3p a été corrélé négativement à celle du granzyme B dans le sang.

Radiotherapy-induced overexpression of exosomal miRNA-378a-3p in cancer cells limits Natural Killer cells cytotoxicity

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Running title: Radiotherapy modulates immune response via exosomal miRNA

Keywords: exosomal miRNA, radiotherapy, miR-378, NK cells, glioblastoma

ABSTRACT

Tumor-derived exosomes act as messagers for the intercellular communication. We here hypothesized that tumor-derived exosomal miRNA (TexomiR) released from irradiated tumors may play a role in the tumor cells escape to NK cells.

Our cellular experimentations indicate that the irradiation of a cancer cells (glioblastoma, cervix, prostate, breast and lung) promotes the TET2-mediated demethylation and overexpression of TexomiR-378a-3p, and induced the decrease of Granzyme B secretion in NK cells (NK92). In end-point blood biopsies of xenograph model, we observed that irradiation induced the TexomiR-378a-3p overexpression. In longitudinal blood biopsies of irradiated glioblastoma and cervix patients, we observed an inverse correlation between the TexomiR-378a-3p and Granzyme B expressions.

Our work identifies TexomiR-378a-3p as a molecular cause associated with the immune escape upon radiotherapy.

INTRODUCTION

Exosomal microRNAs (exomiR) are providing significant mechanism for intercellular communication[1]-[2]-[3]. Thus, tumor-derived exomiR can modulate the functionality of cells of its microenvironment and particularly the immune cells infiltrating or juxtaposing the tumor cells[4]. Besides several tumor-derived exomiRs have been identified to interfere immune cells to promote immune escape such as i) exomiR-203 promotes the dendritic cells dysfunction via the regulation of the TLR4 expression; ii) hypoxic tumor-derived exosomal miR-301a mediates M2 macrophage polarization via PTEN/PI3K to promote pancreatic cancer metastasis[5]-[6]-[7]-[8]; iii) miR-214 transported via EVs from tumor cells to murine peripheral CD4+ T cells participated in the induction of the Treg phenotype by inducing reduction in the PTEN (phosphatase and tensin homolog) levels[9] ; and iv) pancreatic cancer cells-derived exosomes inhibit RFXAP expression via miR-212-3p, which decrease MHC II expression and induce immune tolerance of dendritic cells[10]. Thus, tumor-derived exomiR contributes to cancer progression by affecting the anti-cancer immune response. More interestingly, literature reports that tumor-derived exomiR can participate to the chemotherapy resistance by affecting the anti-cancer immune response [11]. This process can be illustrated by the fact that exomiR-1246 confers chemo-resistance via targeting Cav1/p-gp/M2-type macrophage axis in ovarian cancer[12].

Despite evidences reporting that irradiation increases exosomes release from several tumor cell types[13]-[14]-[15], the role of the irradiation-induced tumor-derived exomiR on anti-cancer immune response is not well-documented. To address this question, we examined if ExomiRs produced by irradiated cancer cells could modulate the cytotoxic activity of NK cells toward non-irradiated cancer cells. We here focused on NK cells since these cells play a crucial role in anti-cancer immunity and particularly in glioblastoma, prostate and cervix cancers, three tumors selected as model in your study [16]-[17]-[18]-[19]-[20]-[21].

MATERIALS and METHODS

Exosome isolation and procedures

Exosome isolation was performed using the ExoQuick-TC ULTRA EV isolation kit (Ozyme, France) from approximately five million U87 cells cultured in 5mL of media for 24 h and transferred them to the same number of recipient NK cells. For this, U87 cells were cultured according to the ATCC's recommendations and Gibco™ Exosome-Depleted FBS (ThermoFisher, France). Exosome pellet was further washed twice with PBS by ultracentrifugation at 100000×g for 70 min, to remove any free dye and finally the exosome pellet was resuspended in PBS at the concentration of ≈1µg protein/µL and used for co-incubation with other cells.

Protein concentrations of exosome fractions were determined using BCA protein Assay kit as recommended by the manufacturer (ThermoFisher, France).

GW4869 is a neutral sphingomyelinase 2 inhibitor that has been previously used to prevent exosome release[22]. In our experiments, cells were treated with 10 µM GW4869 (Sigma-Aldrich, France) or vehicle for 24 h.

Mouse experiments

NMRI nude male mice of 8-week age (Janvier Labs, Saint-Berthevin, France) were engrafted subcutaneously in the left leg with 10^6 PC3-luc cells (Caliper Life Sciences, Villepinte, France) and tumor growth was followed using a digital caliper. When tumors reached 200 mm^3 in average (≈ 21 days), they were irradiated at 10 x 2Gy daily over a period of 2 weeks (= 20 Gy cumulated). Radiotherapy was performed using a small animal irradiator (X-Rad 225cx, Precision X-Ray, North Branford, CT) with an accelerating voltage of 225 kV and a dose rate of 1.78 Gy/min. Animals were sacrificed 12h after the last irradiation. Total blood was collected immediately by aspiration from the heart and stored in Paxgene blood RNA tubes (Qiagen) for nucleic acid preservation. Animal experiments were approved by the Comité d'Ethique en Expérimentation Animale des Pays-de-Loire (C2EA-06) under APAFIS protocol #2336, in accordance with the European Council Directive 2010/63/UE.

Study approval

Specimens were obtained from patients diagnosed with glioblastoma and cervix cancer after IRB approval by the "Institut de Cancérologie de l'Ouest" (ICO). All recruited patients gave signed, informed consent. All the collected samples and associated clinical information were registered in database (N° DC-2018-3321) validated by the French research ministry. Biological resources were stored at the "Centre de Ressource Biologique-Tumorothèque" (Institut de Cancérologie de l'Ouest, Saint-Herblain, F44800, France).

RESULTS

Exosomal miRNA-378a-3p from irradiated U87 cells decreases the cytotoxicity of NK cells by repressing the Granzyme-B expression

First, we analyzed the cytotoxic activity of a NK cell line (MI92) treated with ExomiRs obtained from supernatant of a glioblastoma cell line (U87) irradiated (Ir) (**Figure 1A**). ^{51}Chromium release assays were performed to compare the cytotoxic activity of NK cells exposed, for 24h, to 5 mL of supernatant of U87 cells exposed or not to irradiation treatment (Super and SuperIr) and previously treated or not with GW4869 (a drug that hinders exosome biogenesis by blocking neutral sphingomyelinase 2, nSMase[1]) (SuperIr/GW4869). Similar experiments were carried out in the presence of exosomes purified from 5ml of U87 cells exposed or not to irradiation treatment (ExoIr and Exo). We found that SuperIr, ExoIr and ExomiRIr strongly and significantly decreased the NK-mediated lysis of U87 cells and is limited with SuperIr/GW4869 (**Figure 1B**).

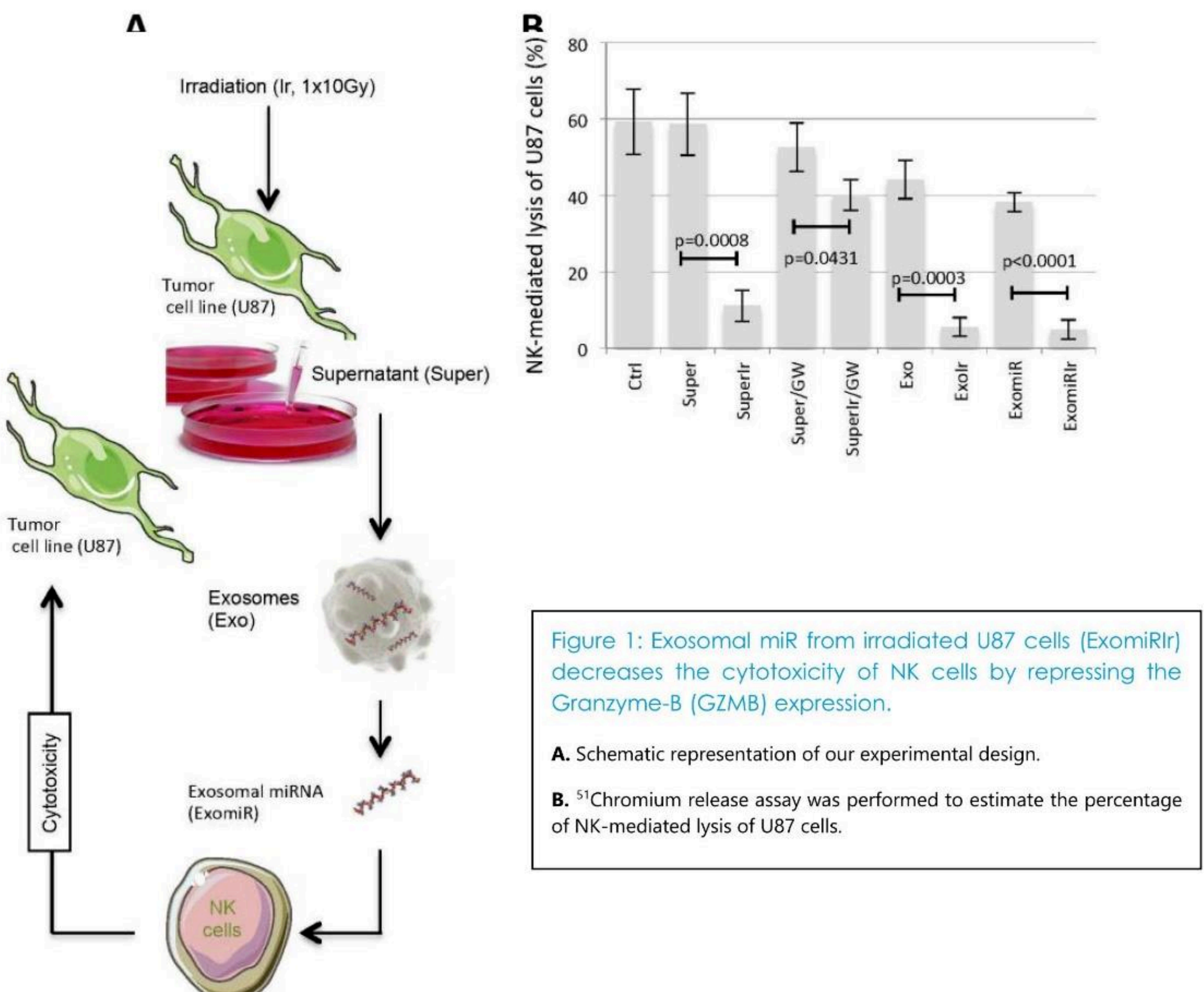


Figure 1: Exosomal miR from irradiated U87 cells (ExomiRIr) decreases the cytotoxicity of NK cells by repressing the Granzyme-B (GZMB) expression.

A. Schematic representation of our experimental design.

B. ^{51}Chromium release assay was performed to estimate the percentage of NK-mediated lysis of U87 cells.

This suggests that ExomiRs were liberated from U87 cells exposed to irradiation with the ability to limit the cytotoxic activity of NK cells. To determine the immune suppression

induced by U87 ExomiRs, Granzyme B/Perforin (GZMB/PRF1) which are the major vectors of NK cell cytotoxic activity were measured by ELISA in NK cell culture supernatants. ELISA revealed that ExomiRIr reduced, in a dose dependent manner, the GZMB expression but not that of PRF1 (**Figures 1C and 1D**).

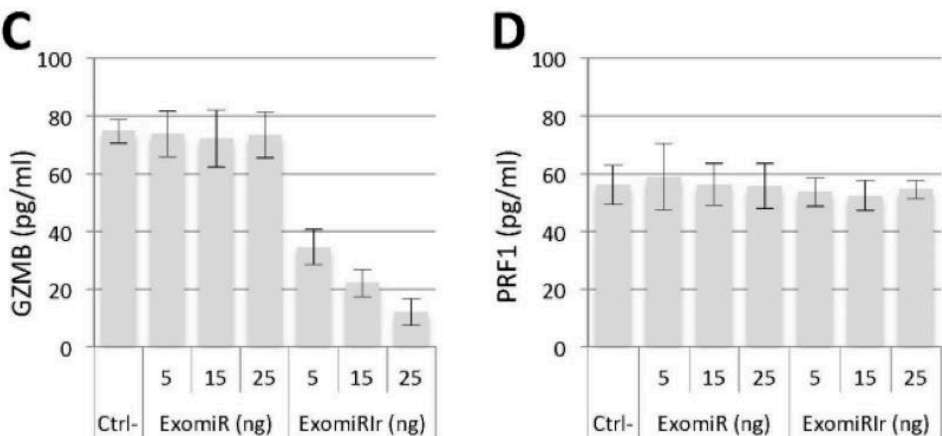


Figure 1: Exosomal miR from irradiated U87 cells (ExomiRIr) decreases the cytotoxicity of NK cells by repressing the Granzyme-B (GZMB) expression.

C. NK cells were transfected with the indicated quantity of exosomal miR derived from U87 cells (ExomiR) or irradiated U87 cells (ExomiR-Ir). Transfections are performed using RNAiMax transfection reagent (ThermoFisher, France). Human Granzyme B ELISA Kit (Abcam, France) was used to estimate the Granzyme-B expression.

D. NK cells were transfected with the indicated quantity of exosomal miR derived from U87 cells (ExomiR) or irradiated U87 cells (ExomiRIr). Transfections are performed using RNAiMax transfection reagent (ThermoFisher, France). Human Perforin ELISA Kit (PRF1) (Abcam, France) was used to estimate the Granzyme-B expression.

Several miRNAs have the ability to repress the GZMB expression (namely miRNA-378a-3p, miR-150, miR-27a, miR-30e[23]-[24]-[25]), and we analyzed by RT-qPCR their expression in exosomes contents. We found that ExomiRIr contained miRNA-378a-3p but not miR-150, miR-27a and miR-30e (**Figure 1E**). A mimic-miR-378a-3p decreased the GZMB expression in dose-dependent manner in contrast to mimic/mutated-miR-378a-3p or mimic-miR-92a, then confirming the functional relational between miR-378-3p and GZMB in NK cells (**Figure 1F**).

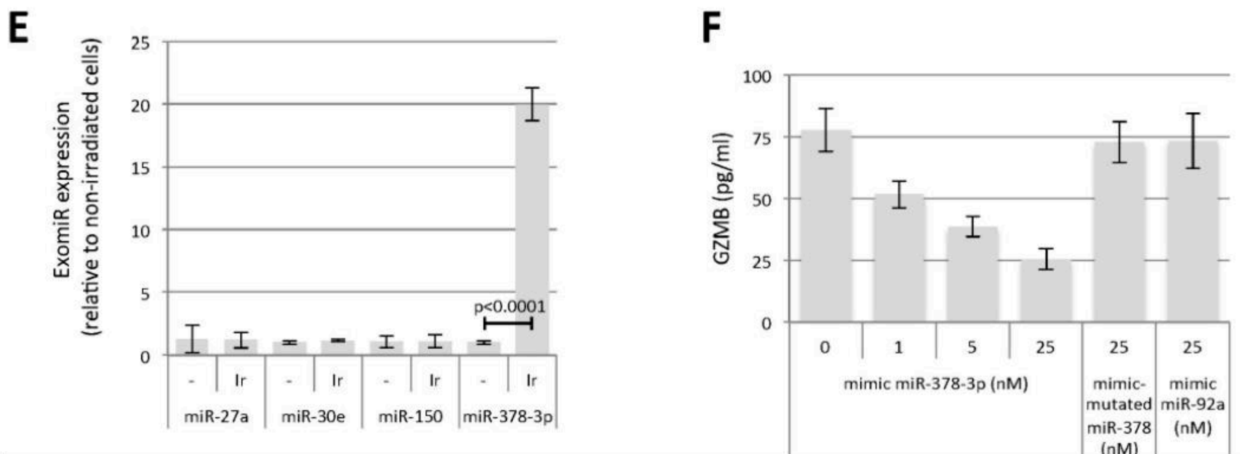


Figure 1: Exosomal miR from irradiated U87 cells (ExomiRIr) decreases the cytotoxicity of NK cells by repressing the Granzyme-B (GZMB) expression.

E. Expression, in exosomes of non-irradiated and irradiated U87 cells, of miRs identified as candidate for the GZMB targeting.

F. Human Granzyme B ELISA Kit (Abcam, France) was used to estimate the Granzyme-B expression in response to the transfection of U87 cells with mimic-miR-378a-3p (acuggacuuggagucagaaggc), mimic-mutated-miR-378a-3p (acuAAGGAAggagucagaaggc) and mimic-miR-92a (uauugcacuugucccggccugu).

Moreover, exosomes purified from non-irradiated U87 cells (Exo) and transfected with mimic-miR-378a-3p decreased the GZMB expression by NK cells, while mimic/mutated-miR-378a-3p and mimic-miR-92a had no effect (**Figure 1G**). In a third experiment, we demonstarted that exosomes purified from irradiated U87 cells transfected with anti-miR-378a-3p restored the GZMB expression in a dose-dependent manner, while anti-miR-92a had no effect (**Figure 1H**).

Next, we investigated the molecular mechanism by which miR-378a-3p regulated on GZMB expression. For this purpose, the enrichments of miR-378a-3p and 3'UTR on GW182 were analyzed by GW182-CLIP-qPCR method (CrossLinking and GW182-ImmunoPrecipitation followed by reverse transcription and quantitative PCR. Here, GW182 is used since it essential for the formation of the miRNA/mRNA duplex[26]). Thus, we found that miR-378a-3p and 3'UTR/GZMB strongly co-immunoprecipitated with GW182 when NK cells were exposed to the U87/irradiated-derived exosomes compared with non-treated U87 derived exosomes (**Figure 1I**).

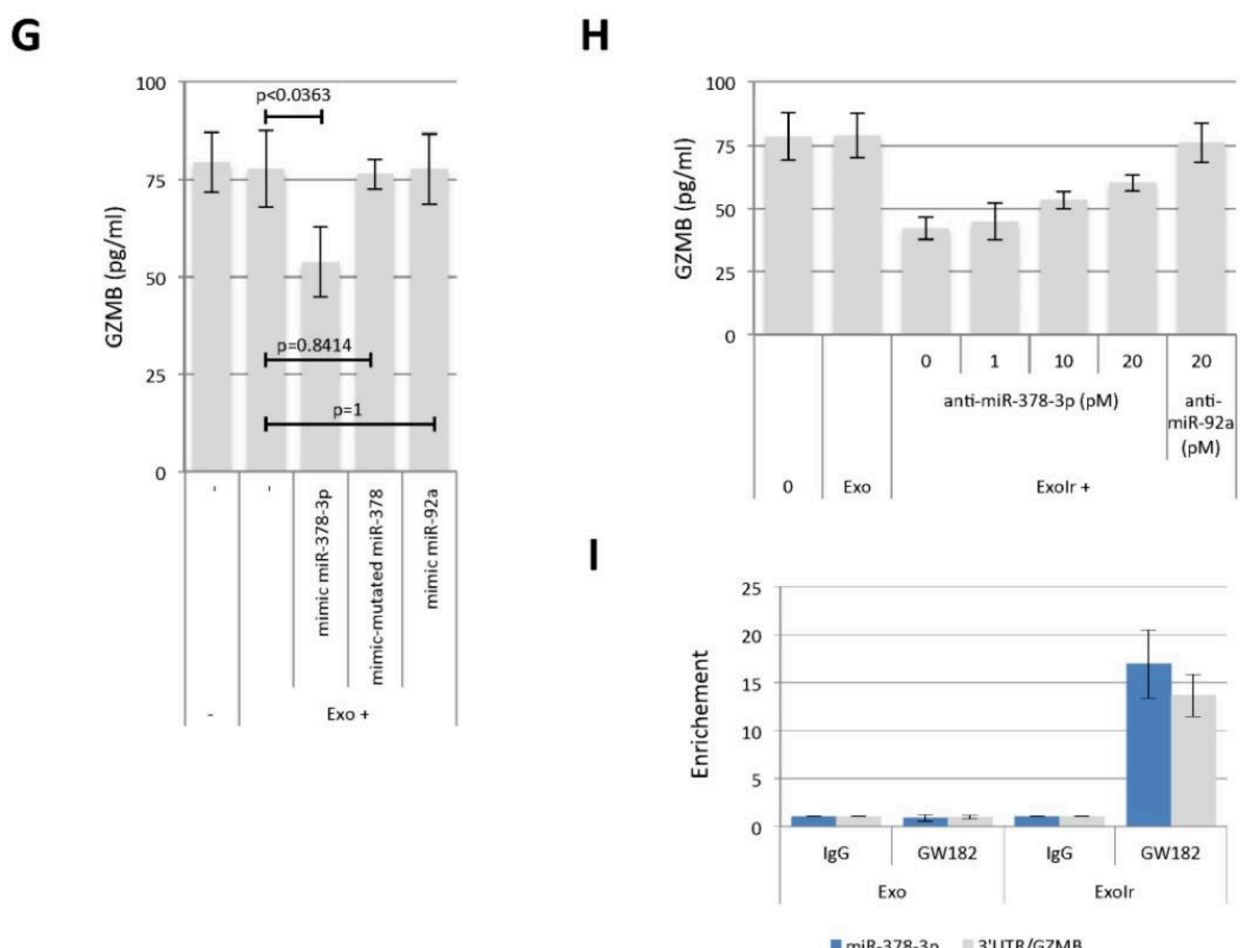


Figure 1: Exosomal miR from irradiated U87 cells (ExomiRIr) decreases the cytotoxicity of NK cells by repressing the Granzyme-B (GZMB) expression.

G. Isolated exosomes (50µg) from non-irradiated U87 cells (Exo) are transfected with indicated mimic-miR (20pmol) according to the Exo-fect Exosome transfection reagent kit (Ozyme, France) and are co-incubated with NK cells (10^5) for 24h. Human Granzyme B ELISA Kit (Abcam, France) was then used to estimate the Granzyme-B expression.

H. Isolated exosomes (50µg) from irradiated U87 cells (ExoIr) are transfected with indicated anti-miR according to the Exo-fect Exosome transfection reagent kit (Ozyme, France) and are co-incubated with NK cells (10^5) for 24h. Human Granzyme B ELISA Kit (Abcam, France) was then used to estimate the Granzyme-B expression.

I. The graph illustrates the miRNA-378a-3p and 3'UTR/GZMB enrichments on GW182 and IgG (negative control). Experiments were performed using the RiboCluster Profiler kit (CliniScience, France) according to manufacturer's instructions.

In addition, **Figure S1** showed that the exomiR 378a-3p was induced by irradiation in 4 additional cell line derived from breast, lung, and cervix cancers and similarly decreased GZMB secretion by NK cells.

TET2-mediated DNA hypomethylation initiated the exomiR-378a-3p overexpression in response to irradiation of glioblatoma cells

As shown in **Figure 2A**, in the presence of GW4869, the amount of cellular miR-378a-3p increased after 24h of irradiation. In parallel, qMSREs performed 2 miR-378a promoter regions indicated that, after 24h of irradiation, the -541/-358 region was hypomethylated, while the 907/-769 region was unchanged (**Figure 2B**). These results suggested that irradiation induced a rapid (less than 24h) and specific DNA hypomethylation of the *miR-378a* promoter. Since DNA hypomethylation process has been catalyzed by TET activities, we monitored both the level and kinetics of TET activity after irradiation. As shown in **Figure 2C**, a peak of TET activity reached a maximum 8h post irradiation and decline rapidly.

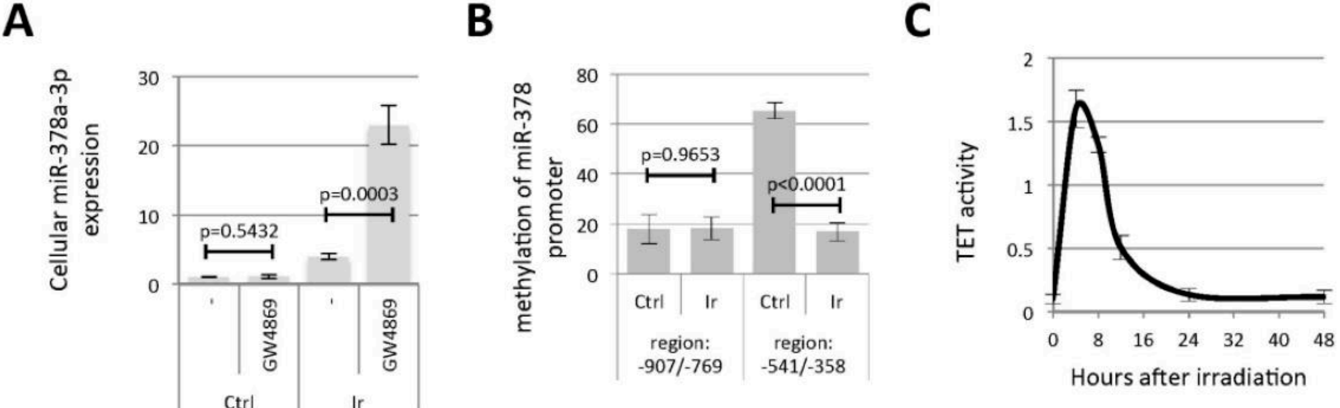


Figure 2: A TET2-mediated DNA hypomethylation process initiates the exomiR-378a-3p overexpression in response to irradiation of glioblastoma cells.

A. 24h after irradiation, RT-qPCRs were performed to quantify the expression of cellular miRNA-378a-3p in presence or not of GW4628, a drug that hinders exosome biogenesis[22].

B. qMSRE investigate the methylation level of 2 regions of the miR-378a promoter.

C. For each indicated time points after irradiation, nuclear extracts from U87 cells were prepared using the Nuclear Extract kit (Active Motif, France). Then, 10 µg of nuclear extract were used to quantify the TET activity according to the Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit recommandations (Euromedex, France).

ChIP experiments indicated that TET2, but not TET1 or TET3, was recruited on the miR-378a$^{-541/-358}$ promoter region in the 6h following irradiation (**Figure 2D**). Among transcription factor with miR-378a$^{-541/-358}$ promoter binding activities (ref), we found that, during this range of time, ATF2 was recruited in this region after irradiation but not p53 or cEBPα. In addition, ReChIP experiments indicated that TET2 and ATF2 were co-recruited on the miR-378a$^{-541/-358}$ promoter, suggesting a coordinated mechanism. Indeed, this was confirmed by TET2 or ATF2 knockdown expressions via RNA interference. siRNA TET2 or ATF2 strongly and

equally decreased the irradiation-induced recruitment of TET2 or ATF2 onto the miR-378a$^{-541/-358}$ promoter and the subsequent DNA hypo-methylation of this region (**Figure 2E**). Finally, kinetics of miR-378a-3p and exomiR-378a-3p expression indicated that the peak of cellular miR-378a-3p expression occurred 8-12h after irradiation, while its expression in exosomes was delayed to 18-24h after irradiation (**Figure 2F**). Our data are consistent with the TET2 controlled overexpression of miR-378a-3p and its incorporation into exosome in U87 after irradiation.

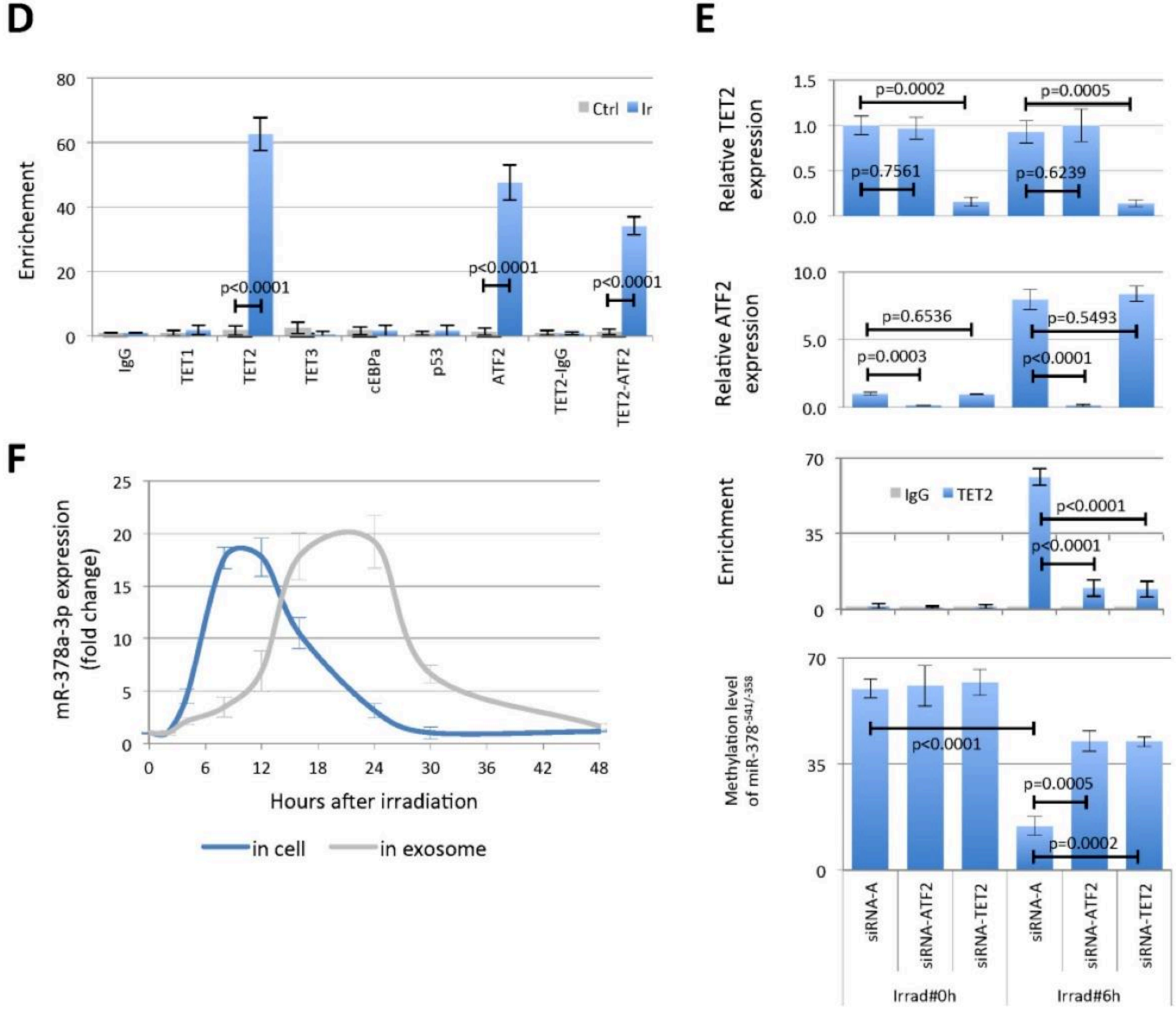


Figure 2: A TET2-mediated DNA hypomethylation process initiates the exomiR-378a-3p overexpression in response to irradiation of glioblastoma cells.

D. Six hours after irradiation, chromatin Immunoprecipitations (ChIP) and ReChIP were performed to estimate the enrichment of TET proteins on the miR-378a$^{-541/-358}$ promoter (ChIP-IT Express and ReCHIP-IT kits, Active Motif, France).

E. RT-qPCR validated the efficiency of the siRNA knockdown directed TET2 and ATF2. ChIP and qMSRE experiments show that these two knockdowns decreased the TET2 recruitment on the miR-378a$^{-541/-358}$ promoter and limit the irradiation-induced demethylation of the miR-378a$^{-541/-358}$ promoter.

F. To investigate the kinetic of cellular and exosomal miR-378a-3p expression, RT-qPCR were performed with miRNA extracted from cells and exosomes at indicated time after irradiation.

The exomiR-378a-3p overexpression is observed in cellular and *in vivo* prostate model of radiotherapy

This study was extended to a model of prostate cancer cell, another radiotherapy treated cancer. Firstly, miR Array (Qiagen, miScript miRNA PCR Array Human Cancer PathwayFinder)) indicated that miR-378a-3p is overexpressed in irradiated PC3 cells (**Figure 3A**). Secondly, we observed that the irradiation of PC3 tumors formed in vivo induced detectable circulating exomiR-378a-3p in the blood samples of 33% (2/6) of mice (**Figure 3B**).

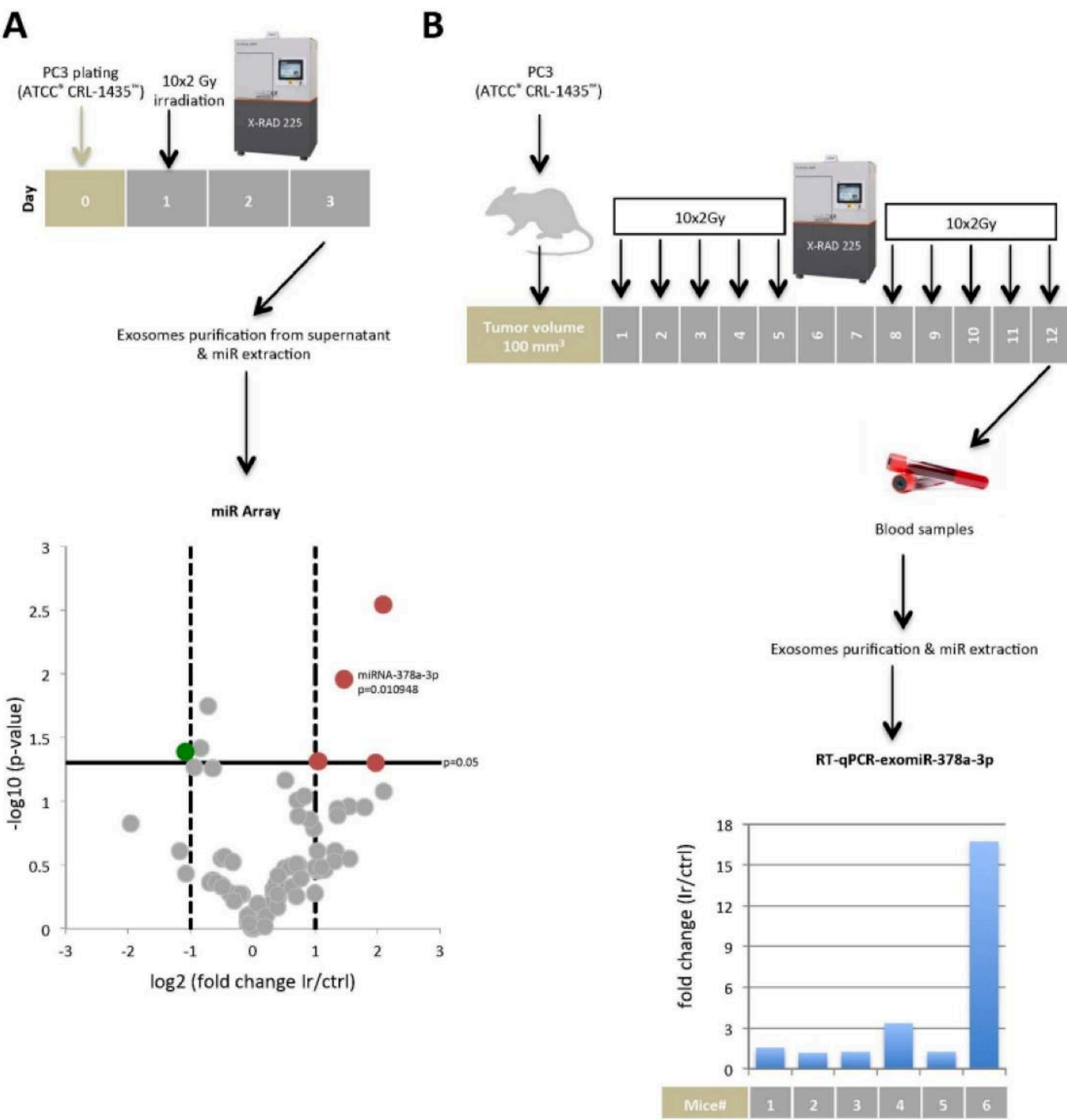


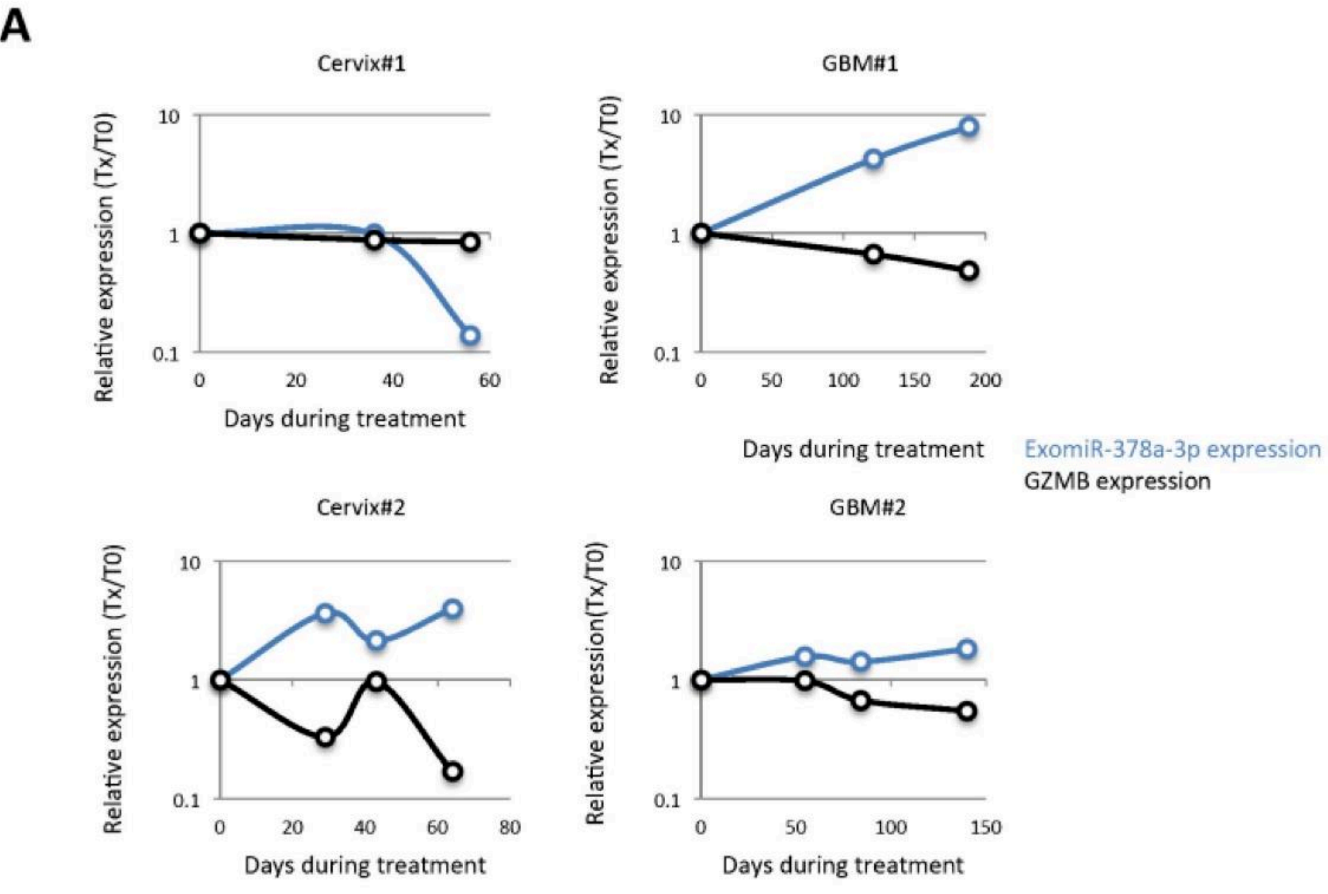
Figure 3: Irradiation induced the exomiR-378a-3p overexpression in the supernatant of PC3 cells and in blood of a PC3-induced model of tumors in mice.

A. miScript miRNA PCR Array Human Cancer PathwayFinder (Qiagen, France) was used to analyze the modification of miRNA expression in irradiated PC3 prostate cancer cells.

B. RT-qPCR quantified the exosomal miR-378a-3p expression.

Circulating exomiR-378a-3p expression is inversely correlated with the blood level of GZMB in patients treated with radiotherapy.

We thus analyzed exomiR-378a-3p and GZMB expression in longitudinal samples of blood biopsies obtained from 2 GBM and 2 cervix cancer patients treated with radiotherapy (**Figure S2**). Blood samples analyses showed that the expression of these two parameters was patient -dependent and followed different patterns (**Figure 4A**). However, we observed that the kinetics of circulating exomiR-378a-3p and GZMB expression were strikingly "mirrored" with an inverse significant correlation between these 2 parameters in all patients (**Figure 4B**).



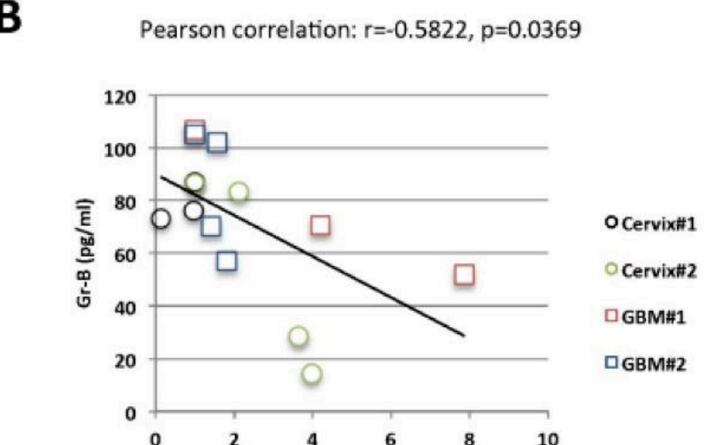


Figure 4: Circulating exomiR-378a-3p expression is inversely correlated with the serum level of GZMB in patients treated with radiotherapy.

A. ExomiR-378a-3p expression (blue square) and GZMB expression (black circle) were been studied in longitudinal blood samples from 4 patients treated by radiotherapy by qPCR and ELISA respectively.

B. An inverse correlation was observed between GZMB and exomiR-378a-3p expressions by Pearson correlation test.

DISCUSSION

Among the non-invasive biomarker, circulating exosomal miRNAs (exomiR) present many advantages as a simple blood sample is enough to recover and analyze them. Several recent results have shown that circulating exomiR often reflect the adaptation of cancer cells in response to the anti-cancer therapy[27]. Several circulating exosomal miRNAs have been identified as putative biomarkers in several cancers: miR-425-3p in NSCLC[28] , miR-1290 in ovarian carcinoma[29] and in prostate cancer[30] or hepatocellular carcinoma[5]. However, the roles of these exomiRs in cancer progression or resistance are not fully understood or even studied.

Here, we show that the irradiation of cancer cells promoted the TET2-mediated demethylation of the miR-378a-3p promoter and the exosomal miR-378a-3p (exomiR-378a-3p) overexpression which, in turn, induced the decrease of Granzyme B (GZMB) secretion from NK cells. The repression of GZMB expression by miR-378a-3p has been already demonstrated in NK cells[25,32], but, to our knowledge, our study is the first to demonstrate that this is also effective with exosomal miR production which followed irradiated cancer cells in vitro and in a preclinical murine model. In addition, a longitudinal study in patients provides a rationale for similar regulations during cancer treatments and thus immune evasion during or following radiotherapy.

Our data suggest the existence of a cascade of events controlling exomiR-378a-3p overexpression: irradiation-induced TET activation (4-8h after irradiation), TET2-mediated demethylation of the *miR-378a* promoter (6h post-irradiation), cellular miR-378a-3p overexpression (8-12h after irradiation) and exomiR-378a-3p overexpression (18-24h after irradiation). Interestingly, the exomiR-378a-3p overexpression took place 18-24h after irradiation i.e. when irradiation increased the exosome release from glioblastoma cells[13]. Besides, the increase of exosome secretion following irradiation was also described for different cancer cell lines including breast and prostate cancer cell lines[14]-[15]. Our data also indicated that the miRNA expression could be modulated over time after irradiation. Similar observation was made for let-7a and let-7b for which a decrease was observed previous a return to baseline expression 24h after irradiation[33].

The fact that the miR-378a-3p overexpression occurs in irradiated cancer cell lines, in irradiated mice and in patients treated with radiotherapy, confirms the hypothesis that the overexpression of this miR is a radio-induced phenomenon such as already reported by several articles[34]-[35]-[36]. However, in nice, we noted that only two in six “PC3-xenografted and irradiated" mice overexpresses exomiR-378a-3p. This result must be put into perspective by the fact that the exomiR-378a-3p overexpression mechanisms we described may be not compatible with an “end point” analysis of exomiR-378a-3p expression in mice. A similar remark also prevails in human in which the exomiR-378a-3p overexpression was observed in 6/9 in human patients (if we compare each point with its previous in a longitudinal study). The half-life of exomiR in blood and the putative radio-sensitivity of tumor cells generating the exomiR are also two other parameters explaining

why the exomiR-378a-3p overexpression is not detected in all considered mice and human samples. This is why the inverse relationship found in human patients blood samples is quite remarkable (Figure 4).

Usually, the link between TET2 and miRNA is reported to mention the existing of TET2-targeting miR (including miR-125b, miR-29b, miR-29c, miR-101, and miR-7[37]), but few investigations report the existing of TET2- and more generally of TET-regulated miR. Among these reports, Pan et al. (2016) reports that TET1 and/or TET3 govern the hydoxymethylation of miR-365-3p promoter and the expression of this miR[38]. Thus, present work is one of the first to dissect the involvement of TET2 in the regulation of miR expression. Besides, our investigations indicated that TET2 promoted the irradiation-induced demethylation of miR-378a$^{-541/-358}$ promoter via its interaction with ATF2. Thus, a process of transcription factor-directed DNA demethylation drives the epigenetic regulation of miR-378a. Literature already reports that TET2 interacts with transcription factors to catalyze the transcription factor-directed DNA demethylation process[39]-[40]-[41].

Due to its anti-tumor role, NK cells are subject to intensive research aiming to restore/increase the NK-mediated cytotoxicity via an innovative drug development. In this context, the understanding of exact mechanisms involved in the decrease of NK cells cytotoxicity is crucial. Our work forms part to this by identifying that the irradiation-induced tumor-derived exomiR-378a-3p as a vector of the decrease of NK cytotoxicity via the GZMB decrease expression. Through this finding, we open a new way for the use of TET inhibitors of which the prescription in patients, dictated by the study of blood expression of GZMB and exomiR-378a-3p, will increase the successful radiotherapeutic anti-cancer strategies.

ACKNOWLEDGMENTS

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CONSENT FOR PUBLICATION

Not applicable.

COMPETING OF INTEREST

The authors declare no competing financial interests.

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SUPPLEMENTARY FIGURES

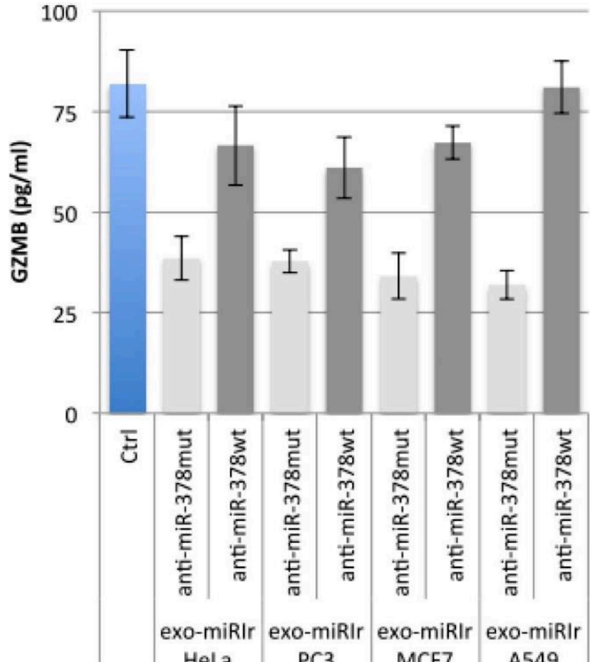

Figure S1. Impact of anti-miR-378 in the granzyme B (GZMB) secretion.

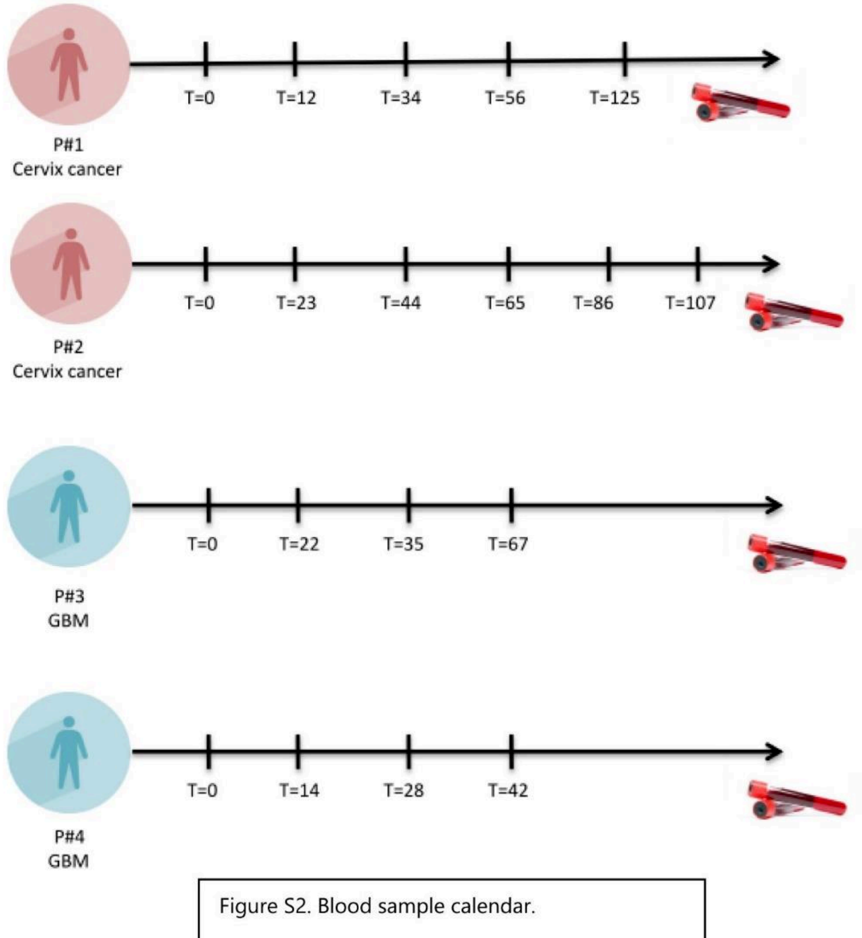

Figure S2. Blood sample calendar.

ADDITIONAL METHODS

^{51}Chromium release assay

The conventional standard 51Cr release assay was performed as follows. U87 target cells (1.10^6) were labeled with 200 µCi (3.7 MBq) of Chromium-51 radionuclide (Perkin Elmer, France) for 1 hour at 37°C. Labeled U87 cells were then washed three times and resuspended at a concentration of 1.105 cells/ml in cultured medium. Labeled U87 target cells(1.104 in a volume of 100 µL) were plated in 96-well V-bottomed plates with NK cells in 200µL of complete medium at an E:T ratio of 4:1. The plate was centrifuged at 1,500 rpm for 1min and incubated for 1 to 4 hours at 37°C under a 5% CO2 atmosphere. The supernatants were collected, and the amount of 51Cr released was measured. Spontaneous release (background) was determined by incubating U87 target cells in medium alone, and maximum release was determined by suspending U87 cells with 0.5% Triton X-100. NK cellcytotoxic activity was calculated as follows: lysis (%) = (experimental 51Cr release − spontaneous 51Cr release) / (maximum 51Cr release − spontaneous 51Cr release) × 100.

Transfections

ExomiR transfections are performed using RNAiMax transfection reagent (ThermoFisher, France) according to the manufacturer's instructions. Isolated exosomes are transfected with mimic-miR (20pmol) according to the Exo-fect Exosome transfection reagent kit (Ozyme, France).

ELISA

Human Granzyme B ELISA Kit (Abcam, France) was used to estimate the Granzyme-B expression. Human Perforin ELISA Kit (PRF1) (Abcam, France) was used to estimate the perforin expression.

ExomiRs extraction

ExomiRs are extracted from exosomes with Total Exosome RNA & Protein Isolation Kit. ExomiRs from 1mL patients' blood are extracted with exoRNeasy Serum Plasma Kits (Cat No./ID: 77044, Qiagen).

Statistical analysis

Expect indications, data are obtained by three independent experimentations. Data in graphs are expressed as the mean ± standard deviation. A t-test is used to determine if there is a significant difference between the mean ± standard deviation of two groups. A Pearson correlation test was used to analyze the relationship between two considered parameters. $p<0.05$ was used as a criteria for statistical significance.

TET activity

TET activity is quantified according to the Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit recommandations (Euromedex, France).

ChIP/ReChIP

Chromatin Immunoprecipitations (ChIP) and ReChIP were performed according to the ChIP-IT Express and ReChIP-IT kits, Active Motif, France.

RT-qPCR

miScript II RT, miScript SYBR Green PCR kits and miScript Primer Assays (Qiagen, France) were used to perform RT-qPCR on the Rotor-Gene Q (Qiagen, France).

miR array

miScript miRNA PCR Array Human Cancer PathwayFinder (Qiagen, France) was used to analyze the modification of miRNA expression in irradiated PC3 prostate cancer cells.

CLIP

The graph illustrates the miRNA-378a-3p and 3'UTR/GZMB enrichments on GW182 and IgG (negative control). Experiments were performed using the RiboCluster Profiler kit (CliniScience, France) according to manufacturer's instructions.

Nuclear extracts

For each indicated time points after irradiation, nuclear extracts from U87 cells were prepared using the Nuclear Extract kit (Active Motif, France).

Article 5 - miR-370-3p is a therapeutic tool in anti-glioblastoma therapy but is not an intratumoral or cell-free circulating biomarker

Pour étudier le potentiel thérapeutique des microARN dans le traitement du GBM, nous nous sommes intéressés au miR-370-3p dans le glioblastome, déjà décrit comme permettant la restauration de la sensibilité au témozolomide *in vitro* en ciblant MGMT [199].

Des inhibiteurs chimiques de la MGMT ont été développé, mais s'ils se sont révélés efficaces *in vitro*, les essais cliniques ont démontré une trop grande toxicité. Le risque avec ces inhibiteurs étant que la MGMT ne répare plus efficacement l'ADN dans les cellules normales, ce qui pose problème étant donné son importance dans les mécanismes de réparation de l'ADN.

C'est pourquoi de plus en plus de traitements cherchent à être le plus précis possible, pour éviter les effets off-target et limiter les effets secondaires indésirables.

Dans cet article, nous avons essayé de déterminer la potentielle utilisation du miR-370-3p en tant que biomarqueur permettant de prédire la réponse au traitement, mais aussi son utilisation comme outil thérapeutique. Pour l'utilisation en tant que biomarqueur, le miR-370-3p pourrait permettre de prédire la survie des patients. Pour l'utilisation en thérapie, le fait qu'il cible MGMT est une piste intéressante.

Au moment de l'intervention chirurgicale, nous n'avons pas pu corréler l'expression du miR-370-3p à la survie du patient, que ce soit dans la tumeur ou circulant dans le sang.

Cependant, ce miR-370 pourrait avoir un rôle thérapeutique. En effet, en diminuant l'expression de MGMT il sensibilise les cellules tumorales au TMZ. Le miR-370-3p, en combinaison avec le traitement au TMZ permet, chez la souris, une diminution de la taille des tumeurs.

Molecular Therapy
Nucleic Acids
Original Article

miR-370-3p Is a Therapeutic Tool in Anti-glioblastoma Therapy but Is Not an Intratumoral or Cell-free Circulating Biomarker

Arulraj Nadaradjane,[1,2,3,4,5] Joséphine Briand,[1,2,3,4,5] Gwenola Bougras-Cartron,[1,2,3,4,5] Valentine Disdero,[7] François M. Vallette,[1,2,3,5,6] Jean-Sébastien Frenel,[7] and Pierre-François Cartron[1,2,3,4,5,6]

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In the last decade, microRNAs (miRs) have been described as biomarkers and therapeutic agents. Based on this finding, our aim here is to know if (1) miRNA-370-3p can be used as a biomarker associated with a favorable survival and if (2) miRNA-370-3p can be used as a therapeutic tool that increases the efficiency of standard anti-GBM treatment. A first approach using the data available on the "Prognostic miRNA Database" indicated that the expression level of miRNA-370-3p in GBM (T-miR-370-3p) is not associated with a prognosis value for survival. A second approach quantifying the expression level of cell-free circulating miRNA-370-3p (cfc-miR-370-3p) also indicated that cfc-miR-370-3p is not associated with a prognosis value for survival. To investigate whether miR-370-3p can be used *in vivo* to increase the anti-GBM effect of TMZ, we then used the model of LN18-induced GBMs in mice. Our data indicated that the miRNA-370-3p/TMZ treatment was two times more efficient than the TMZ treatment for decreasing the tumor volume. In addition, our study correlated the decrease of tumor volume induced by the miRNA-370-3p/TMZ treatment with the decrease in FOXM1 and MGMT (i.e., two targets of miR-370-3p).
Our data thus support the idea that miR-370-3p could be used as therapeutic tool for anti-glioblastoma therapy, but not as a biomarker.

INTRODUCTION

With an incidence of 2–3 per 100,000 people in Europe and the United States, glioblastoma multiforme (GBM) accounts for 12%–15% of all intracranial tumors and is the most deadly malignant primary brain tumors in adults (http://braintumor.org/). The standard treatment for GBM is based on surgery followed by combined radiation and chemotherapy with temozolomide. Despite these treatments, overall survival ($\approx$15 months) and the 5-year survival rate ($\approx$4%) remain very low, and GBM treatment is in a situation of unmet medical need. To remedy this, intense research into the biology and treatment of GBM has been carried out in the last few decades. One of the most promising strategies provided by this research[1–6] is the use of microRNA (miRNA).[7–9]

miRNAs are small (18–25 nt) endogenous non-coding mRNA. These molecules have the ability to inhibit gene expression by binding to target mRNA, thereby promoting a process of translational silencing and/or mRNA degradation. In a clinical setting, there are two advantages to miRNAs: miRNAs can be used as a therapeutic agent or a biomarker.

The literature usually mentions the clinical trial (ClinicalTrials.gov: NCT01829971) using a liposomal formulation of an miRNA-34 mimic (MRX34) as an example. To our knowledge, no clinical trials using therapeutic miRNAs have been reported for GBM, despite robust data supporting a potential therapeutic role for several miRNAs in the treatment of GBM. Of these miRNAs,[10] there is miRNA-370-3p. Gao et al.[11] report that the upregulation of miRNA-370-3p restores the sensitivity of GBM cell lines to temozolomide by influencing O-6-methylguanine-DNA methyltransferase (MGMT) expression.

Concerning the biomarker role played by miRNAs in GBM, the literature reports one clinical trial (ClinicalTrials.gov: NCT01849952) evaluating the expression levels of miRNA-10b, as this miRNA regulates the invasion, angiogenecity, and apoptosis of GBM cells.[12]

Based on these findings, we investigated here whether (1) miRNA-370-3p can be used as an intratumoral and/or cell-free circulating

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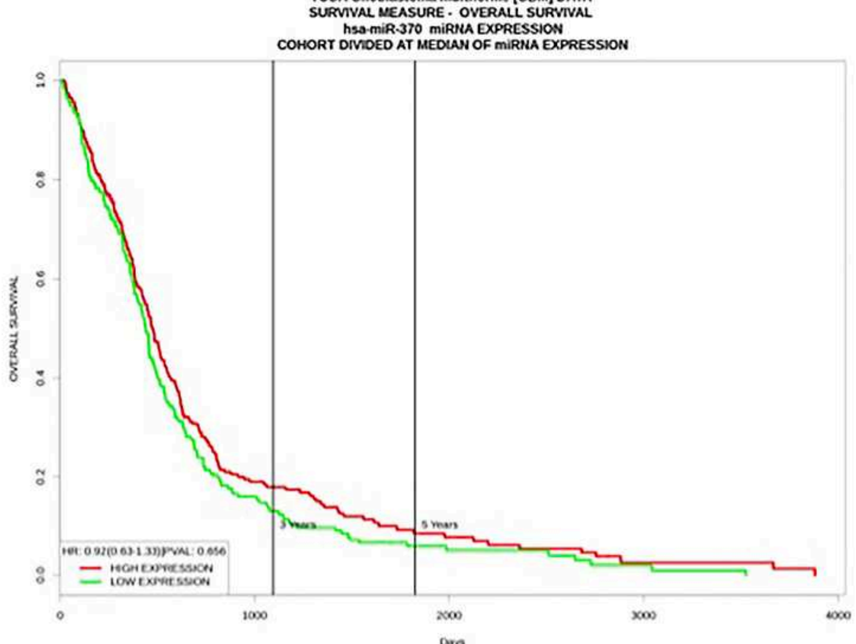


Figure 1. The Tumor Expression Level of miRNA-370-3p Is Not Associated with a Prognosis Value

Kaplan-Meier curves illustrate the overall survival of 471 GBM patients divided into two subgroups based on their median value of miRNA-370 expression. Patients whose miRNA-370 expression was greater than the median value are in red (median survival [days] = 476). Patients whose miRNA-370 expression was less than or equal to the median value are in green (median survival [days] = 441).

biomarker associated with favorable survival and (2) whether miRNA-370-3p can be used as a therapeutic tool that increases the efficiency of the standard anti-GBM treatment.

RESULTS

The Intratumoral Expression Level of miR-370-3p Is Not a Biomarker in GBM Patients

To investigate whether miR-370-3p can be used as a biomarker associated with favorable survival, we first analyzed the data available in the Prognostic miRNA Database (http://xvm145.jefferson.edu/progmir/index.php). We observed that the expression level of miRNA-370-3p in GBM (T-miR-370-3p) is not associated with either a prognosis value for survival or for overall survival (OS) (Figure 1A).

Table 1. Characteristics of the GBM Patients

Characteristics	Patients (n = 23)	Log-Rank Test
Age (years) median [min;max]	61 [36;80]	p = 0.42
Gender (n)		
Male	11	p = 0.28
Female	12	
Survival Time (Days)		
Median [min;max]	376 [85;656]	
Extent of Surgery		
Biopsy or partial resection	7	p = 0.37
Complete resection	16	

The Cell-free Circulating Expression Level of miR-370-3p Is Not a Biomarker in GBM Patients

In a second approach, we considered the cell-free circulating expression level of miR-370-3p (cfc-miR-370-3p). The cfc-miR-370-3p expression level was analyzed from the plasma of 23 GBM patients treated at the Institut de Cancérologie de l'Ouest. The main characteristics of these patients are summarized in Table 1. qRT-qPCRs were performed to estimate the expression level of cfc-miR-370-3p (Figure 2A). Our cohort of 23 samples was then divided into two subgroups using the median value as threshold. Survival curves were visualized in a Kaplan-Meier plot. A log-rank test indicated a lack of difference between the overall survival of GBM patients with a high level of cfc-miR-370-3p and those with a low level of cfc-miR-370-3p (Figure 2B).

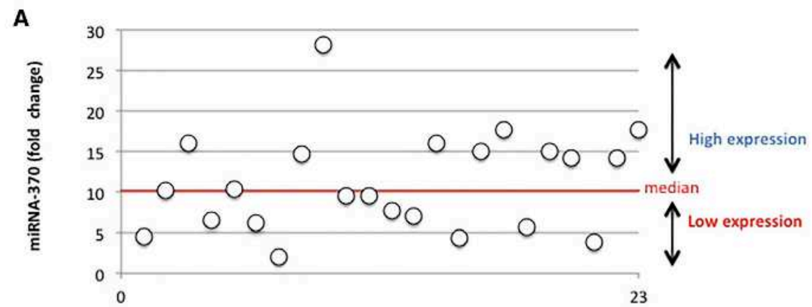
miR-370-3p Downregulates the MGMT Expression and Increases the Temozolimide-Induced Cell Death

To investigate whether miR-370-3p can be used as a therapeutic tool increasing the efficacy of the standard anti-GBM treatment, we first investigated whether miRNA-370-3p affects the MGMT expression level and increases the sensitivity of GBM cells to temozolomide, as previously reported.

To perform this investigation, we used LN18 cell lines, i.e., a cell line expressing MGMT. As expected, a first set of experimentations confirmed that miR-370-3p affected the $MGMT^{mRNA}$ and MGMT expression levels in a dose-dependent manner (Figures 3A and 3B).

A second set of experimentations indicated that miR-370-3p increased temozolomide (TMZ)-induced cell death on LN18 cells in a dose-dependent manner (Figures 3C and 3D).

To determine whether the miR-370-3p-mediated increase of TMZ-induced cell death is not a phenomenon specifically associated with the consideration of LN18 cells, we then asked whether miR-370-3p could increase TMZ-induced cell death on a panel of primary-cultured tumor cells (PCTCs). Figure 3E shows that miR-370-3p increased TMZ-induced cell death in 4/5 PCTCs. Interestingly, we



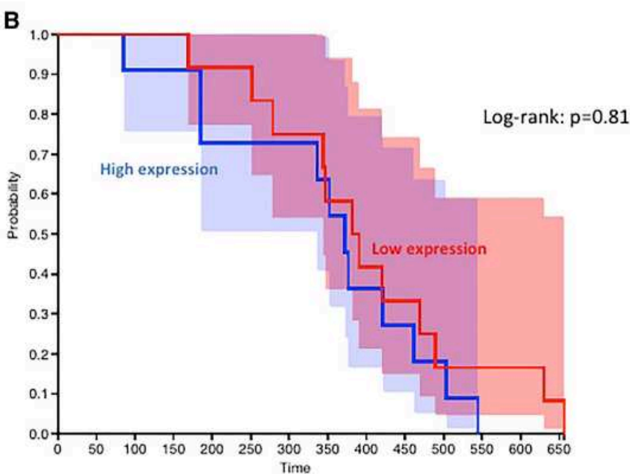


Figure 2. Cell-free Circulating miRNA-370 Is Not Associated with a Prognosis Value

(A) Expression of cell-free circulating (cfc) miRNA-370-3p was estimated by qRT-PCR for each patient (n = 23). Each open circle symbolizes one patient. (B) Kaplan-Meier curves illustrate the overall survival of 23 GBM patients divided in two subgroups based on their median value of cfc-miRNA-370 expression. Patients whose cfc-miRNA-370 expression was greater than the median value are in blue (n = 11, median survival [days] = 372). Patients whose cfc-miRNA-370 expression was less than or equal to the median value are in green (n = 12, median survival [days] = 386).

noted that the PCTCs with no gain of miR-370-3p-mediated sensitivity to TMZ-induced cell death is a PCTC with a high level of TMZ-induced cell death. Our data thus suggest that the miR-370-3p-mediated increase of TMZ-induced cell death is a general phenomenon, not restricted to LN18 cells.

miR-370-3p Also Targets FOXM1, but Not FOXO1 or TGFβ–PII

As miRNA has multiple targets, we considered three other targets for miR-370: forkhead box protein M1 (FOXM1), FOXO1, and transforming growth factor β (TGFβ)-RII (Table 2). ELISA and qRT-PCR indicated that only FOXM1 expression was affected at the mRNA and protein level by miR-370-3p (Figure 4).

miR-370-3p Increases Temozolomide Sensitivity *In Vivo*

We investigated whether miR-370-3p can be used *in vivo* to increase the anti-GBM effect of TMZ. For this purpose, LN18-induced GBMs were generated by xenograft in mice. When the volume of the LN18-induced GBMs was close to 100 mm^3, four mice were randomly untreated or treated with TMZ and/or miR-370-3p at two concentrations (Figure 5A). By comparing the effect of the TMZ treatment with the effect of the TMZ/miR-370-3p treatment, we could see clearly that the latter treatment was more efficient than the TMZ treatment (Figures 5B and 5C).

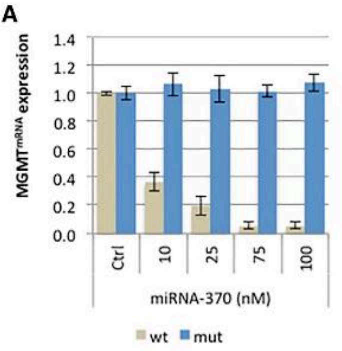
The Decrease in Temozolomide/miR-370-3p-Induced Tumor Volume Correlates with the Decrease in Expression of MGMT and FOXO1, Two of the Targets of miR-370-3p

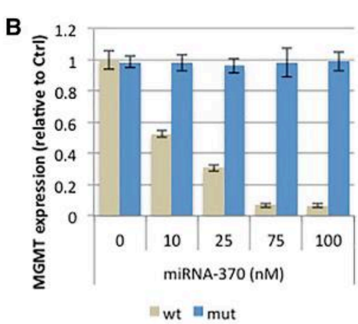
We next analyzed the putative correlation between the TMZ/miR-370-induced reduction in tumor volume and the TMZ/miR-370-induced reduction in MGMT expression. This last parameter was estimated by calculating the difference in MGMT expression at protein levels between the MGMT expression seen in LN18 cells and in resected tumors. ELISA was used to do this. By considering the eight tumors previously treated with TMZ/miR-370-3p, we noted a correlation between the miR-370-induced reduction in tumor volume and the miR-370-induced reduction in MGMT expression (Figure 6A).

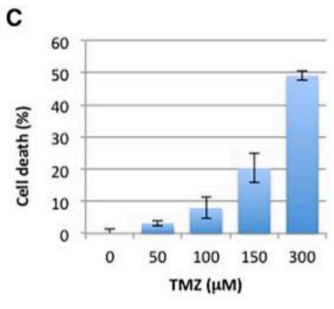
Interestingly, we noted that the miR-370-induced reduction in tumor volume was also correlated with the miR-370-induced reduction in FOXM1 expression (Figure 6B). However, the miR-370-induced reduction in tumor volume was not correlated with the miR-370-induced reduction in FOXO1 or TGFβ-RII expressions (Figure 6C).

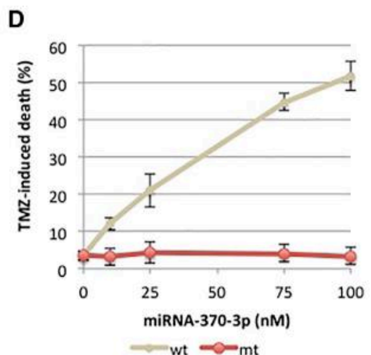
Study of the Longitudinal Expression of miR-370-3p during Standard Anti-GBM Treatment

Our data paradoxically indicated that expression of miR-370-3p is not associated with a prognosis value of response to standard anti-GBM, whereas the addition of this miR increased sensitivity to the standard anti-GBM treatment in *in cellulo* and *in vivo* models of GBM. Based on this point, we postulated that miR-370-3p could be dynamically modulated (up- and downregulated) during the administration of standard anti-GBM treatment. To investigate this point, we analyzed the expression level of cfc-miR-370-3p in longitudinal blood samples of three GBM patients treated with the standard anti-GBM treatment. Figure 7A shows that miR-370-3p expression was dynamic during standard treatment of GBM patients. This last fact suggests that the consideration of miR-370-3p expression as a biomarker requires the realization of a longitudinal study.









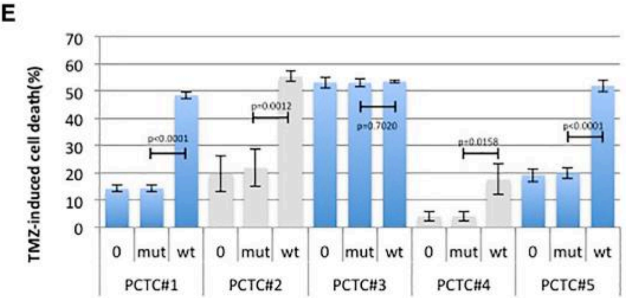


Figure 3. miRNA-370-3p Downregulates the Expression of MGMT and MGMT^{mRNA} and Increases Sensitivity to TMZ *In Cellulo*

(A and B) qRT-PCR (A) and ELISA (B) indicate that miRNA-370-3p decreases MGMT expression at the mRNA and protein levels, respectively. Mimetic wild-type (gccug cuggggguggaaccuggu) or mutated (gAAugcAAggguggaa AAuggu) miR-370-3p were transfected using HiPerFect transfection reagent (QIAGEN, France) according to the manufacturer's instructions. (C) Response of the LN18 cell line to treatment with TMZ for 72 hr was assessed by cytotoxicity assay (Abcam, ab197010, France). (D) The impact of dose-escalation miRNA-370 on TMZ (50 µM)-induced cell death was estimated by cytotoxicity assay (Abcam, ab197010, France). (E) The impact of TMZ (50 µM)-induced miR-370-3p-mediated cell death was estimated by cytotoxicity assay (Abcam, ab197010, France) on a panel of five distinct primary-cultured tumor cells (PCTC). Mimetic wild-type or mutated miR-370-3p (25 nM) were transfected using HiPerFect transfection reagent (QIAGEN, France) according to the manufacturer's instructions. A t test (GraphPad software) compares the mean ± SD of indicated values. Histograms represent average ± SD of 3 independent experiments of the considered parameters.

DISCUSSION

The aim of an extensive amount of research is to identify miRNA as a biomarker and/or therapeutic agent. Our article is also a part of this research axis as it investigated the biomarker and/or therapeutic agent role that miRNA-370-3p might play in GBM.

From our data, we can conclude that miR-370-3p is a therapeutic tool in anti-glioblastoma therapy but not an "in initial tumor" or initial cell-free circulating biomarker. The absence of a biomarker value for miRNA-370-3p in initial GBM or blood is supported by consideration of 471 samples in the data available in the Prognostic miRNA Database and consideration of 23 samples in our cohort of samples. To data, Hayes et al.[13] and Li et al.[14] have reported that miRNA-370-3p expression is protective in neural subtype of GBM. Gao et al.[11] reported that miRNA-370-3p has significantly lower expression in GBM tissue compared to paired non-cancerous tissue, and this independently of the GBM subtypes. However, these studies did not investigate the prognosis value of miRNA-370-3p expression in GBM. In tumors other than GBM, miRNA-370-3p expression had a prognosis value in hepatocellular carcinoma[15] and in pediatric acute myeloid leukemia[16] for example, but also in non-cancer diseases such as coronary artery disease[17] and hyperlipidemia.[18] The longitudinal analysis of miR-370-3p from blood collected during tandard anti-GBM therapy received by patients revealed a relationship between the miR-370-3p expression changes and the time of patient survival before relapse: the longer miR-370-3p is overexpressed, the longer the patient survival before relapse is long. This observation is consistent with the fact that our work identifies miRNA-370-3p as a therapeutic agent potentiating the anti-GBM effect of temozolomide on GBM cells. By showing that 50% of cell death is induced by 300 µM of TMZ or by 50 µM of TMZ + miR-370-3p, our data introduce the idea that using miR-370-3p could make it possible to reduce the quantity of temozolomide and by extension reduce the secondary effects associated with using of temozolomide. In addition, longitudinal miR-370-3p monitoring in blood during the anti-GBM treatment could make it possible to observe that miR-370-3p expression is dynamic during standard anti-GBM treatment. Consequently, our study needs to include more patients to determine whether the time period characterized by elevated miR-370-3p expression is associated with a favorable prognosis of response.

Table 2. Prognosis Value of miRNA-370-3p Targets

	Validation Methods			Prognosis Value	
	Reporter Assay	Western Blot	qPCR	Log-Rank Test	Expression Associated with a Favorable Prognosis
TGFβ-RII	✓	✓	✓	p = 0.336	–
FOXO1	✓	✓	✓	p = 0.248	–
FOXM1	✓	✓	✓	p = 0.0219	low

Here, the targets for miRNA-370-3p considered to be identified by the miRTarBase website. We considered targets validated by three strong pieces of evidence, i.e., targets validated by the use of three different methods (reporter assay, western blot, qPCR) commonly used to analyze the effect of miRNA on its targets (according to miRTarBase website criteria). Prognosis values were calculated using the Betastasis website and the REMBRANDT (repository for molecular brain neoplasia data) bioinformatics knowledgebase available for GBM (http://www.betastasis.com/glioma/rembrandt/kaplan_meier_survival_curve).

Gao et al.[11] have reported that miRNA-370-3p sensitizes the response of GBM cells to temozolomide via the downregulation of MGMT. Our study also supports this data and is more advanced by providing *in vivo* results. For the first time in GBM, our study shows that miRNA-370-3p potentiates the anti-GBM effect of temozolomide in *in vivo* models of xenograft GBM. However, this effect has already been described for tumors other than GBM. For example, Liu et al.[19] report that miRNA-370 inhibits the growth and metastasis of lung cancer. The tumor-suppressive function of miRNA-370 is also reported in both acute myeloid leukemia[20] and laryngeal squamous cell carcinoma.[21] While these results and our data support the idea of using miR-370 as anti-cancer agents, several other publications report that miR-370-3p plays an oncogenic role. Thus, Lo et al.[22] show that overexpression of miRNA-370-3p contributes to gastric carcinoma, and Wei and Ma[23] report that miR-370-3p acts as an oncogene in melanoma.

The tumor suppressor or oncogene roles played by miRNA-370-3p could be due to its targets. When miRNA mainly represses the expression of oncogenes, it is considered to be a tumor suppressor, while an miRNA that mainly represses the expression of tumor-suppressor genes is considered to be an oncogene (also named oncomiR). With regard to miR-370, Zhang et al.[20] demonstrated that the tumor-suppressive role of this miRNA in acute myeloid leukemia is associated with the targeting of FOXM1, and FOXM1 is mainly considered an oncogene in the literature.[24] We and Gao et al.[11] have associated the tumor-suppressive role of miRNA-370 in GBM with the targeting of MGMT. On the contrary, Lo et al.[22] have associated the progression of gastric carcinoma with the miRNA-370-3p-induced downregulation of TGFβ-RII, and TGFβ-RII is associated with a tumor-suppressive pathway.[25] In our *in vivo* study, miRNA-370-3p plays a tumor-suppressive role, and this role is associated with the MGMT and FOXM1 downexpressions, while the expressions of TGFβ-RII and FOXO1 remain unchanged.

In conclusion, our study supports the idea of using miR-370-3p in an miR-based treatment of GBM.

MATERIALS AND METHODS

Plasma Samples

Plasma was collected from GBM patients treated at the Institut de Cancérologie de l'Ouest (ICO, http://www.ico-cancer.fr). In accordance with the regulations, all subjects signed a specific informed consent form for this biocollection, approved by an Ethics Committee (CPP OUEST IV, no. 18/16), the French State Department for National Education, Higher Education and Research (Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, no. DC-2015-2457) and the CNIL (compliance commitment to MR 001).

Cell Culture Conditions

LN18 cells were cultured in high-glucose DMEM with addition of 10% of heat-inactivated fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 U/mL), and 2 mmol/L L-glutamine. Cells were cultivated in a 5% CO_2 incubator at a temperature of 37°C. Cells reaching sub-confluency were detached from the culture dishes using 0.05% trypsin 0.02% EDTA in calcium-free PBS and counted in a Scepter cell counter (Millipore).

PCTCs

Fresh brain-tumor tissue obtained from the neurosurgery department of the Laennec Hospital (Nantes/Saint-Herblain, France) was collected and processed within 30 min after resection. The clinical protocol was approved by the French laws of ethics with informed consent obtained from all subjects. The primary cultured tumor cells were obtained after mechanical dissociation using the technique previously described.[26] In brief, tumor tissue was cut into pieces of 1–5 mm^3 and plated in a 60-mm^2 tissue culture dish with DMEM with 10% FBS and antibiotics. Additionally and in parallel, minced pieces of tumor were incubated with 200 U/mL collagenase I (Sigma, France) and 500 U/mL DNaseI (Sigma, France) in PBS for 1 hr at 37°C with vigorous constant agitation. The single-cell suspension was filtered through a 70-mm cell strainer (BD Falcon, France), washed with PBS, and suspended in DMEM-10% FBS. Cell cultures were subsequently split 1:2 when confluent and experiments were carried out before passages 3–5. During this period, cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 air.

miRNA Extraction and qRT-PCR

A QIAcube automate and miRNEasy Serum/Plasma Kit (QIAGEN, France) were used to isolate circulating miRNA. miScript II RT, miScript SYBR Green PCR kits, and miScript Primer Assays (QIAGEN, France) were used to perform the qRT-PCR on the Rotor-Gene Q (QIAGEN, France). Quantification and purity were analyzed using Qubit (Thermo, France) and Agilent 2100 (Agilent Small RNA kit) according to the manufacturer's instructions, respectively.

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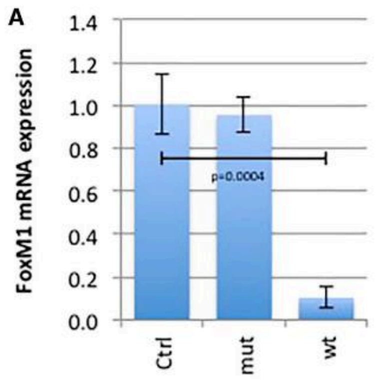

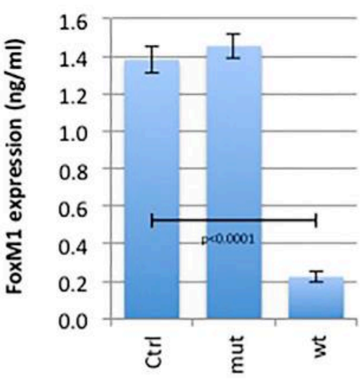

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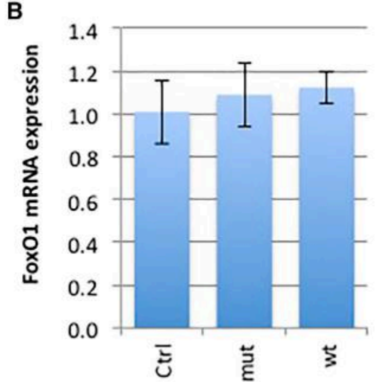

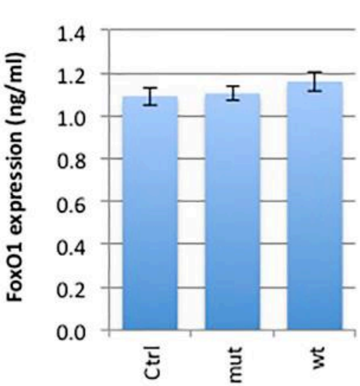

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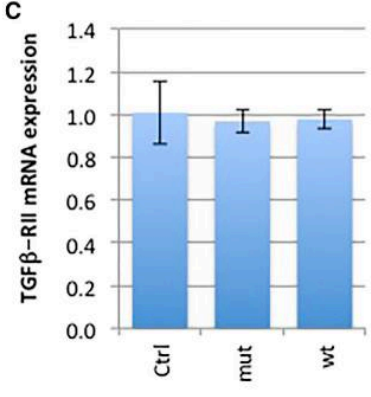


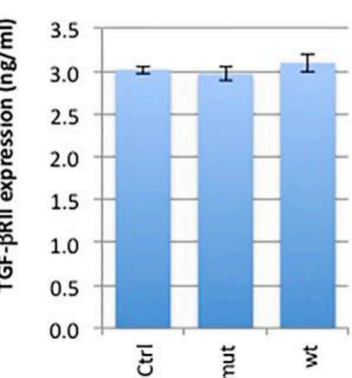


Figure 4. miR-370-3p Also Targets FOXM1, but Not FOXO1 and TGFβ-RII

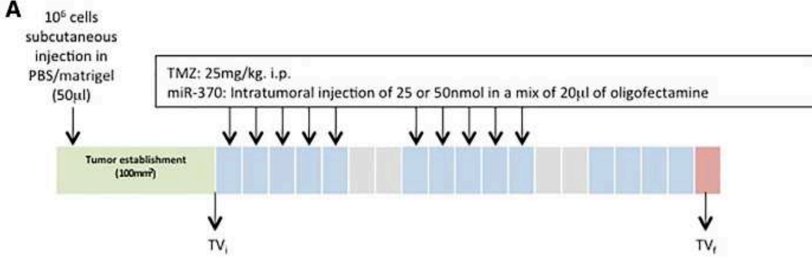
LN18 cells were untreated or treated with mimetic miR-370-3p or mutated mimetic miR-370-3p. qRT-PCR and ELISA analyzed the expression level of FOXM1 (A), FOXO1 (B), and TGFβ-RII (C) at mRNA and protein levels, respectively. Mimetic wild-type or mutated miR-370-3p (25 nM) were transfected using HiPerFect transfection reagent (QIAGEN, France) according to the manufacturer's instructions. A t test (GraphPad software) compares the mean ± SD of indicated values. Histograms represent average ± SD of 3 independent experiments of the considered parameters.

In Vivo Experiments

Cultured LN18 cells were harvested by trypsinization, washed and resuspended in saline buffer. Cell suspensions were injected subcutaneously (s.c.) into the flank of 7- to 8-week-old mice (Janvier, France).

Tumor volume based on caliper measurements was calculated using the modified ellipsoidal formula (tumor volume = 1/2(length × width^2)) according to previous data. At the end of the 21-day observation period, the mice with xenograft tumors were euthanized, and the tumor tissues were removed for analysis.

The experimental procedures using animals were in accordance with the guidelines of Institutional Animal Care and the French National Committee of Ethics. In addition, all experiments were conducted according to the Regulations for Animal Experimentation at the



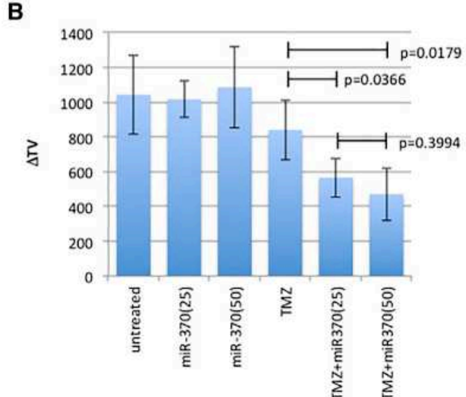


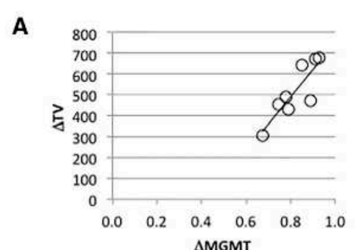
Figure 5. miRNA-370 Increases the Sensitivity of TMZ *In Vivo*

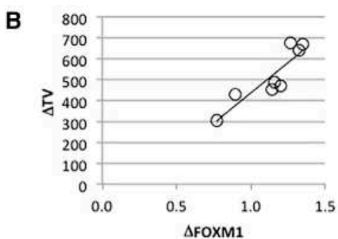
(A) Schematic representation of the protocol used to treat mice. BALB/c nude mice (female, 5–6 weeks old) with LN18 tumors were separated into six treatment groups (four mice per group); None, miR-370(25), miR-370(50), TMZ, TMZ + miR-370(25), and TMZ + miR-370(50). TMZ (25 mg/kg) and/or miR-370-3p were administered intraperitoneally (i.p.) and intratumorally (i.t.) on days 1, 2, 3, 4, and 5 of each week for 2 weeks. Tumor volumes were measured *in situ* with digital calipers at the indicated time in order to evaluate initial tumor volume (TVi) and final tumor volume (TVf). (B) The growth in tumor volume (ΔTV) was calculated as follows: ΔTV = TVf − TVi. Each bar represents mean ± SD calculated from four mice. A t test (GraphPad software) compares the mean ± SD of indicated values. Histograms represent average ± SD of 3 independent experiments of the considered parameters.

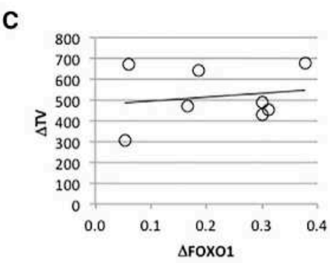
Plate-forme Animalerie in the Institut de Recherche en Santé de l'Université de Nantes (IRS-UN) and approved by the French National Committee of Ethics.

mRNA Extraction and qRT-PCR

A QIAcube automate and RNeasy Mini QIAcube Kit (QIAGEN, France) were used to isolate the mRNA. QuantiTect Reverse







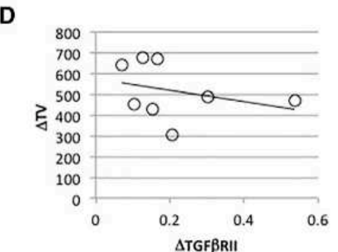


Figure 6. Study of the Correlation between the TMZ/miR-370-3p-Induced Decrease in Tumor Volume and the Modulation in Expression of Four miRNA-370-3p Targets

MGMT (A), FOXM1 (B), FOXO1 (C), and TGFβ-RII (D).

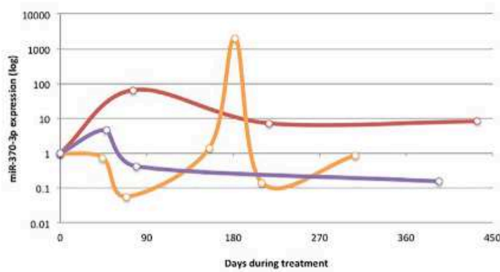


Figure 7. Study of the Longitudinal Expression of miR-370-3p during the Standard Anti-GBM Treatment

The graph illustrates the changes in miR-370-3p expression during the standard anti-GBM therapy received by seven patients. Each curve symbolizes one patient. Each date of sampling is symbolized by a circle on a curve. T = 0 represents the first day of standard anti-GBM treatment received by a considered patient.

Transcription, QuantiFast SYBR Green PCR Kits, and QuantiTect Primer Assays (QIAGEN, France) were used to perform the qRT-PCR on the Rotor-Gene Q (QIAGEN, France).

Protein Analysis: ELISA

Protein extracts were obtained using RIPA Lysis and Extraction Buffer (Thermo Scientific, France) in accordance with the manufacturer's instructions. ELISAs were performed according to the manufacturer's instructions (MyBiosource, USA).

AUTHOR CONTRIBUTIONS

P.-F.C. designed and coordinated the project. A.N., J.B., and P.-F.C. performed all the experiments. G.B.-C., V.D., and J.S.-F. coordinated the obtaining and use of patient samples. G.B.-C., F.M.V., J.-S.F., and P.-F.C. interpreted and discussed the data. P.-F.C. wrote the first version of the manuscript and all authors reviewed and approved it.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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Article 6 - N6-adenosine methylation of mature miRNA-200b-3p influences its functionality and appears as a theranostic tool

Bien que les miRNA soient des cibles et outils thérapeutiques prometteurs, il est crucial de réussir à limiter les effet off-target, tout comme pour la chimiothérapie. La modification chimique de ces miR est une piste prometteuse : ainsi, seules les cellules tumorales seraient capables de restaurer leur activité de répresseurs transcriptionnels.

Dans cet article, nous avons confirmé l'existence de l'adénosiméthylation des miRNA matures et décrit son effet sur l'activité de répression transcriptionnelle des miRNA ainsi que le potentiel thérapeutique de cette découverte. Le miR-200b-3p est méthylé sur l'adénosine par METTL3 et perd son rôle de répresseur transcriptionnel. Il est déméthylé par FTO en combinaison avec l'αcétoglutarate. Le miR-200b-3p inhibe les XIAP, des inhibiteurs de l'apoptose. Ce miR aurait donc un rôle anti-tumoral et pourrait être utilisé comme pro-drogue, pour n'avoir un rôle dans les cellules tumorales et donc une toxicité limitée voire nulle dans les cellules saines.

N6-adenosine methylation of mature miRNA-200b-3b influences its functionality and is a theranostic tools

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Running title: Adenosine methylation miR-200b-3p as a prodrug?

Keywords: miRNA methylation, glioblastoma, Adenosine methylation, prodrug, biomarker

ABSTRACT

Micro-RNAs (miRNAs or miR) plays crucial roles in biological and pathological processes. Certain miRNAs also appear as promising biomarkers and therapeutic tools. However, the epitranscriptomic regulation of miRNAs is not yet fully elucidated in all their fields of application.

We report that the adenosine methylation of mature miR-200b-3p inhibits its repressive function toward its targets such as XIAP by blocking the formation of the miRNA/3'UTR^{mRNA} duplex.

Our data indicate that the adenosine methylation of mature miR-200b-3p is associated with the survival outcome of glioblastoma patients.

All together, our data support the idea that the adenosine methylation of miR-200b-3p can be used as a prodrug whose the cytotoxicity is selective of cancer cells (while excluding PBMC, astrocytes, neuron and hepatocytes).

INTRODUCTION

MicroRNA (miRNA) are short non-coding RNAs that regulate protein expression toward their function of translational repressor. Thus, miRNA are crucial regulators of many cellular processes including proliferation, apoptosis, immunogenicity, development and differentiation. miRNA biogenesis can be epigenetically regulated in both physiological and pathological conditions toward the DNA methylation of miRNA genes. Wang et al. report that the expression of approximately 50 % of miRNA genes is putatively regulated by DNA methylation since they are associated with CpG islands [1]. As for genes, a variety of DNA methylation-specific methyl-CpG-binding domain proteins (MBD) were found to transcriptionally regulate miRNA [2]. Finally, Malumbres et *al.* also report that the miRNA genes expression is also regulated through histone modifications, such as methylation and deacetylation [3]. Two recent publications report that chemical modifications can occur in miRNA and that these modifications regulate the miRNA processing or functionality [4-5]. Despite these undeniable advances, further study of the molecular mechanisms governing the chemical modifications of miRNA in a tumor context is required in order to increase the understanding of the role played by these modifications in tumors.

We here focus our study on the impact of presence of N6adenosine methylation in miRNA-200b-3p in samples of patients suffering from glioblastoma multiforme (GBM).

RESULTS

The m6A methyltransfesrase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6adenosine methylation of miR-200b-3p

Literature reports that miR-200 and particularly miR-200b-3p play a role in GBM [6-7-8-9]. Berulava et *al.* (2015) have identified the presence of m6A in certain miRNAs such as miR-200b-3p[5]. In agreement with these findings, we have investigated the miR-200b-3p level expression (miR-200b-3p^{exp}) and the percentage of miRNA-200b-3p containing m6A (miR-200b-3p$^{\%m6A}$) in a collection of 32 GBM samples (**Table S1**). RT-qPCR experiments indicated a high level of heterogeneity in miR-200b-3p^{exp} with a max/min ratio equal to 37.6 (**Figure 1A**). RNA immunoprecipitation performed with an anti-m6A antibody followed by qPCR analysis (miRIP^{6mA}-qPCR) indicated that 10/32 tumors contained a miR-200b-3p$^{\%m6A}$>10% (**Figure 1B**). In addition, we observed a correlation between miR-200b-3p$^{\%m6A}$ and miR-200b-3p^{exp} (p=0.0022) (**Figure 1B**). This finding could suggest that the presence of m6A affect the biogenesis and/or stability of miR-200b-3p in GBM samples.

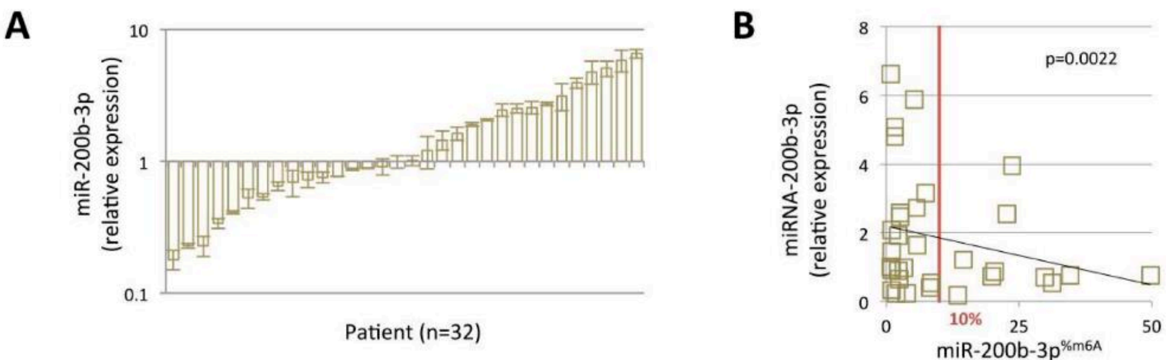


Figure 1: The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6adenosine methylation of miR-200b-3p

A. Each bar represents the relative expression level of miR-200b-3p (miR-200b-3p^{exp}) in 32 samples of GBM. After miR extraction from tumors, qRT-PCR ("miScript/Qiagen" products range) were performed to evaluate the relative expression level of miRNA-200b-3p by using non tumor brain samples as reference and SNORD6.1 as housekeeping miR.

B. Correlation between the relative expression level of miR-200b-3p and the percentage of N6-adenosine methylation in miR-200b-3b (miR200b-3p$^{\%6mA}$). This percentage was calculated via the realization of RNA immunoprecipitation performed with an anti-m6A antibody followed by qPCR analysis (miRIP^{6mA}-qPCR) (as previously described by Berulava et al. 2015).

We next hypothesized that FTO and alpha-ketoglutarate (αKG) could be involved in the N6-adenosine methylation of miR-200b-3 since FTO is an adenosine demethylase that requires alpha-ketoglutarate (αKG) to catalyze the adenosine demethylation[10]. In our collection of 32 GBMs, Pearson's correlation tests show an absence of significant correlation FTO expression level with miR-200b-3p$^{\%m6A}$ (p=0.0689) (**Figure 1C**) and between αKG and miR-200b-3p$^{\%m6A}$ (p=0.0668) (**Figure 1D**). To consider these two parameters, we isolated GBM samples harboring a low FTO expression level (lower than median) and a low αKG level (lower than median) (FTO^{Low}/αKG^{Low}) from the other GBM samples (**Figure S1**). Based on this subdivision,

we noted that GBM samples harboring $FTO^{Low}/\alpha KG^{Low}$ were more m6A-methylated than other GBM samples (p=0.0042) (**Figure 1E**).

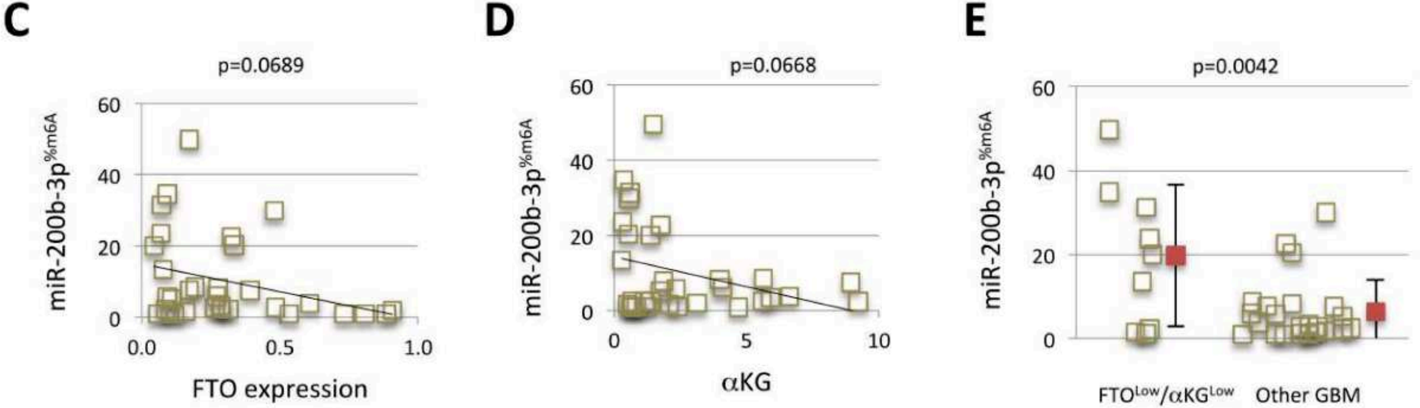


Figure 1: The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6adenosine methylation of miR-200b-3p

C. Absence of correlation between the relative expression level of FTO and the percentage of N6-adenosine methylation in miR-200b-3b. Human FTO ELISA Kit (Tebu-Bio, France) was used to estimate the FTO expression.

D. Absence of correlation between the relative presence of alpha-ketoglutarate (αKG) and the percentage of N6-adenosine methylation in miR-200b-3b. Alpha-ketoglutarate Assay Kit (Abcam, France) was used to estimate the relative presence of αKG.

E. High percentage of N6-adenosine methylation in miR-200b-3b is observed in GBM harboring a low level of FTO and αKG.

Thus, we conclude that both FTO and αKG affects the m6A-methylation level of miR-200b-3p. The involvement of FTO and αKG in the N6-adenosine methylation of miR was also supported by the fact that siRNA directed against FTO increased miR-200b-$3p^{\%m6A}$ (**Figure 1F and Figure S2**), αKG treatment decreased miR-200b-$3p^{\%m6A}$ (**Figure 1G**), meclofenamic acid (MA, a selective FTO inhibitor [12]) decreased the miR-200b-$3p^{\%m6A}$ (**Figure 1H**). In addition, we noted that the knock-down of ALKBH5 (a RNA adenosine demethylase) unchanged the miR-200b-$3p^{\%m6A}$ (**Figure S3**).

Alarcon et *al.* (2015) having identified that methyltransferase-like 3 (METTL3) methylates pri-miRNA in mammalian cells [13], we hypothesized that METTL3 could be implicated in the adenosine methylation of miR-200b-3p. To support this hypothesis, we first observed a significant correlation between miR-200b-$3p^{\%m6A}$ and the METTL3 expression level (p=0.0010) (**Figure 1I**).

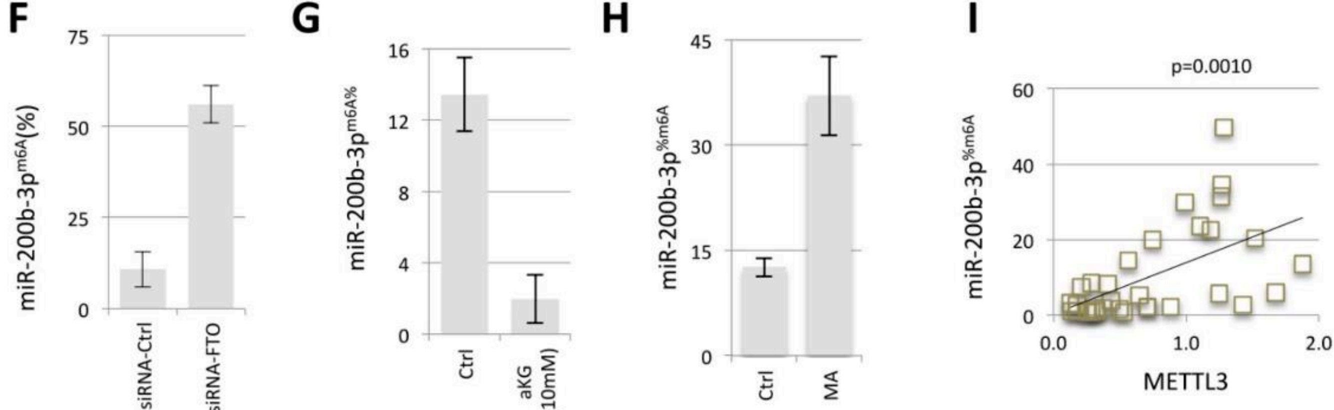


Figure 1: The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6adenosine methylation of miR-200b-3p

F. The impact of the down-regulation of FTO on the adenosine methylation percentage of miR-200b-3p$\%^{m6A}$ was calculated through the realization of miRIP^{6mA}-qPCR as previously described.

G. The impact of the alpha-Ketoglutarate (aKG) treatment on the adenosine methylation percentage of miR-200b-3p (miRNA-200b-3p$^{\%m6A}$) was calculated through the realization of miRIP^{6mA}-qPCR as previously described.

H. Impact of the Meclofenamic acid (MA) treatment on miRNA-200b-3p$^{\%m6A}$ in U87 cells. After MA treatment (15µM/24h, Santa Cruz, France), the miRNA-200b-3p$^{\%m6A}$ was calculated through the realization of miRIP^{6mA}-qPCR as previously described.

I. Correlation between the relative expression level of METTL3 and the percentage of N6-adenosine methylation in miR-200b-3b.

Secondly, dot blot experiments indicated that the products of METTL3 immunoprecipitation (i.e. METTL3-including complexes) promoted the presence of m6A in miRNA-200b-3p^{mimetic} (**Figure 1J**). Thirdly, METTL3 knock-down (siRNA method) decreased the level of m6A in miR-200b-3p (**Figures 1K et S4**).

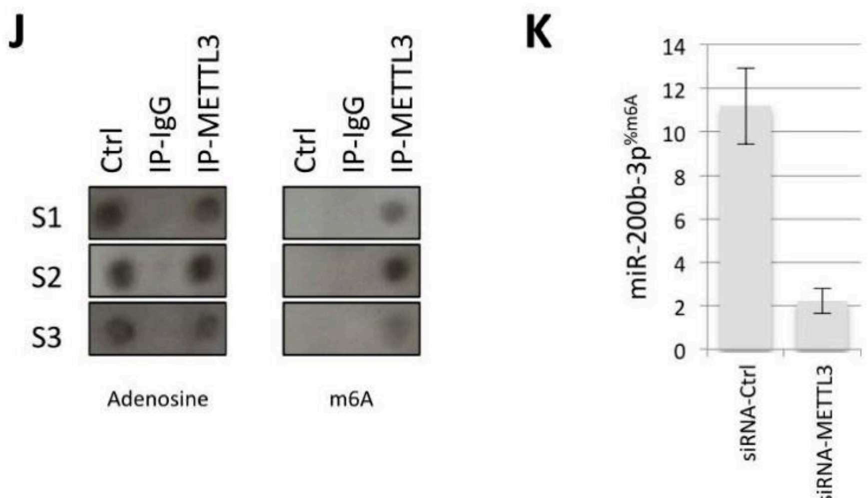


Figure 1: The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6adenosine methylation of miR-200b-3p

J. Dot blot illustrating the presence of adenosine methylation in mimetic miR-200b-3b in presence of METTL3 immunoprecipitation product. Adenosine detection is used as control. S1/S2/S3 are three independent experiments.

K. The METLL3 knock-down (by siRNA approach) decreases the percentage of adenosine methylation of miR-200b-3p.

All the above results suggesting that αKG, FTO and METTL3 collectively influence the presence of m6A in miR-200b-3p, we have calculated the αFM^{score} as reflecting of the consideration of these three parameters. For each samples, +1 was affected when the expression of αKG, FTO and METTL3 influences the N6-adenosine methylation i.e. when the αKG and FTO expressions are lower or equal to the median value of our cohort and when METTL3 expression is higher than the median value of our cohort. -1 was affected when the expression of αKG, FTO and METTL3 influences the N6-adenosine demethylation i.e. when the αKG and FTO expressions are higher than the median value of our cohort and when METTL3 expression is lower or equal to the median value of our cohort. Thus, we noted that the αFM^{score} and the percentage of presence of m6A in miR-200b-3p were significantly correlated in our collection of 32 GBM (p=0.0006) (**Figure 1L**).

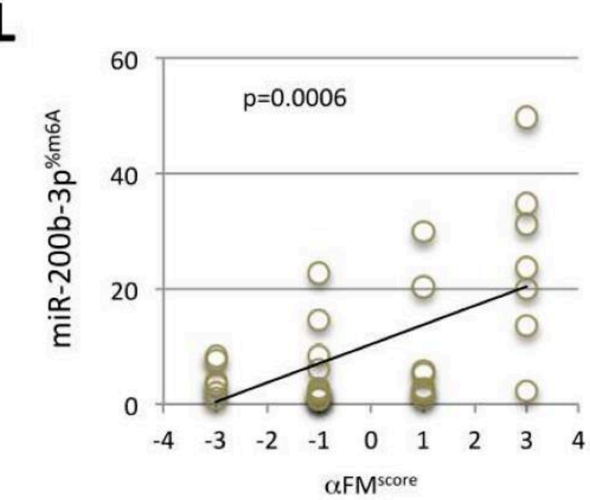


Figure 1: The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6adenosine methylation of miR-200b-3p

L. αFM^{score} reflects the METTL3, FTO and αKG expression levels in the 32 GBM samples. More αFM^{score} is higher more the percentage of adenosine methylation of miR-200b-3p is higher.

Taken together, our data support the idea that METTL3, FTO and αKG are involved in the regulation of the N6-adenosine methylation of miR-200b-3p.

The N6-adenosine methylation of miR-200b-3p limits its translational repressor function towards anti-apoptotic players and confers poor prognosis in GBM patients

XIAP being identified as a target of miR-200b-3p (according to the miRTarBase website), we next investigated whether there is a link between miR-200b-3p^{exp}, miR-200b-3p$^{\%m6A}$ and the XIAP expression in our collection of 32 GBM samples.

Our study did not correlate miR-200b-3p^{exp} and the XIAP expression when all GBM samples were considered (p=0.8803) (**Figure 2A**).

We then extended our study by dividing our samples in 3 groups by taking into consideration the adenosine methylation percentage of miR-200b-3p (**Figure 2B**). Group#1 included samples with miR-200b-3p$^{\%m6A}$ >10%. Group#2 included samples with a percentage miR-200b-3p$^{\%m6A}$<10% and miR-200b-3p^{exp} inferior to the median (miR-200b-3p$^{exp\text{-}low}$). Group#3 included samples with miR-200b-3p$^{\%m6A}$<10 and an expression level of miR-200b-3p superior to the median (miR-200b-3p$^{exp\text{-}high}$).

For all samples having miR-200b-3p$^{\%m6A}$<10 (group#2 and #3), we noted that XIAP expression is inversely associated/correlated with miR-200b-3p^{exp} (**Figures 2B and S5**). This data is consistent with the dogma saying that miRNA is a post-transcriptional repressor.

Surprisingly, we noted that the average of XIAP expression of group#1's samples is higher than the ones of the two other groups (**Figure 2B**). These results can be interpreted as signals suggesting that miR-200b-3p regulates XIAP expression when its sequence does not contain m6A (or a level inferior to 10%) and that the m6A presence in miR-200b-3p could abrogate the post-transcriptional repressor function of this miRNA.

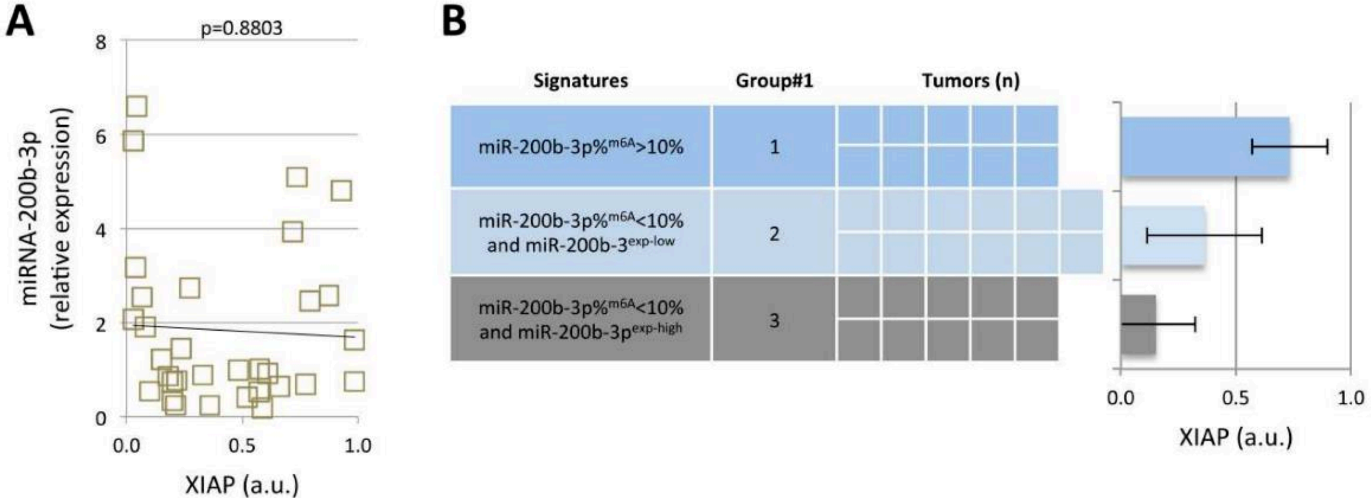


Figure 2: The N6-adenosine methylation of miR-200b-3p limits its translational repressor function toward anti-apoptotic players and confers poor prognosis in GBM patients.

A. Absence of correlation between the relative expression level of XIAP and miRNA-200b-3b (miR200b-3p^{exp}) in all 32 patients included in our study. Human XIAP ELISA Kit (Abcam, France) was used to estimate the relative expression level of XIAP.

B. Samples were stratified according to the miR-200b-3p^{exp} and miR-200b-3p%^{m6A} parameters in order to distinguish the 3 indicated groups. Each box represents a sample/patient. For each group, the average of XIAP expression Human XIAP ELISA Kit (Abcam, France) was calculated and represented on the graph.

To investigate this hypothesis, U251 cells were treated with an unspecific oligonucleotide (negative control), miR-200b-3p^{mimetic} or m6A-modified miR-200b-3p^{mimetic}. As expected, we did not observe any change in XIAP expression when cells were treated with unspecific oligonucleotide, while XIAP expression strongly decreased when cells were treated with miR-200b-3p^{mimetic} (**Figure 2C**). Interestingly, we noted that this decrease is less efficient when cells were treated with the same quantity of m6A-modified miR-200b-3p^{mimetic} (**Figure 2C**). Thus, it appears that the presence of m6A in miR-200b-3p abrogates the post-transcriptional repressor function of this miRNA toward XIAP.

We next performed Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR) analyses to determine whether the adenosine-methylation of miR-200b-3p influences the endogenous formation of 3'UTR-XIAP/miR-200b-3p duplex. In our assays, immunoprecipitation is performed via an antibody directed against GW182 (a protein of a RISC complex with a central

role in miRNA-mediated silencing), and qPCRs were performed to detect the enrichment/presence of miRNA and 3'UTR^{mRNA} on the GW182-mediated co-immunoprecipitation products. CLIP-qPCRs were performed from samples with knock-down of METTL3 in order estimate the impact of the loss of adenosine-methylation on the GW182-mediated co-immunoprecipitation of miRNAs and mRNAs. The miR-150-5p/3'UTR-EP300 duplex was considered as control. The choice of this control was dictated by the fact that miR-150-5p is not adenosine-methylated [5] and the fact that miR-150-5p targets 3'UTR-EP300 [14].

We first noted that miR-150-5p and 3'UTR-EP300 were present in GW182-mediated co-immunoprecipitation products, and this independently of the METLL3 knock-down (**Figure 2D and Figure 2E**). Secondly, we noted that the METLL3 knock-down increased the presence of miR-200b-3p and 3'UTR-XIAP on the GW182-mediated co-immunoprecipitation products (**Figure 2D and Figure 2E**). Thus, these last results indicate that the METLL3-mediated adenosine-methylation status of miR-200b-3p influences the endogenous formation of 3'UTR-XIAP/miR-200b-3p duplex.

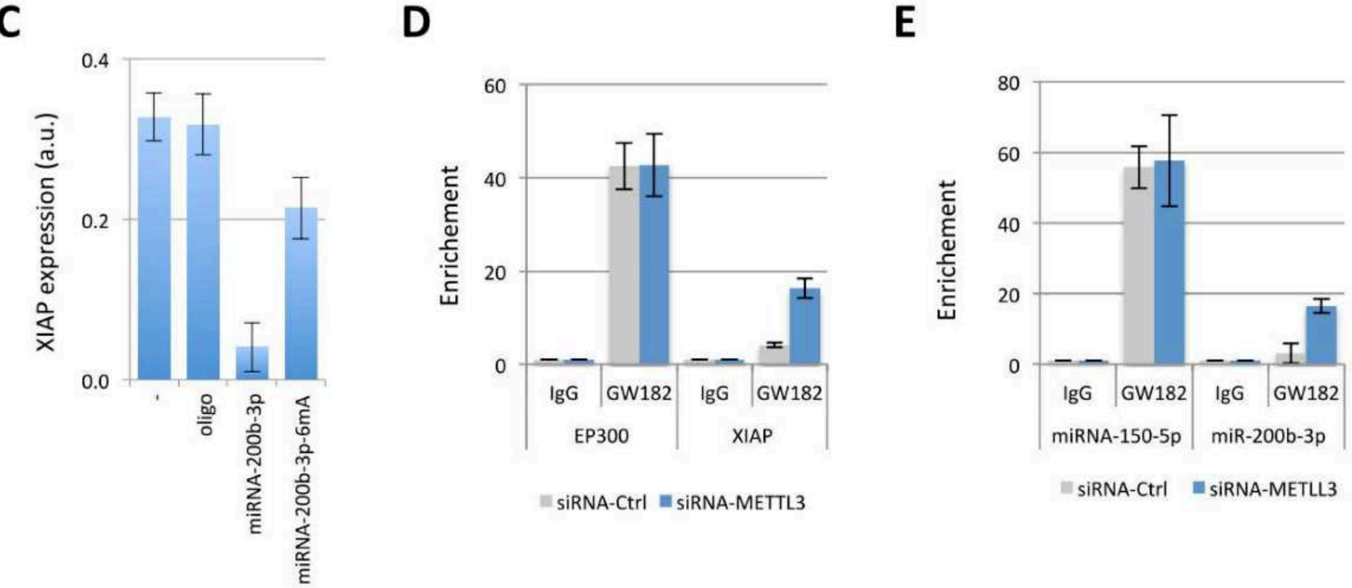


Figure 2: The N6-adenosine methylation of miR-200b-3p limits its translational repressor function toward anti-apoptotic players and confers poor prognosis in GBM patients.

C. Impact of the *in vitro* N6-adenosine methylation of miRNA-200b-3p^{mimetic} on the expression of the XIAP (Human XIAP ELISA Kit (Abcam, France).

D. Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR) investigate the 3'UTR/XIAP and 3'UTR/EP300 (internal control) enrichments on GW182 and IgG (negative control). Experiments were performed using the RiboCluster Profiler kit (CliniScience, France) according to the manufacturer's instructions.

E. Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR) investigate the miR-150-5p (internal control) and miR-200b-3p enrichments on GW182 and IgG (negative control). Experiments were performed using the RiboCluster Profiler kit (CliniScience, France) according to manufacturer's instructions.

By affecting the expression of XIAP, an apoptotic player, our data suggest that the expression level and the N6-adenosine methylation level of miR-200b-3p could affect the intrinsic apoptosis level of tumors. To investigate this hypothesis, we analyzed the Caspase/DEVDase activity as a marker of the intrinsic apoptosis level of tumors. Our work indicates that tumors harboring the miRNA-200b-3p$^{exp-low}$ signature or the miR-200b-3p$^{\%m6A}$>10 signature have a lower intrinsic apoptosis level (**Figure 2F**).

Finally, we observed that patients whose tumors harbored the miRNA-200b-3p$^{exp-low}$ signature or the miR-200b-3p$^{\%m6A}$>10 signature have a lower survival outcome than the other GBM patients (**Figure 2G**).

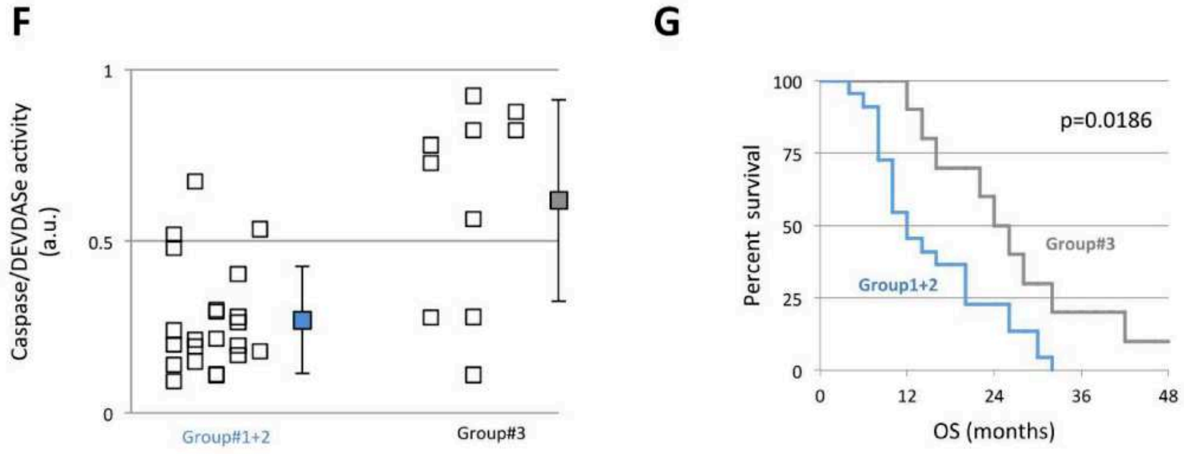


Figure 2: The N6-adenosine methylation of miR-200b-3p limits its translational repressor function toward anti-apoptotic players and confers poor prognosis in GBM patients.

F. For each samples, DEVDase activity was estimated such as previously described. Each open square symbolizes a sample. Blue square symbolizes the average of samples having miR-200b-3p^{m6A}>10% or miRNA-200b-3p$^{exp-low}$. Grey square symbolizes the average of samples having miR-200b-3p^{m6A}<10% or miRNA-200b-3p$^{exp-high}$.

G. Kaplan-Meier representation of survival curves for GBM patients those tumors are characterized by a miR-200b-3p^{m6A}>10% or a miRNA-200b-3p$^{exp-low}$ (in blue) and by a miR-200b-3p^{m6A}<10% and a miRNA-200b-3p$^{exp-high}$ (in grey).

m6A-miR-200b-3p appears as promising tool in anti-GBM therapy

Based on the fact that the miR-200b-3p affects the intrinsic apoptosis level, we extended our study by investigating whether miR-200b-3p and m6A-miR-200b-3p could be used as a therapeutic tool. For this purpose, the miR-200b-3p- and m6A-miR-200b-3p-induced cell death was measured from a panel of cells representing human brain cells (astrocytes (HAST40), neuron (RN33b) and astrocytomas (U87). We included in this panel U87^{IDH1mut} cells since the presence of IDH1 mutation decreased αKG and limited the FTO adenosine-demethylase activity. Meclofemalic acid was also used as a FTO inhibitor [12]. Because peripheral blood is the place where exposure to chemicals occurs PBMC (peripheral blood mononuclear cells) were also included in our study. Firstly, our data indicated that miRNA-200b-3p induced cell death in all cells with the exception of neuron (RN33b cell line) (**Figure 3A**). Secondly, we observed that m6A-miR-200b-3p induced cell death in U87 cells, but not in

U87^{IDH1mut}, U87^{Meclofemalic}, PBMC, neurons and astrocytes (**Figure 3A**). In other terms, these data suggest that the ability of m6A-miR-200b-3p to induce cell death occurs in cancer cells and not in no-cancerous cells that are PMBC, neurons and astrocytes. Based on our knowledge, the absence of massive m6A-miR-200b-3p-induced cell death in U87^{IDH1mut} could be associated to the fact that these cells have a lower quantity of αKG i.e. a lower quantity of the enzyme co-factor (FTO) catalyzing the adenosine demethylation of miR-200b-3p (**Figure S6**). Besides, the fact that the meclofemalic acid treatment abrogated the m6A-miR-200b-3p-induced cell death in U87 cells confirmed the involvement of FTO in this process (**Figure 3A**).

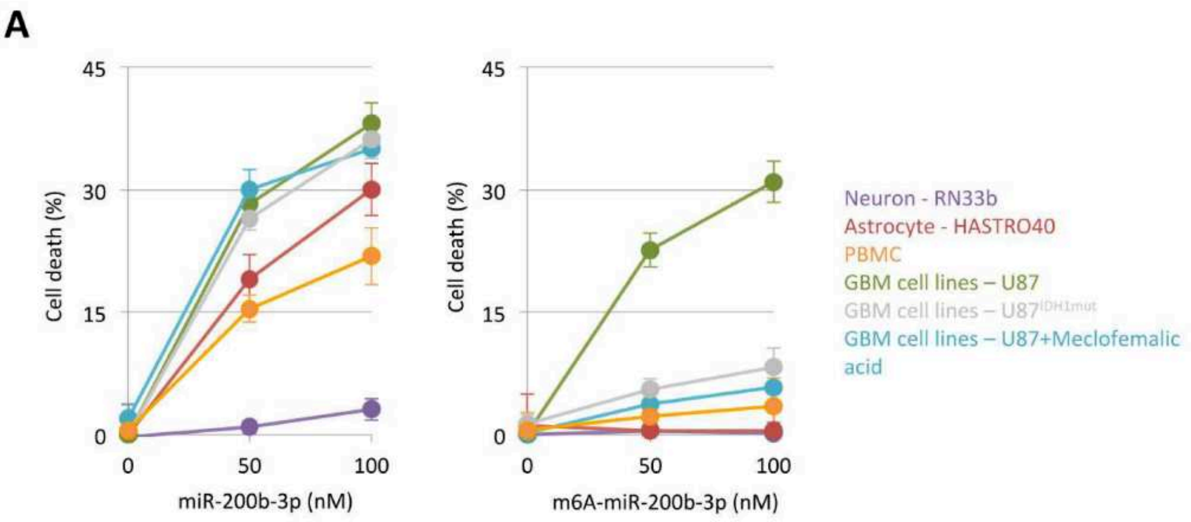
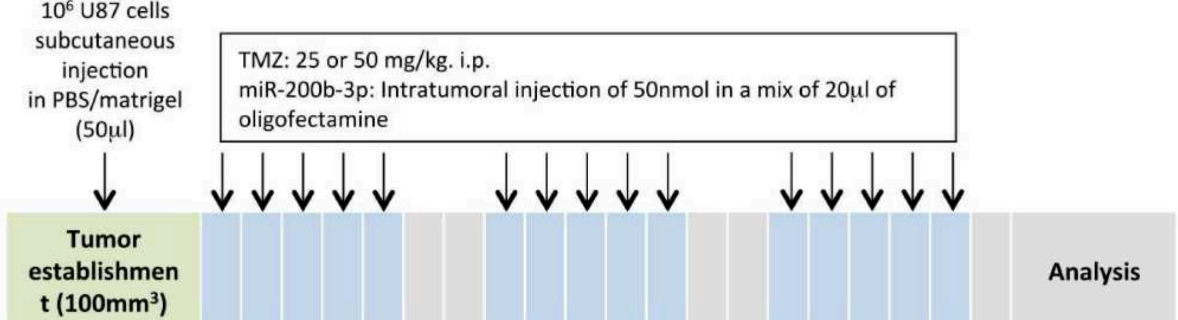


Figure 3: The N6-adenosine methylation of miR-200b-3p selectively induces apoptosis in cancer cells and has an anti-tumor growth effect.

A. miR-200b-3p promotes cell death by itself in cancerous and no-cancerous cells (excepted neuron RN33b), while miR-200b-3b induced apoptosis by itself in U87 cells, only. The LDH-Cytotoxicity Assay Kit (Abcam, France) is used to estimate the cell death 24h after the m6A-miR-200b-3b incubation.

Our last set of data indicating that miR-200b-3p induces cell death of PBMC, neurons and astrocytes, we concluded that this miR has an elevated toxicity toward no-cancerous cells despite its promising ability to promote the cell death of tumor cells. Consequently, we have then investigated the putative anti-GBM effect of m6A-miR-200b-3p in an *in vivo* model of GBM. For this purpose, U87-induced GBMs were generated by xenograft in mice. When the volume of the U87-induced GBMs was close to 100mm^3, three mice were randomly untreated, treated with TMZ or m6A-miR-200b-3p (**Figure 3B**). By comparing the effect of the TMZ treatment with the effect of the m6A-miR-200b-3p treatment, we could see clearly that the m6A-miR-200b-3p treatment has similar efficiency than the TMZ-25mg/kg treatment (**Figure 3C**). We also noted that the m6A-miR-200b-3p+TMZ-25mg/kg treatment has the same efficiency than the TMZ-50mg/kg treatment (**Figure 3C**).



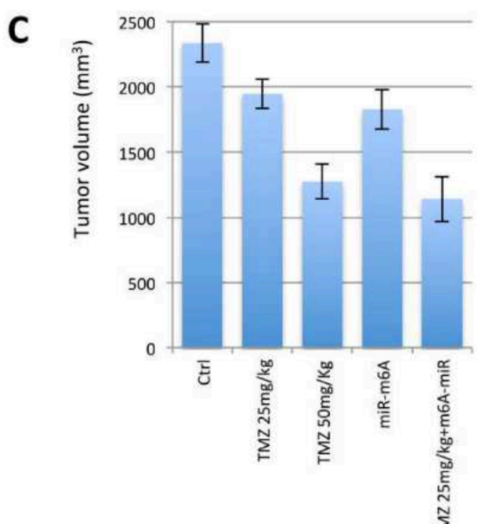


Figure 3: The N6-adenosine methylation of miR-200b-3p selectively induces apoptosis in cancer cells and has an anti-tumor growth effect.

B. Representation of our *in vivo* model of U87-induced GBM.

C. Impact of the adenosine-methylated form of miR-200b-3p on the tumor growth in mice model.

Thus, these two last results are consistent with the fact that m6A-miR-200b-3p appears as promising tool in anti-GBM therapy.

DISCUSSION

Recent investigations concerning the description of the molecular mechanisms of bases modification of miRNAs have provided meaningful progresses in the understanding of regulation of the miRNAs biogenesis and functionality. Thus, after the studies of Alarcon et al. (2015) and Berulava et al. (2015), our study is, to date, the third to report the presence of m6A in miRNAs [135]. Despite this posterior position, our study harbors several innovative points.

The findings of Alarcon et al. (2015) show that the METTL3-induced m6A mark in pri-miRNA, while our findings also incriminate METTL3 for the N6-adenosine methylation of mature miRNAs [13]. Alarcon et al. (2015) also report that m6A mark act as a key post-transcriptional modification that promotes the initiation of miRNAs biogenesis, while our data show that the m6A marks in mature miRNAs acts as a crucial regulator of the post-trancriptional repressor function of miRNAs. In addition to XIAP, our work inducates that the adenosine methylation of miR-200b-3p abrogates its translational repressor function towards putative targets of this miR such as Bcl-2 and CD274/PD-L1 (**Figure S8**). The works published by Alarcon et al. (2015) and Berulava et al. (2015) report that the existence of 2 different consensus sequences for the m6A methylation in pri-miRNAs (UGAC) and in mature miRNAs (ADRA). Interestingly, we noted that the miRNA-200b-3p sequence contains a sequence replying to these 2 sequences (**Table S2**). We also noted that the miR-200b-3p sequence contains a sequence replying to the consensus sequence binding by METTL2/WTAP defined by Ping et al. (2014) [15] (**Table S2**). From a certain perspective, this last point can also constitute an argument supporting the role of METTL3 in the adenosine methylation of mature miRNAs.

The works of Berulava et al. (2015) indicate that FTO plays a crucial role in the demethylation of mature miRNAs [5]. Our data complete this by indicating that the presence of αKG also acts as a non-negligible player in the demethylation of miRNAs.

In addition to these 2 initial reports, our study indicated that the presence of 6mA acts as an inhibitor of the post-transcriptional repressor function of miRNAs. Mechanistically, our data indicate that the presence of 6mA limits the formation of miR/mRNA duplex. Our study is also distinguished from the first 2 studies by its character of "clinical translational study" in a cohort of cancer patients. Indeed, our study is the first to mention that the level of N6-adenosine methylation of a miRNA (in association with the expression level of this miRNA) acts as a biomarker characterizing GBM patients with a poor survival.

The involvement of METTL3 and FTO/αKG in the methylation/demethylation of miRNA-200b-5p is the first evidence describing the existence of an adenosine methylation process replying to a demethylation process for the mature miRNA. Thus, as the DNA methylation regulated by the DNMTs and TETs proteins or the histone methylation regulated by the EZH2 and KDM proteins, the adenosine methylation of mature miRNAs, trough the example of miRNA-200b-3p, appears as a reversible and transmissible process. Thus, our work contributes to open a new epigenetic field: the epigenetic regulation of mature miRNAs and more generally the epitranscriptomic. The role of FTO as a demethylase is debated since Mauer et al. (2017)

report that FTO preferentially demethylates m6Am rather than N6-methyladenosine [16]. However, this article did not indicate that FTO is devoid of N6-methyladenosine demethylase activity, just that FTO prefers m6Am to m6A as a substrate. Besides the N6-methyladenosine demethylase activity of FTO is supported by several other recent publications [17][18]. The fact that the presence of an elevated level of αKG is required to permit the FTO-mediated demethylation of m6A-miR-200b3p also introduced the idea that IDH1/2 mutations act as limiting factors for this FTO-mediated demethylation, since IDH1/2 mutations promote the 2-hydroxyglutarate accumulation at the expense of the αKG accumulation. Besides, the experiments performed with $U87^{IDH1mut}$ confirmed this point since these cells have a higher level of adenosine-methylated miR-200b-3p than U87 cells (**Figure S7**).

By reporting that m6A methylation of miRNAs could act as a biomarker characterizing GBM patients with a poor survival, our data open the idea that the molecular actor writing this epimark (METTL3 according to our data) could be used as a target for the development of epidrugs. Indeed, this point of view is already discussed since METTL3 promote oncogene translation [19][20].

During the last decade, miRNA mimics and molecules targeted at miRNAs (antimiRs) have shown promise in preclinical development [21] [22]. Four arguments strongly support the idea that the adenosine-methylated form of 200b-3p could be used as a promising therapeutic tool. First, m6A-miR-200b-3p is apoptogenic by itself via the repression of two anti-apoptotic proteins (XIAP and Bcl-2) without promoting the inhibition of predicted pro-apoptotic targets such as Caspase-2 (**Figure S8**). Secondly, our data indicate that m6A-miR-200b-3p promotes cell death in cancerous cells such as U87 (but also in other cancer cell lines) and not in non-cancerous cells such as neuron, PBMC, astrocytes and hepatocytes (**Figure S9**). Thirdly, our *in vivo* data indicate that m6A-miR-200b-3p has an anti-tumor growth in an *in vivo* model of GBM. Fourthly, our *in vivo* data also indicate that the m6A-miR-200b-3p/TMZ combination permit to limit the dose of TMZ since the m6A-miR-200b-3p/TMZ-25mg/kg combination has the same anti-tumor growth effect than the use of the TMZ-50mg/kg treatment.

Thus, all these arguments define the adenosine-methylated form of miR-200b-3p as the prodrug from of this miR. More interestingly, our data indicate that its conversion under an active form occurs in cancer cells but not in no-cancerous cells. This observation is a highly promising since it can be translated such as the fact that only cancerous cells have the "tools" (FTO and αKG) to activate the prodrug form of miR-200b-3p. Thus, the adenosine-methylated form of miRs could be considered such as a manner to limit the off-targets effect of miR therapy associated with the relative lack of addressing of miRNA-based therapy against the cancer cells [23]. Our data also introduce the idea that the presence of IDH1 mutations could be considered such as a biomarker excluding the use of adenosine-methylated form of miRs since cells presenting IDH1 mutations have a low level of αKG. Concretely, the first reading of this idea might exclude the use of m6A-miR-200b-3p treatment in less than 10% of primary GBM and in 6-10% of de novo AML, as example [24] [25]. However, this point is available when the m6A-miR-200b-3p treatment is envisioned as single treatment since its combination with

BAY1436032 (a pan-mutant IDH1 inhibitor [26]) restored its ability to promote cell death (**Figure S10**).

In conclusion, our study opens a new area in the understanding of epigenetic modifications concerning miRNA and in the development of innovative epidrugs. Indeed, since several years chemical modifications of RNAs (i.e. epitranscriptomic) are defined such as central players in the control of messenger and ncRNA activity [27]. Our data reinforce this idea by showing that the adenosine methylation of mature miRNAs abrogates their post-transcriptional repressive function. By initiate the idea that adenosine-methylated miRNA could be used as a prodrug, our work provides the base for the development of a new pathway of anti-cancer therapeutic strategies targeting miRNA. Thus, in the future years, the understanding of the mechanisms involved in the epigenetic regulation of miRNA could improve patient stratification and the development of successful therapeutic strategies aiming to limit the off-target of miRNA.

MATERIALS AND METHODS

miRNA extraction

miRNA extractions were performed using the NucleoSpin® miRNA kit (Macherey Nagel , France) according to the manufacturer's instructions.

RNA-immunoprecipitation for miRNA

For immunoprecipitation of RNA, two rounds using 5 µg of anti-m6A antibody (Abcam, France) and 5 µg of small RNA were performed. The reaction was carried out using Dynabeads Protein G Immunoprecipitation kit with some modifications (ThermoFisher Scientific, France). First, the antibody was coupled to Dynabeads Protein G in 500 µl of Binding and Washing Solution for 2 hours at 4°C followed by 15 minutes incubation at room temperature. Beads were washed three times in Washing Buffer. Small RNA was added to the previous mixture in IP buffer (140 mM NaCl, 10 nM sodium phosphate, 0.05% Triton-X) and incubate at 4°C on the rotating platform for 18h. After 5 washes with IP buffer, beads were treated with 250 µl of Elution Buffer (5 mM Tris pH 7.5, 1 mM EDTA, 0.05% SDS) supplemented with 2.1 µl Proteinase K (20 mg/ml) for 1.5 h on a heating block at 50°C at 1100 rpm. Immunoprecipitated RNA was recovered with Trizol LS reagent according to the manufacturer's instructions (ThermoFisher Scientific, France). As a control, immunoprecipitation was performed using IgG instead of anti-m6A antibody. The rest of experimental parameters were kept identical.

Quantitative PCR of miRNA

For miRNA expression analysis and detection from product of RIP performed with anti-6mA antibody, RNA was reverse transcribed using miRScript II RT kit and analyzed by qPCR with the miScript SYBR Green PCR Kit using the specific hsa-miR miScript Primer Assays (Qiagen, France) according to the manufacturers' instructions.

ELISA

Proteins extracts were obtained by using RIPA Lysis and Extraction Buffer (Thermo Scientific, France) in accordance with the manufacturer's instructions. XIAP (Human) Cell-Based ELISA Kit (Abnova, Taiwan), Alpha Ketoglutarate (alpha KG) Assay Kit (ab83431) (Abcam, France) Human FTO ELISA Kit (68ELH-FTO) (Tebu-Bio, France) Methyltransferase like 3 (METTL3), ELISA Kit (MBS9326769) (My BioSource, USA), CST - PathScan® Total Ezh2 Sandwich ELISA Kit (Ozyme, France), EpiQuik Dnmt1 Assay Kit (EpiQuik Dnmt1 Assay Kit, Euromedex/EpiGentek, France), Human Bcl-2 ELISA Kit (Abcam, France), Caspase-2 ELISA Kit (Tebu-Bio, France) and PathScan® Total PD-L1 Sandwich ELISA Kit (Ozyme, France) were performed according to the manufacturer's instructions.

Cell lines

U87^{IDH1mut} and A549 cells were obtained from the American Type Culture Collection (ATCC, USA). OE21 cells were obtained from Sigma (France). HEP10 cells were obtained from ThermoFisher (France). MCF7 and T47D cells were provided by the P Juin's lab. SKOV3 cells were provided by the E Scottet's lab. OV90 cells were provided by the R Spisek's lab.

AUTHOR CONTRIBUTIONS

PFC designed and coordinated the project.

JB, AS, AN, GCB and PFC performed all experiments.

FMV, AS, BO, DH and PFC interpreted and discussed the data.

PFC wrote the first version of the manuscript and all authors reviewed and approved it.

CONFLICT OF INTEREST

Authors declare that they have no financial relationship with the organization that sponsored the research.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental procedures using animals were in accordance with the guidelines of Institutional Animal Care and the French National Committee of Ethics. In addition, all experiments were conducted according to the Regulations for Animal Experimentation at the "Plate-forme Animalerie" of "Institut de Recherche en Santé de l'Université de Nantes (IRS-UN) and approved by the French National Committee of Ethics (Agreement number: B44278).

Patient material as well as records (diagnosis, KPS, age, sex, date of death) was used with confidentiality according to French laws and recommendations of the French National Committee of Ethic. In addition, patient material and experiments using this material are conducted according to the regulations of "the Réseau des tumorothèques du Cancéropôle Grand-Ouest" and more particularly with the regulations of "Réseau Gliome". All patients provided informed consent in accordance with the Helsinki Declaration.

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SUPPLEMENTARY FIGURES

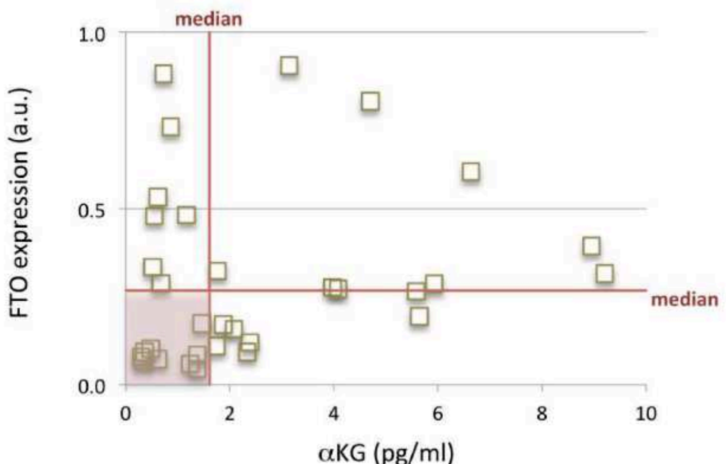


Figure S1. Patients stratification based on the FTO and αKG expression seen in their GBM.

By using the median value of the two considered parameters, we identified a sub-population of patients harboring a low level of FTO and a low level of αKG (in red area). Each square symbolizes a GBM sample (n=32).

ELISA were used to quantified the FTO and αKG expression.

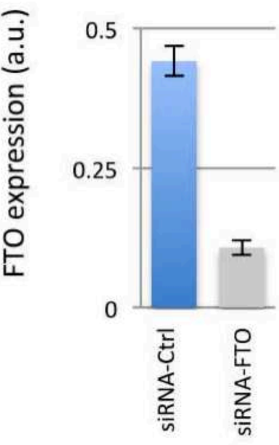
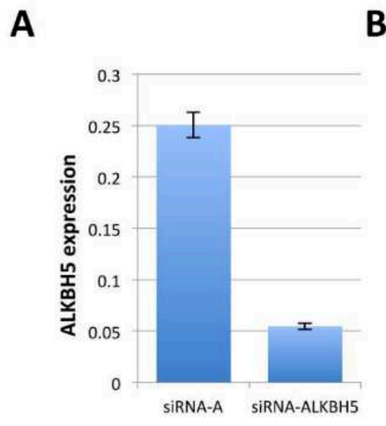
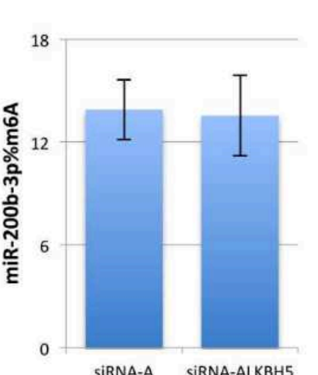


Figure S2. Impact of the siRNA-mediated FTO invalidation on the adenosine methylation percentage of miR-200b-3p (miR-200b-3p%^{m6A}) in U87 cells.

Human FTO ELISA kit was used to estimate the impact on FTO expression of cell treatment with si-RNA control Silencer® Negative Control #1 siRNA and si-RNA directed against FTO Silencer® FTOsiRNA (ThermoFisher Scientific) . Experiments were performed according to the manufacturer' instructions.

A **B**





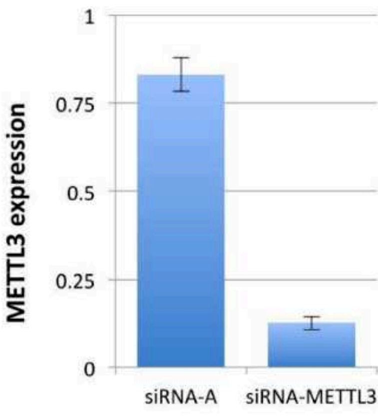


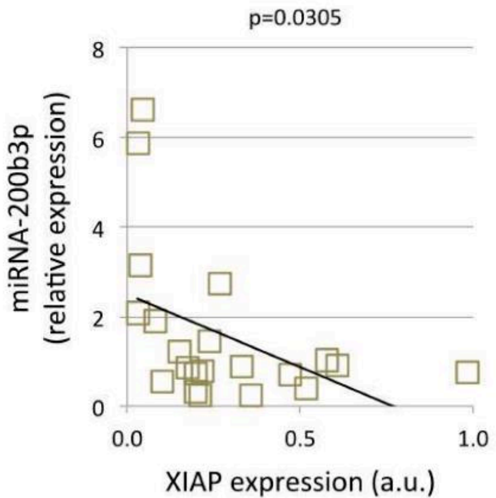
Figure S3. Impact of the ALKBH5 invalidation the adenosine methylation percentage of miR-200b-3p (miR-200b-3p%^{m6A}) in U87 cells.

A. Human ALKBH5 ELISA kit was used to estimate the impact on ALKBH5 expression of cell treatment with si-RNA control Silencer® Negative Control #1 siRNA and si-RNA directed against ALKBH5 Silencer® FTOsiRNA (ThermoFisher Scientific, France). Experiments were performed according to the manufacturer' instructions.

B. miR-200b-3p%m6A was calculated trough the realization of miRIP6mA-qPCR as previously described.

Figure S4. siRNA-induced invalidation of METTL3) in U87 cells.

Human METLL3 ELISA kit was used to estimate the impact on METLL3 expression of cell treatment with si-RNA control Silencer® Negative Control #1 siRNA and si-RNA directed against METLL3 Silencer® METLL3siRNA (ThermoFisher Scientific, France) . Experiments were performed according to the manufacturer' instructions.



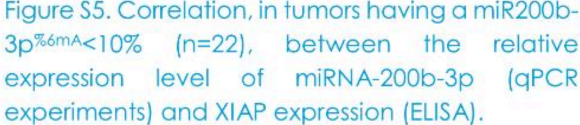


Figure S5. Correlation, in tumors having a miR200b-3p$^{\%6mA}$<10% (n=22), between the relative expression level of miRNA-200b-3p (qPCR experiments) and XIAP expression (ELISA).

p-value is estimated from the Pearson Correlation Coefficient Calculator (r=-0.4618).

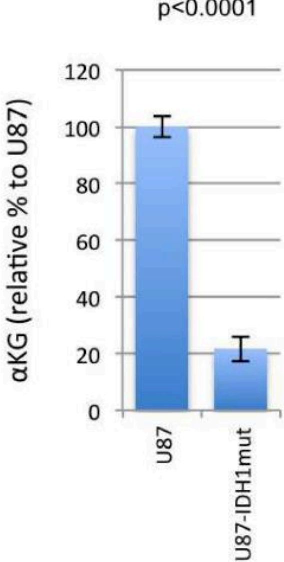


Figure S6. αKG expression in U87 and U87^{IDH1mut} cells (ELISA).

p-value is estimated from t-test.

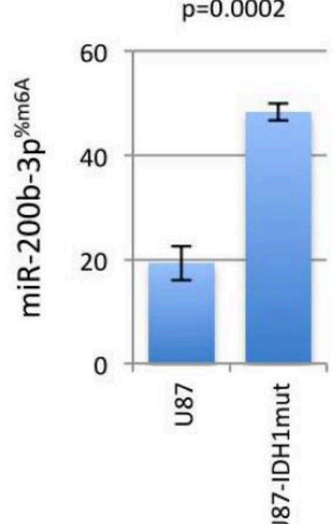


Figure S7. Adenosine-methylation of miR-200b-3p in U87 and U87^{IDH1mut} cells.

miRNA-200b-3p$^{\%m6A}$ was calculated through the realization of miRIP^{6mA}-qPCR as previously described and p-value is estimated from t-test.

Apoptotic players

Epigenetic players

Immune players

miRTarBase

Figure S8. Impact of m6A-miR-200b-3p and miR-200b-3p on the expression of putative protein targets according to miRTarBase website.

ELISA indicated that the presence of m6A in miRNA-200b-3p also abrogates the translational repressor function of miRNA-200b-3p toward Bcl-2 and PD-L1/CD274, but not Caspase-2, EZH2 and DNMT1.

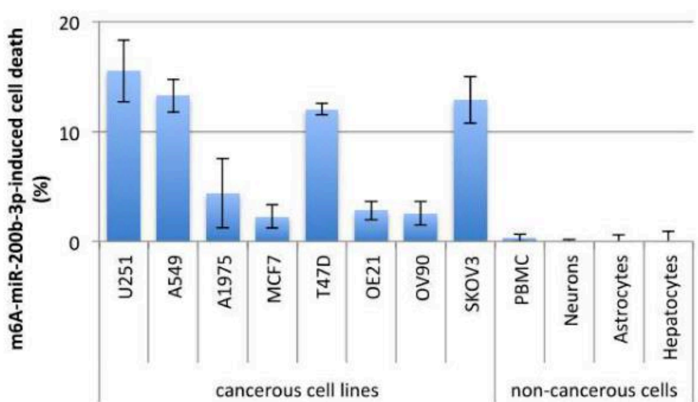


Figure S9. m6A-miR-200b-3p promotes cell death in several cancer cell lines but not in non-cancerous cells.

The LDH-Cytotoxicity Assay Kit (Abcam, France) is used to estimate the cell death 24h after the m6A-miR-200b-3b incubation.

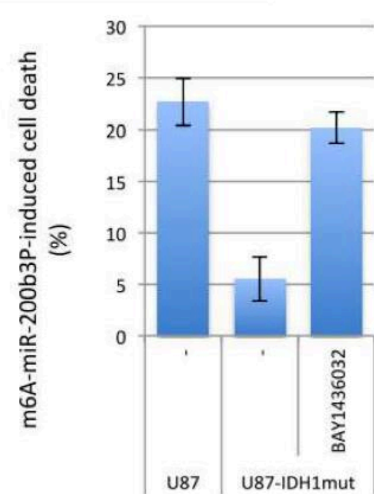


Figure S10. IDH1 inhibitor (50µM, BAY1436032) restores cell death in U87IDH1mut cells.

The LDH-Cytotoxicity Assay Kit (Abcam, France) is used to estimate the cell death 24h after the m6A-miR-200b-3b and/or BAY1436032 incubations.

Characteristics		Patients (n=32)
Age (years) Median (range)		59 (43;76)
Sex		
	Male (%)	19 (59)
	Female (%)	13 (41)
Karnofsky performance score		
	60-80 (%)	22 (69)
	90-100 (%)	10 (31)
Survival time (months)		
Median (range)		16(6;42)
Extent of surgery (%)		
	Biopsie	0
	Partial resection	0
	Complete resection	32 (100)

Table S1. Clinical characteristics of GBM's patients included in our study.

	Reference	-3	-2	-1	0	+1	+2	Homology (%)
m6A in mRNA and lncRNA	Berulava et al. PMID: 25723394		G/A	G/A	A	C	A/U/G	60
Discriminated motif for m6A in miRNA	Berulava et al. PMID: 25723394	A	A/G/U	G/A	A			100
METTL3/WTAP motif	Ping et al. PMID: 24407421	A/U	G/U	G/U	A	C/U	U/G	100
FIRE motif discovery analysis of the METTL3 HITS-CLIP binding sites in pri-miRNA	Alarcon et al. PMID: 25799998		U	G	A	C		75
miRNA-200b-3p	miRBase	A	U	G	A	U	G	

Table S2. Alignment of the miRNA-200b-3p sequence with consensus sequences of interest.

4. Discussion

Le cancer est une pathologie évolutive et en constante modification au cours de la prise en charge du patient. Ici, nous avons étudié différentes phases de la pathologie, dans le but de mieux la comprendre pour mieux la guérir ; en amont puis au moment du diagnostic, et au cours du traitement. Pour finir, nous nous sommes intéressés au développement de nouvelles thérapies.

Cette étude a été réalisée majoritairement dans le glioblastome multiforme, une tumeur cérébrale particulièrement agressive.

L'étude et le traitement des cancers étant un sujet très vaste, nous avons décidé de l'aborder avec un angle épigénétique. En effet, cet axe d'étude qui s'intéresse principalement à la régulation de l'expression des gènes est un champ de recherche important.

Au-delà de l'étude des cellules tumorales, nous avons aussi travaillé sur deux types de cellules immunitaires : les cellules dendritiques plasmacytoïdes et les cellules Natural Killer, afin de s'intéresser à un phénomène important, l'immunosubversion.

Dans ce travail de thèse, nous nous sommes intéressés à différents aspects de la lutte contre le glioblastome :

- Les facteurs de risques, avec notamment l'exposition au diuron, un herbicide
- Les facteurs pronostiques suite à une biopsie, via l'expression de TET2
- Les épimarques circulantes, en nous concentrant sur les microARN
- L'utilisation de nouveaux traitements tels que les miRNA.

4.1) ÉPIGÉNÉTIQUE, GLIOBLASTOME ET PRÉVENTION

4.1.1) SUR LE DÉVELOPPEMENT DE LA GLIOBLASTOMAGÉNÈSE

En premier lieu, nous avons cherché à améliorer la prévention, en caractérisant des facteurs de risques et en essayant d'identifier des signatures précoces du développement d'une tumeur. Pour cela, nous nous sommes intéressés au diuron, un herbicide couramment utilisé jusqu'à son interdiction pour un usage agricole en 2003, mais toujours retrouvé dans l'eau de par sa présence dans la peinture des coques de bateaux, et à son impact sur la gliomagenèse (article 1). À partir d'un modèle de gliomagenèse induite développé par le laboratoire d'E. C. Holland [302], nous avons pu démontrer *in vivo* que la surexpression d'Akt couplée à l'exposition au diuron pouvait induire le développement de glioblastomes, tout comme la surexpression simultanée de Ras et d'Akt. Nous avons prouvé que ce mécanisme passait par une déméthylation globale de l'ADN (c'est-à-dire une diminution du taux de 5mC sur le génome), due à la surexpression d'APOBEC3γ et à la rupture du complexe de maintien de la méthylation de l'ADN DNMT1/UHRF1/PCNA. Parmi les zones déméthylées étudiées, trois ont été identifiées comme impliquées dans la croissance tumorale : PD-L1, LLT1 et Bcl-w. Les deux premiers gènes sont impliqués dans l'échappement tumoral au système immunitaire, le troisième est un inhibiteur de l'apoptose (Figure 36). En clinique, malgré un nombre peu élevé de patients, nous avons remarqué que la déméthylation concomitante de ces trois gènes était présente uniquement chez les patients ayant déclaré avoir été exposés au diuron.

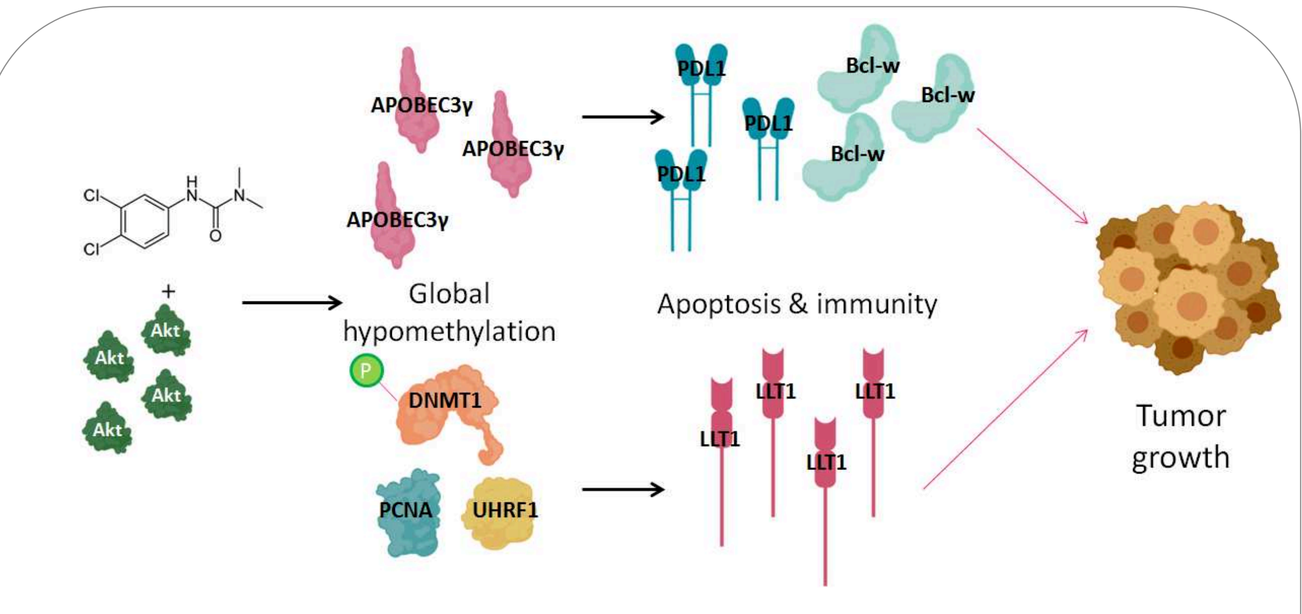


Figure 36 : Abstract graphique de l'article "Diuron exposure and Akt overexpression promote glioma formation through DNA hypomethylation"

Cette étude démontre bien la pertinence du modèle des hits oncogéniques, où plusieurs phénomènes oncogéniques sont nécessaires pour induire le développement d'une tumeur. Dans le cas de la gliomagenèse, deux hits sont nécessaires : la surexpression d'Akt couplée à celle de Ras ou à l'exposition du diuron (Figure 37). Ce même phénomène a été décrit par notre laboratoire avec le glyphosate : dans le cancer du sein, l'exposition à cet herbicide n'est pas suffisante seule mais induit la transformation d'une cellule normale en cellule tumorale lorsqu'elle est couplée à la surexpression du miR-182-5p [242]. La théorie selon laquelle un seul hit oncogénique n'est pas suffisant pour l'initiation tumorale est largement décrite et a été mise en évidence en premier lieu par Knudson en 1971 dans le rétinoblastome [303]. Le même mécanisme est impliqué dans le lien entre la pathologie *Xeroderma pigmentosum* et le développement de cancers de la peau. Le premier hit correspond à la mutation (notamment de XPA) induisant la pathologie, le second à l'exposition aux UV.

L'hypothèse des « two-hits » oncogéniques amène à deux considérations. La première est qu'une personne ayant subi une seule altération peut très bien ne jamais développer de cancer au cours de sa vie. La deuxième, découlant directement de la première, est qu'il est

possible de mettre en place une surveillance accrue chez les personnes étant identifiées comme ayant subi le premier hit afin d'anticiper la survenue d'un cancer (Figure 37).

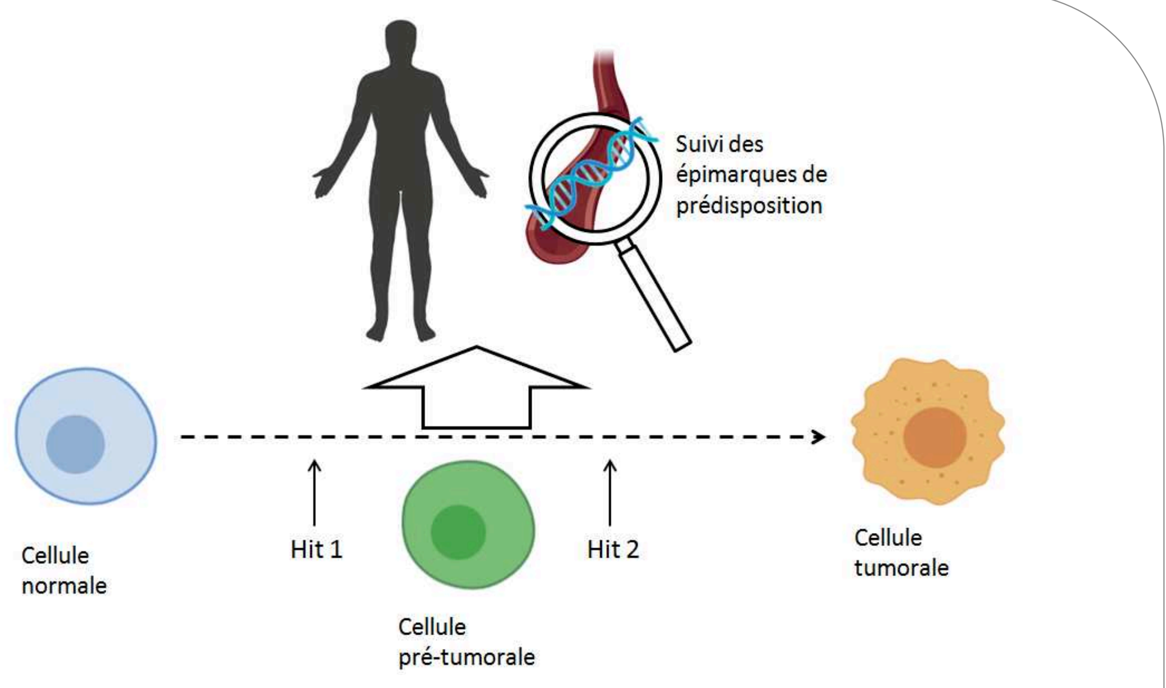


Figure 37 : Schéma de l'hypothèse des deux hits oncogéniques

Basé sur l'hypothèse de Knudson, le modèle des deux hits oncogéniques est confirmé dans cet article, ce qui pourrait avoir un impact dans le diagnostic précoce des patients exposés au diuron.

Par exemple, chez les personnes ayant été exposées au diuron de manière importante, notamment de par leur activité professionnelle, une surveillance accrue pourrait être mise en place soit par des examens cliniques réguliers tels que l'IRM, ou par des biomarqueurs circulants. Dans ce dernier cas, plusieurs articles ont mis en évidence des biomarqueurs pronostics mais pas prédictifs [253, 304]. Dans ce modèle, bien que les résultats restent à confirmer au vu du faible nombre de patients exposés au diuron inclus, nous proposons la signature composée de la déméthylation simultanée des promoteurs de Bcl-w, PD-L1 et LLT1. Le faible nombre de patients exposés au diuron dans cette étude s'explique par le fait qu'étant interdit depuis plusieurs années, il est possible que les patients ne sachent pas exactement quand ni s'ils ont été exposés, et encore moins en quelle quantité.

Ces trois gènes sont impliqués dans l'échappement tumoral au système immunitaire (PD-L1 et LLT1) et dans l'inhibition de l'apoptose (Bcl-w). D'autres pesticides ont été décrits comme modifiant ces deux fonctions biologiques. Par exemple, l'Intervix, un herbicide, induit une dégénérescence des cellules du foie en modifiant leur apoptose par clivage de la caspase-3 [305]. L'HCB (un fongicide) quant à lui diminue la production d'IFN-γ [306].

Il est important de garder à l'esprit que même si le diuron est un herbicide de moins en moins utilisé en France suite à son interdiction, des personnes y sont exposées depuis des années. De plus, nous nous intéressons aux impacts épigénétiques de ce pesticide, et les marques épigénétiques peuvent être héritées de manière transgénérationnelle entre individus. Cela veut dire que l'exposition d'un des parents pourrait avoir un impact sur le développement de pathologies chez l'enfant. De plus, chez une femme exposée lors de sa grossesse, des modifications épigénétiques peuvent apparaitre chez son enfant à naitre mais aussi dans les gamètes de ce dernier, ce qui veut dire que trois générations peuvent être impactées par l'exposition d'une seule, comme cela a déjà été démontré pour le glyphosate [307].

Comme décrit précédemment, les études épidémiologiques sont compliquées à mettre en place. La tumorogénicité du diuron a donc été testée sur des modèles animaux. Il a été montré comme étant carcinogène pour la vessie [288] chez le rat, les glandes mammaires chez la souris [287] et est classé comme carcinogène humain possible par l'agence de protection environnementale des USA [308]. Aucun de ces articles ne s'est intéressé à l'impact du diuron sur l'épigénétique, un champ d'études pourtant primordial, notamment de par son côté héritable. Nous sommes les premiers à nous être à l'impact de l'exposition au diuron sur l'apparition de glioblastomes multiformes et à avoir démontré l'implication de mécanismes épigénétiques.

Dans les modèles *in vitro* et *in vivo*, la surexpression d'Akt a été induite artificiellement, mais c'est un phénomène couramment observé dans plusieurs types de cancers, dont les gliomes [309–311]. De plus, l'exposition au diuron a eu lieu après la surexpression d'Akt. Mais que se passe-t-il lorsque chronologiquement ces deux phénomènes sont inversés, dans le cas où un agriculteur par exemple aurait été exposé au diuron puis aurait développé

une surexpression d'Akt ? L'article de Jain et al. indique que l'ordre d'activation des oncogènes n'a pas d'importance et que c'est l'accumulation d'oncogènes actifs qui conduit à l'initiation tumorale [312].

Notre article reconfirme la capacité d'APOBEC3γ à déméthyler l'ADN, remise en cause par l'article de Wijesinghe et Bhagwat [313]. Dans cet article, les auteurs montrent que APOBEC3γ est capable de convertir les cytosines en uraciles, mais que lorsque le substrat est une 5mC cette activité de conversion est fortement diminuée. Les arguments avancés sont (i) le fait que le substrat favori de cette famille d'enzyme seraient les cytosines, et qu'il y a 30 fois plus de cytosines non méthylées que de cytosines méthylées dans le génome des mammifères, (ii) que ces enzymes préfèrent l'ADN simple brin et (iii) que l'utilisation systématique du système BER après la désamination d'une cytosine en uracile et le mésappariement induirait un risque de mutations trop important. Dans cet article, la capacité de désamination des 5mC par APOBEC3γ a été testée chez E. Coli et *in vitro*. Cela reste moins pertinent que dans des cellules humaines, puisque tous les acteurs protéiques et régulateurs ne sont pas présents. Néanmoins, nos résultats, notamment avec les siRNA, montrent un rôle de APOBEC3γ dans la déméthylation de l'ADN induite par le diuron.

Au-delà du fait que la déméthylation de l'ADN par APOBEC3γ induise en partie la glioblastomagenèse, le fait qu'elle ai lieu via cette enzyme et non par une enzyme de la famille TET induit un autre problème. En effet, la déméthylation de l'ADN médiée par APOBEC3γ est plus susceptible d'induire des mutation génétiques C>T [314], auquel cas une anomalie épigénétique réversible induirait une mutation génétique irréversible. Toujours dans cette idée d'une mutation épigénétique induisant une mutation génétique, l'hypométhylation des transposons, en induisant une instabilité chromosomique peut provoquer leur transposition et induire là encore une mutation insertionnelle irréversible.

Suite à cet article, une question reste tout de même en suspens : le diuron est-il capable d'induire lui-même la surexpression d'Akt ? Des articles ont démontré une hyperméthylation du promoteur de PTEN, régulateur d'Akt dans les GBM [315, 316]. D'autre part, nous avons déjà démontré que le diuron induit une hypométhylation globale de l'ADN [317] : ce phénomène d'hypométhylation locale mais d'hyperméthylation ciblée

est appelé paradoxe de la méthylation. Même si une hypométhylation globale massive peut être à elle seule oncogénique [59, 318], elle n'est pas toujours suffisante puisque dans le cas du diuron un autre évènement doit avoir lieu. Il existe peut-être un seuil de déméthylation à atteindre pour que celle-ci soit oncogénique seule.

À propos de la méthylation locale, classiquement dans le glioblastome, la méthylation de *MGMT* est étudiée. Dans cet article, la méthylation de MGMT n'est pas modifiée, alors que lorsque nous avons étudié l'impact du diuron sur une lignée de GBM une déméthylation était observée. Le fait que MGMT ne semble pas impliquée dans la glioblastomagenèse peut s'expliquer par le fait que cette enzyme joue un rôle dans la réparation de l'ADN suite aux lésions induites par le traitement TMZ/IR, donc une fois la transformation en cellule tumorale effectuée. Bien que nous ayons identifié trois gènes déméthylés par l'exposition au diuron couplée à la surexpression d'Akt, nous n'avons pas regardé la méthylation de l'ensemble du génome. Un MeDIP-seq par exemple pourrait permettre d'approfondir la compréhension de l'impact de l'hypométhylation globale observée. Cependant, les trois gènes identifiés pourraient suffire pour mettre au point un test de détection précoce du développement d'un glioblastome.

4.1.2) Sur l'échappement au système immunitaire

Nous avons démontré que le diuron était capable de diminuer l'efficacité de la cytotoxicité des cellules dendritiques plasmacytoïdes, et que ce phénomène avait lieu via des mécanismes épigénétiques. En effet, une diminution du taux de 5mC sur le génome est observée, due à la surexpression de TET2. Celle-ci entraine la déméthylation des promoteurs de TRAIL et ILT7, ayant respectivement une fonction d'immunostimulation et d'immunotolérance. La déméthylation d'un promoteur induit généralement le rétablissement de sa transcription, comme c'est le cas pour ILT7. Cependant, le mécanisme pour TRAIL est différent. En effet, la déméthylation du promoteur de TRAIL induit le recrutement de CTCF qui peut agir comme répresseur transcriptionnel [319]. Ces deux phénomènes induisent la diminution de la cytotoxicité des pDC (Figure 38).

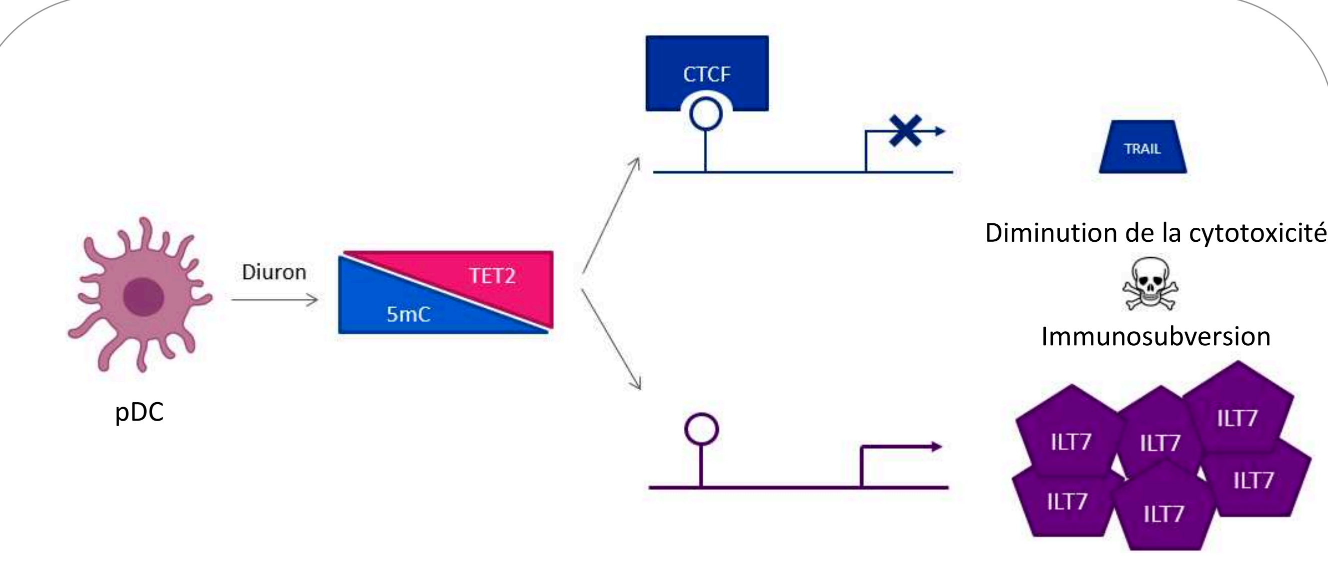


Figure 38 : Abstract graphique de l'article "Diuron modulates the DNA methylation status of the ILT7 and TRAIL/TNFSF10 genes and decreases the killing activity of plasmacytoid dendritic cells"

Le fait que la déméthylation d'un promoteur induise la répression de l'expression du gène a déjà été décrit auparavant. En effet, les effets de la méthylation de l'ADN ne sont pas aussi simples que "la méthylation empêche l'expression". Les deux phénomènes jouant majoritairement sur l'effet de la méthylation sont : le facteur de transcription exprimé et recruté, et le lieu de la méthylation.

Tout d'abord, il existe des répresseurs transcriptionnels. Par exemple, les enzymes se liant à des modifications épigénétiques, telles MeCP2 qui se lie aux CpG méthylés empêchent la fixation des facteurs de transcription [320]. Mais dans cet article 2, le mécanisme est différent, puisque la déméthylation d'un promoteur induit une diminution de son expression. Dans le cas de TRAIL dans les pDC, même avec un CpG méthylé le gène était transcrit, mais la déméthylation de ce CpG a induit le recrutement de CTCF, un répresseur transcriptionnel se liant préférentiellement à l'ADN non méthylé [321]. Un autre mécanisme de répression de la transcription correspond à la méthylation du promoteur dirigée par un facteur de transcription tel que ZEB1. Par exemple, il est capable de réprimer l'expression de l'E-cadhérine et d'induire la transition épithélio-mésenchymateuse, en interagissant avec DNMT1 et donc en maintenant la méthylation du promoteur de l'E-cadhérine [322].

Comme décrit dans l'introduction, le lieu de méthylation a aussi son importance. Au niveau du génome, la méthylation de l'ADN a lieu principalement hors des îlots CpG [323]. Même si elle peut se produire au niveau des promoteurs des gènes, elle peut également survenir sur les régions distales (enhancers, silencers), zones pauvres en CpG mais présentant toutes les formes de cytosines [324]. Le lien entre l'état de méthylation des enhancers et silencers n'est pas aussi claire que pour les promoteurs [38].

De plus, si un CpG est compris dans la séquence consensus d'un facteur de transcription, il y a plus de chances que sa méthylation ait un effet important sur la transcription, puisqu'elle risque d'empêcher la fixation du facteur de transcription.

Nous sommes les premiers à démontrer que TET2, ILT7 et TRAIL sont surexprimés dans les pDC suite à l'exposition au diuron.

Les deux articles sur l'impact du diuron mettent en évidence deux phénomènes différents : l'immunosélection et sur l'immunosubversion [219]. Le premier correspond à la perte de capacité des cellules tumorales à être reconnues par le système immunitaire, et le second à la perte de la capacité des cellules immunitaires à tuer les cellules tumorales. Ces deux phénomènes combinés ont la même finalité mais sont tous deux très utiles à la survie de la cellule tumorale. Donc même si les polluants ne sont pas forcément

carcinogènes seuls et nécessitent un second hit oncogénique, le fait qu'ils jouent un rôle dans l'immunosubversion confirme leur effet néfaste sur l'organisme.

Nous avons donc démontré que l'exposition aux pesticides pouvait avoir un double rôle négatif aussi bien sur les cellules tumorales que sur des cellules du système immunitaire, les pDC. Au laboratoire, des études ont prouvé un impact des pesticides sur les cellules du microenvironnement, elles-mêmes modifiant les cellules tumorales [167, 325] (Figure 39). Cependant, le diuron induit seulement une une diminution du taux de 5mC sur le génome des cellules tumorales mais nous n'avons pas déterminé de modifications transcriptomiques ou phénotypiques associées. En effet, nous avons étudié l'impact de l'exposition au diuron sur une lignée de GBM, les U251. Une déméthylation globale de l'ADN a été observée après 72h de traitement, mais nous n'avons pas trouvé de surexpression associée au niveau des acteurs apoptotiques, immunitaires ou des miRNA. Une hypométhylation du promoteur de *MGMT* a également été observée, mais sans augmentation de la transcription du gène ni de résistance au traitement. Le diuron seul ne semble donc pas augmenter l'agressivité des cellules tumorales (Poster "Aspect épigénétique de l'impact du diuron sur le glioblastome" présenté lors des Journées du Cancéropôle Grand Ouest en 2017).

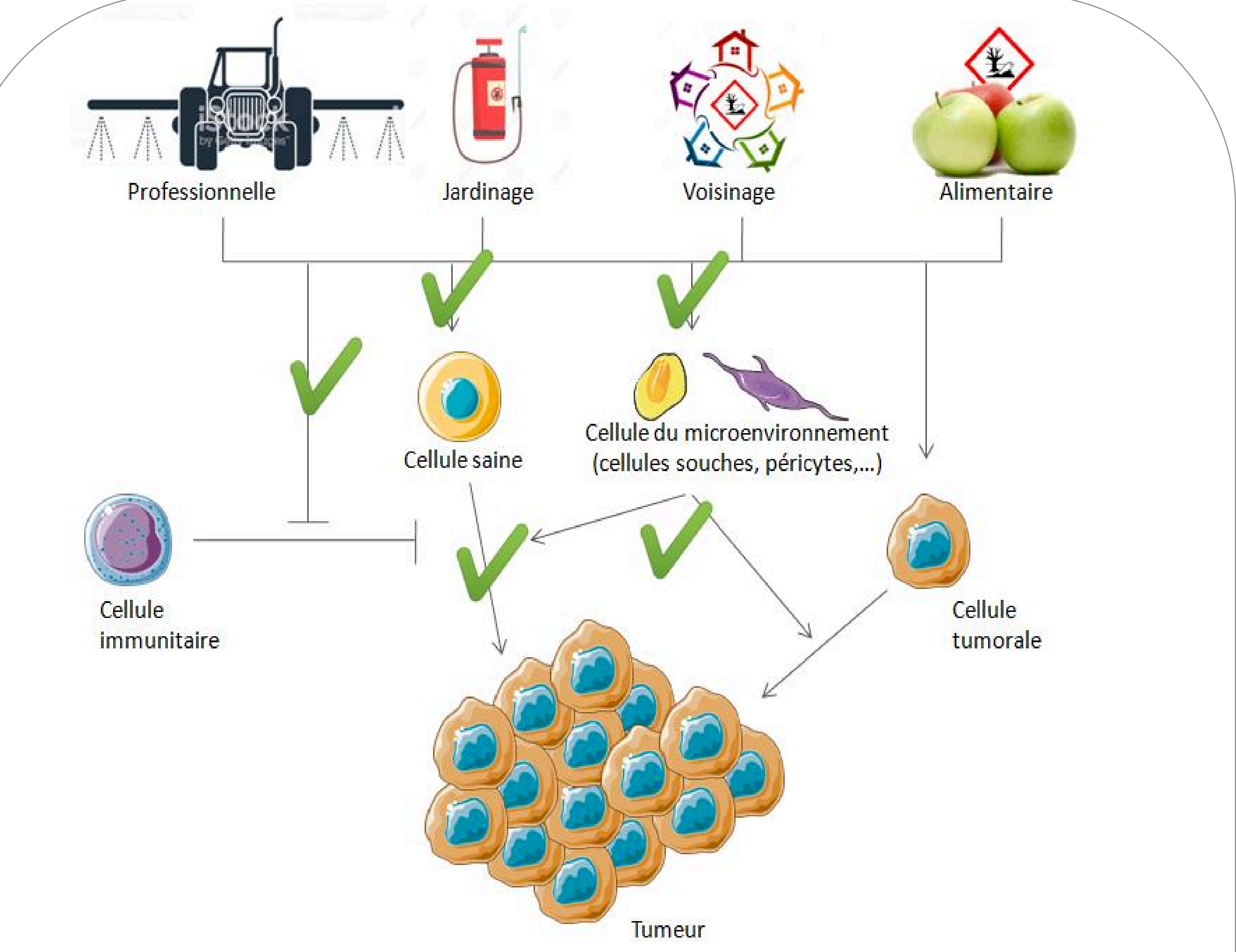


Figure 39 : L'exposition aux pesticides a différents impacts sur l'Homme

L'exposition aux pesticides par différents biais a des répercussions sur différents types cellulaires. Elle empêche la reconnaissance des cellules tumorales par le système immunitaire, elle induit la transformation d'une cellule normale en cellule tumorale, elle impacte les cellules du microenvironnement qui elles-mêmes vont jouer sur les cellules tumorales.

Le diuron induit donc une diminution du taux de 5mC sur le génome aussi bien dans les pDC que dans les astrocytes, mais par des mécanismes moléculaires différents. Afin de contrer ces effets néfastes, il est donc nécessaire de s'intéresser aux acteurs impliqués et pas seulement au résultat final. Une suite possible au projet pourrait être de surveiller ces changements de profils épigénétiques au cours du temps chez des gens exposés ou non à des pesticides afin de détecter le plus précocement possible une modification délétère pouvant entrainer le développement d'un cancer.

4.2) Épigénétique et pronostique

Dans un second temps, nous nous sommes intéressés aux facteurs pronostiques suite à une biopsie, et plus particulièrement à TET2. Dans le glioblastome, l'hypométhylation de l'ADN a été identifiée comme étant un facteur pronostic négatif de la survie [190]. De plus, TET2 est connu comme étant muté dans de nombreuses hémopathies, mais plusieurs altérations ont aussi été décrites dans le GBM [208, 209]. Nous nous sommes donc intéressés à l'expression de TET2 dans le GBM. Dans un premier temps, nous avons étudié son expression au niveau protéique au moment de la première résection, mais elle n'est pas pronostique à ce moment-là. Ensuite nous avons eu l'opportunité d'accéder à des tumeurs plus rares, puisqu'elles proviennent de patients dont la résection tumorale a été effectuée deux fois. Cette configuration est inhabituelle puisque la survie étant courte chez les patients atteints de glioblastome multiforme, il est rare de les opérer deux fois consécutives, bien que cela améliore la survie [299–301]. Nous avons donc établi une cohorte de 10 patients ayant été opérés deux fois, chez lesquels l'expression protéique de TET2 a été analysée et corrélée au temps entre les deux résections. Lorsque l'expression de TET2 augmente entre les deux résections, le temps entre les deux opérations diminue. Ces résultats semblent montrer une implication de TET2 dans l'agressivité tumorale suite à la première résection.

TET2 est une enzyme de régulation épigénétique largement décrite dans différents cancers. Cependant, son rôle est ambivalent, car les mutations de TET2 qui entrainent souvent un troncage de la protéine et donc une perte de fonction sont bien décrites dans les leucémies [208], ce qui irait dans le sens d’une fonction suppresseur de tumeur, alors que nous avons démontré que dans le glioblastome multiforme l’augmentation de son expression induit une rechute plus rapide. Cependant dans les GBM aucun lien n'a été démontré entre une perte de fonction de TET2 et une modification du taux de 5hmC [209]. Il n’est donc pas possible de conclure sur le rôle de TET2 pour toutes les pathologies, les études doivent être menées au cas par cas.

Les questions en suspens suite à cette étude restent tout de même nombreuses, mais seront explorées prochainement. La première question est : quels sont les effets de TET2

qui induisent une augmentation de l'agressivité ? Cela est probablement lié à son activité de déméthylation active de l'ADN, mais dans ce cas, est-ce dû à une déméthylation globale induisant une instabilité chromosomique, ou plutôt spécifique d'oncogène ? Un ELISA 5mC et un MeDIP-seq permettrait de répondre à ces deux questions.

Et surtout, pourquoi le taux de TET2 n'est pas pronostique au moment du diagnostic, mais seulement entre deux résections ? Le traitement témozolomide/irradiation a-t-il un impact sur l'expression de TET2 ? Il est décrit depuis longtemps que l'utilisation de la chimiothérapie induit la résistance de certaines cellules tumorales, cette dernière pourrait être due entre autres à la surexpression de TET2. En effet, la surexpression de TET2 pourrait être un mécanisme de défense mis en place par les cellules tumorales pour résister au traitement TMZ/IR, son expression ne serait alors pas pronostique dans des cellules n'ayant pas encore reçu le traitement. La question globale derrière cette problématique est donc : TET2 est-il une cause ou une conséquence de la récurrence ?

Il semble donc pertinent de suivre l'évolution de TET2 dès le début du traitement. Bien évidemment, il est impossible d'effectuer une biopsie à chaque cure de chimiothérapie/radiothérapie, la biopsie liquide est donc une piste plus qu'intéressante. Une étude portant sur le dosage de TET2 au niveau ARNm et protéine dans le sang permettrait d'apporter des pistes de réflexions sur la potentielle utilisation de cette enzyme comme biomarqueur de la réponse thérapeutique.

Si les résultats de l'étude confirment l'hypothèse que TET2 est de mauvais pronostic dans le GBM, cette enzyme pourrait alors être utilisée comme cible thérapeutique. A l'heure actuelle, il n'existe pas d'inhibiteurs spécifiques de TET2, mais seulement le DMOG qui inhibe les hydroxylases dépendantes de l'αKG [326] ou les hydrazones, des chélateurs de Fe^{2+} [327]. Alors pourquoi ne pas envisager l'utilisation d'un miRNA, par exemple le miR-22-3p ou encore le mir-29a-3p [94, 328] ? De plus, le miR-29a-3p possède un CG, ce qui veut dire qu'il est potentiellement méthylable, et donc pourrait être utilisé comme prodrogue. Cette partie sera plus longuement décrite dans la partie 3.4, qui traite des épithérapies.

Cependant, comme toutes les drogues touchant à l'ADN au sens large (enzymes de réparation, enzymes épigénétiques, etc…), un trop large spectre d'activité est bien souvent néfaste à l'effet thérapeutique. Cet argument peut bien évidemment être objecté par quelques contre-exemples où des inhibiteurs d'enzymes épigénétiques sont efficaces, notamment l'utilisation de la 5-azacytidine dans le traitement des syndromes myélodysplasiques [329], ou celle des HDAC inhibiteurs tel que le SAHA dans le lymphome cutané à cellules T [274] . Malheureusement, ces drogues ne sont pas efficaces dans tous les types de cancers.

C'est pourquoi au lieu de cibler directement une enzyme épigénétique, voire une classe complète d'enzymes (comme dans le cas des HDACi par exemple), cibler des complexes précis enzymes épigénétiques/facteurs de transcription décrits comme étant pronostic est une nouvelle piste thérapeutique.

4.3) ÉPIGÉNÉTIQUE ET SUIVI DE LA RÉPONSE AU TRAITEMENT

Afin d’améliorer la prise en charge des patients, il est indispensable de suivre la réponse de la tumeur à la thérapie au cours du traitement, et les épimarques circulantes sont des outils prometteurs. Dans cette partie, nous nous sommes intéressés non pas à un type de cancer à proprement parler, mais à des pathologies touchant plusieurs organes mais ayant le même traitement : les cancers traités par irradiation. *In vitro*, nous avons travaillé sur des lignées cellulaires de GBM et de cancer de la prostate, alors que chez les patients nous avons eu accès à des biopsies liquides de patients traités pour des GBM et des cancers du col de l’utérus. De plus, nous nous sommes concentrés sur un type d’épimarques circulantes : les miRNA contenus dans les exosomes, appelés exomiR. En effet, ces exomiR reflètent assez bien l’évolution de la tumeur et peuvent permettre de détecter la résistance au traitement [330]. Après avoir démontré que les exosomes issus de cellules irradiées induisaient une diminution de la cytotoxicité des NK envers les cellules tumorales, nous avons identifié le miR responsable de cet effet : le miR-378a-3p. En effet, ce dernier a pour cible décrite le granzyme B, acteur majeur de la cytotoxicité des NK. Dans la lignée de GBM utilisée, les U87, la diminution de la cytotoxicité des NK est due à une cascade d’étapes :

les cellules tumorales irradiées surexpriment TET2, qui déméthyle le promoteur du miR-378. ATF2, un facteur de transcription est recruté sur le promoteur, induit alors la surexpression du miR-378. Il est alors exporté dans les exosomes et arrive jusqu'aux NK, où il se fixe à l'ARNm codant pour le granzyme B et empêche sa traduction. Le granzyme B est une enzyme qui clive après une asparagine, notamment les caspases 3, 7, 9 et 10, inductrices d'apoptose. Cette lyse est donc aspécifique et peut avoir lieu dans n'importe quel type de cellule. Le NK perd alors son activité cytotoxique et n'est plus capable d'induire la mort des cellules tumorales. Chez les patients, nous avons observé une évolution en miroir de l'exomiR-378a-3p et du granzyme B dans le sang chez 75% des patients. De plus, une corrélation inverse a été établie entre l'expression du miR et celle du granzyme B. Le miR-378a-3p pourrait donc avoir une double utilité : biomarqueur et cible thérapeutique.

Les cellules tumorales déploient de nombreux moyens pour parvenir à survivre malgré l'éventail thérapeutique utilisé. Au-delà de l'acquisition de la résistance au traitement, elles mettent aussi en place des stratagèmes leur permettant d'échapper au système immunitaire. Pour cela, deux grands types de réponses sont utilisés : l'échappement au système immunitaire, notamment via l'expression de checkpoints inhibiteurs, mais aussi, comme ici, la réduction de la cytotoxicité des cellules immunitaires.

Ici, nous avons démontré que par un phénomène de communication intercellulaire les cellules tumorales irradiées sont capables de diminuer la cytotoxicité des NK en augmentant leur production de miR-378-3p et son exportation dans les exosomes afin qu'ils puissent atteindre leur cible (Figure 40).

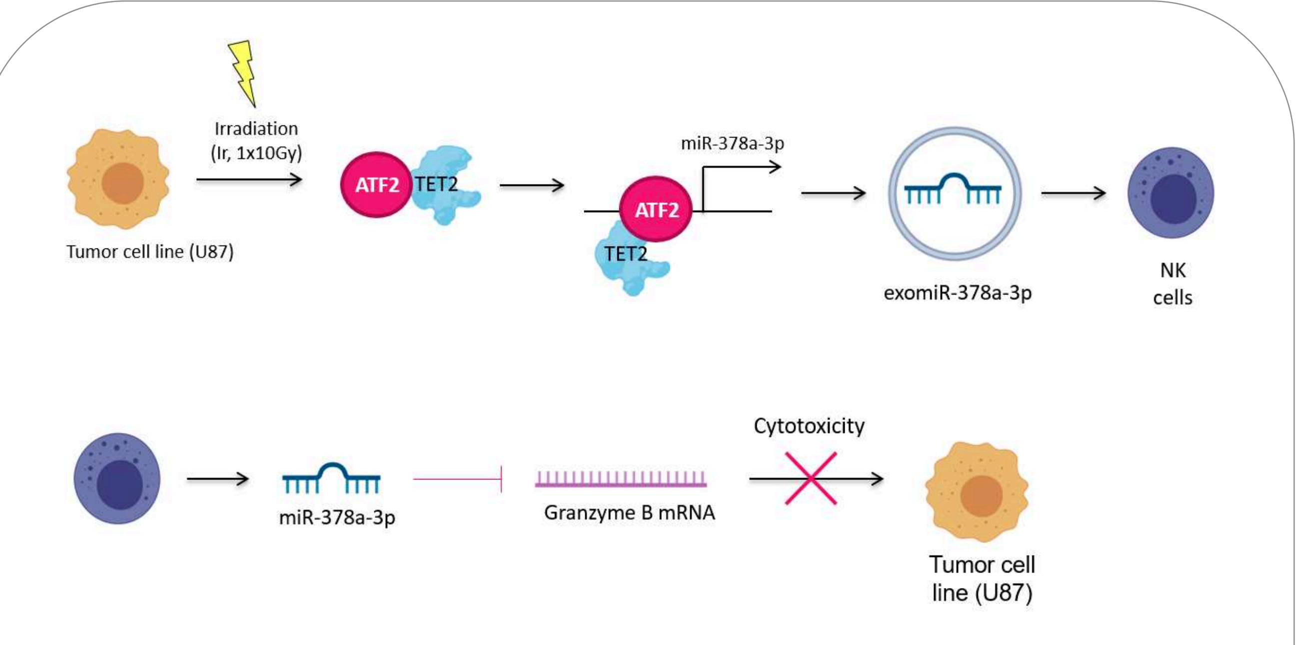


Figure 40 : Abstract graphique de l'article "Radiotherapy-induced overexpression of exosomal miRNA-378a-3p in cancer cells limits Natural Killer cells cytotoxicity via the downregulation of granzyme B"

Nous ne sommes pas les premiers à démontrer l'effet des traitements sur la modification de l'expression des miRNA. En effet, Ma *et al.* ont démontré que le miR-622 était surexprimé suite à l'irradiation de cellules de cancer colorectal et que celui-ci induisait une radiorésistance [331]. Le miR-770-5p est quant à lui surexprimé dans des cellules de cancer du sein suite à l'irradiation de ces cellules [332]. De même, une signature de miRNA surexprimés suite à l'irradiation de fibroblastes a été établie [333]. Le point fort de notre article est de démontrer le mécanisme épigénétique sous-jacent à la surexpression du miR-378 induite par l'irradiation : la déméthylation du promoteur du miR-378a par TET2 et ATF2.

L'effet du miR-378 sur l'expression du granzyme B dans les NK était déjà connu [334], mais nous sommes parmi les premiers à démontrer le rôle d'une communication intercellulaire dans ce mécanisme. Il serait également pertinent d'étudier l'impact de l'exomiR-378-3p sur les lymphocytes T cytotoxiques car ces cellules produisent aussi du granzyme B [335], il est donc possible que l'exomiR agissent de la même manière sur ces cellules que sur les NK,

diminuant fortement leur cytotoxicité et donnant un avantage considérable aux cellules tumorales.

Dans le sang, le dosage du granzyme B est facilement réalisable, mais reflète-t-il vraiment la réponse immunitaire ? Chez des patients infectés par *Plasmodium falciparum*, une augmentation de la quantité de granzyme circulant a été observée [336], ainsi que chez des patients avec une infection bactérienne [337]. De plus, la chimiothérapie induit aussi une augmentation du taux de granzyme B circulant [338]. Le granzyme B circulant est donc bien révélateur d'une activité immunitaire.

La question de la cinétique est également intéressante. En effet, la surexpression du miR-378 est concommittante à l'augmentation du relargage des exosomes. Comme décrit dans l'introduction, l'exportation de miRNA dans les exosomes n'a pas seulement pour objectif d'exporter le surplus à l'extérieur de la cellule. Dans cet article, nous ne nous sommes pas intéressés à l'expression des RNA-binding protéines, responsables de l'export des miRNA dans les exosomes, mais l'exomotif de hnRNPA2B1 est présent dans la séquence du miR-378a-3p. Une étude plus poussée au niveau des mécanismes épigénétiques (méthylation de l'ADN et modifications des histones) permettrait de comprendre comment l'irradiation est capable de modifier l'expression des miR et leur relargage. À ce sujet, une thèse débute au laboratoire.

Les enzymes de la famille des TET ont déjà été décrits comme modifiant l'expression des miR. Par exemple, la surexpression de TET1 induit une expression aberrante des miR dans la leucémie myéloïde aigüe [339]. TET1 et TET3 quant à eux régulent l'expression du miR-365-3p [340].

Dans cet article, ATF2 a été décrit comme le facteur de transcription responsable de la transcription du miR-378 suite à la déméthylation de son promoteur par TET2. Dans les carcinomes rénaux, l'expression d'ATF2 est de mauvais pronostic [341]. Il faudrait déterminer si l'interaction TET2/ATF2 est directe et si elle est de mauvais pronostic, auquel cas casser les complexes TET2/ATF2 grâce à un peptide pourrait être une piste thérapeutique, comme décrit dans la partie « épithérapies » de l'introduction. De plus, ce

travail tend à renforcer les preuves d'existence de mécanismes de transcrition factor-directed demethylation.

La dernière question que soulève ce travail est : quelles sont les causes de la surexpression de TET2 suite à l'irradiation ? Est-ce un rétrocontrôle positif, ce qui voudrait dire que TET2 déméthylerait lui-même son promoteur, permettant la fixation d'un facteur de transcription ? Ces questions pourront ouvrir un nouvel axe de travail et être explorées en profondeurs dans les prochaines années.

Nos articles suggèrent donc un rôle de TET2 positif pour le glioblastome multiforme, puisque sa surexpression est corrélée à une rechute plus rapide mais aussi à l'échappement au système immunitaire suite à l'irradiation.

Bien que ce travail soulève des questions, il permet tout de même d'esquisser le développement d'un nouveau type de protocole thérapeutique. En effet, afin de limiter les phénomènes de résistance au traitement (ici la radiothérapie), il est possible d'imaginer un protocole où l'expression de l'exomiR-378a-3p dans le sang serait monitorée au cours du temps, et lorsqu'elle dépasse un certain seuil, la radiothérapie est interrompue et/ou un anti-miR-378 est administré (Figure 41). Lorsque l'expression de l'exomiR-378 repasse sous le seuil fixé, la radiothérapie peut reprendre si elle a été interrompue, et l'anti-miR n'est plus administré puisqu'il n'est plus utile, n'ayant plus de cible. Derrière cette idée, le but est aussi de limiter l'administration de médicaments à des moments inopportuns, ayant possiblement l'effet inverse de celui attendu.

Par exemple, notre laboratoire a démontré que poursuivre l'administration d'anticorps anti-PD1 alors que 90% des récepteurs présents sur les LT sont saturés induisait la surexpression de l'exomiR-4315, ciblant Bim, une molécule pro-apoptotique dans les cellules tumorales. Ce qui veut dire que l'administration non raisonnée d'anti-PD1 peut aller au contraire des intérêts du patient (article en révision).

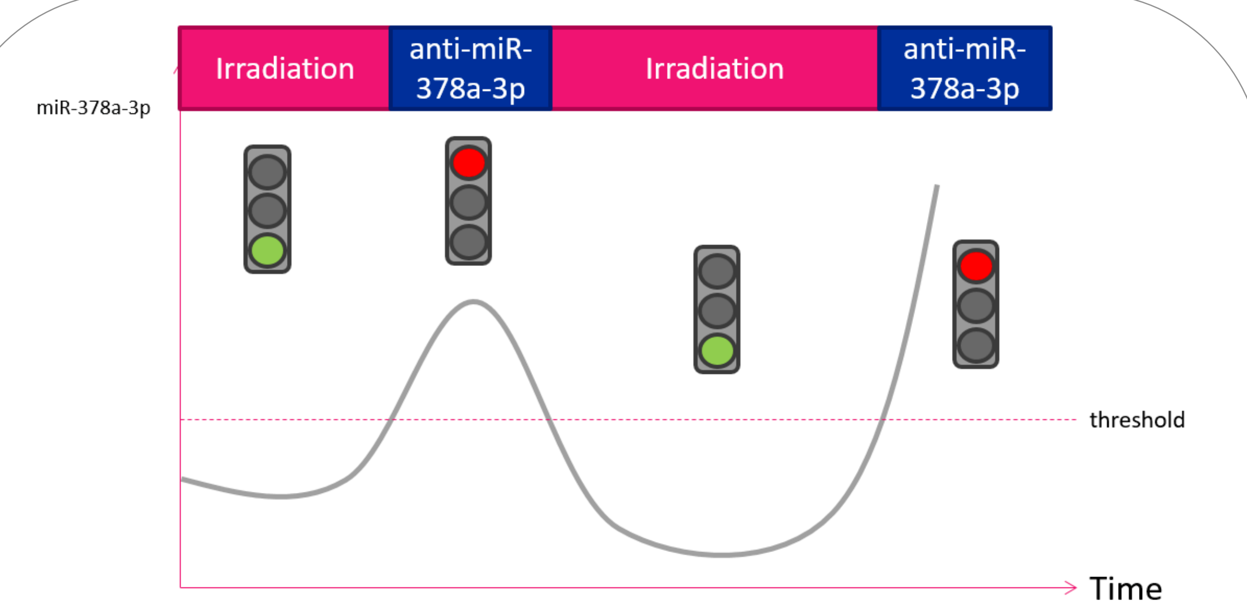


Figure 41 : Suivi de l'expression du miR-378a-3p au cours du temps chez un patient traité par radiothérapie

Afin d'améliorer la prise en charge des patients atteints d'un cancer traité par irradiation, l'expression du miR-378a-3p pourrait être monitorée au cours du temps, et un anti-miR-378a-3p pourrait être administré à partir d'un certain seuil. Cette nouvelle stratégie thérapeutique pourrait permettre l'amélioration de l'efficacité de la radiothérapie, en utilisant la médecine personnalisée.

4.4) Épithérapies

4.4.1) Avec un miRNA

Enfin, nous nous sommes penchés sur l'utilisation de nouveaux traitements tels que les miRNA. Afin d'étudier le potentiel thérapeutique des microARN dans le traitement du cancer, nous nous sommes intéressés au miR-370-3p dans le glioblastome, déjà décrit comme permettant la restauration de la sensibilité au témozolomide *in vitro* en ciblant MGMT [199]. Dans le premier article de cette partie, nous avons essayé de déterminer l'utilisation potentielle du miR-370-3p en tant que biomarqueur permettant de prédire la réponse au traitement, mais aussi son utilisation comme outil thérapeutique. Pour l'utilisation en tant que biomarqueur, le miR-370-3p ne permet pas de prédire la survie avant le début du traitement. En effet, au moment de l'intervention chirurgicale, nous

n'avons pas pu corréler l'expression du miR-370-3p à la survie du patient, que ce soit dans la tumeur ou circulant dans le sang. Peut-être que son évolution au cours du traitement a valeur pronostique, comme démontré dans l'article 3 pour TET2. Cependant, le miR-370 pourrait avoir un rôle thérapeutique. En effet, en diminuant l'expression de MGMT il sensibilise les cellules tumorales au TMZ. Le miR-370-3p, en combinaison avec le traitement au TMZ permet, chez la souris, une diminution de la taille des tumeurs (Figure 42).

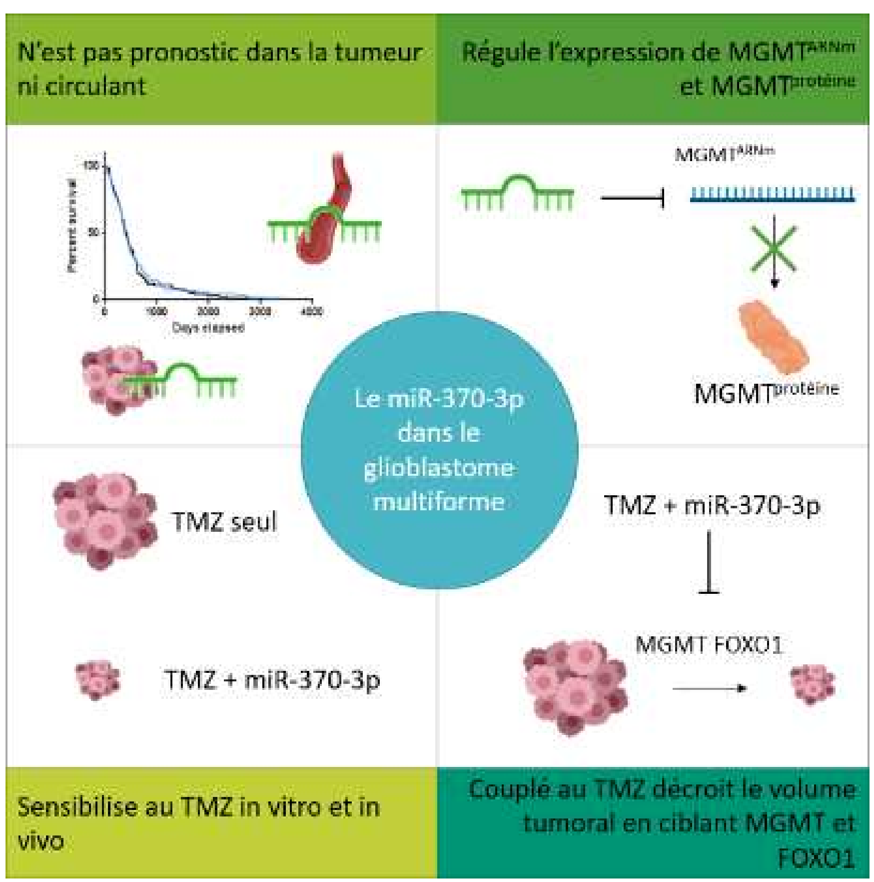


Figure 42 : Abstract graphique de l'article « miR-370-3p is a therapeutic tool in anti-glioblastoma therapy but is not an intratumoral or cell-free circulating biomarker »

Bien que le miR-370-3p ne soit pas pronostic de la survie au moment de l'intervention chirurgicale, d'autres miRs ont été identifiés comme ayant valeur pronostique. La diminution de l'expression du miR circulant -125b-5p dans le cancer du pancréas est corrélée à une diminution de la survie globale, et une signature de 6 miR dans le cancer colorectal permettent de définir deux groupes : haut risque et bas risque de progression, par exemple. De plus, le miR-370-3p a été démontré comme réprimant l'expression de WNT7a, protéine activant la voie WNT et promeuvant invasion des cellules tumorales de vessie. Au contraire, dans le cancer du sein, il contre l'effet suppresseur de tumeur de GRM4. Cela montre une fois de plus le caractère versatile des miRNA. Dans le glioblastome, Gao et al. avaient déjà démontré que le miR-370-3p restaurait la sensibilité au TMZ en ciblant MGMT, mais seulement in vitro.

Cet article est une sérieuse piste thérapeutique pour le traitement du glioblastome, même si bien évidemment l'utilisation des miRNA en thérapie reste discutable et discutée. En effet, les miRNA, bien qu'étant de potentiels nouveaux traitements ne sont pas encore largement utilisés de par leur manque de spécificité et leur administration problématique. En effet, un même miR est capable de cibler aussi bien des oncogènes que des gènes suppresseurs de tumeurs, ils peuvent donc avoir un rôle ambivalent voire versatile. Cependant, en 2018, le tout premier siRNA a été approuvé pour le traitement de l'amylose héréditaire à transthyrétine [342, 343]. Cette approbation ouvre un champ des possibles dans l'utilisation d'ARN interférents pour le traitement de différentes pathologies.

Plusieurs essais cliniques sont en cours à propos de l'utilisation des miRNA dans le cadre de cancers. L'utilisation du miR-34 est à l'étude dans le traitement du cancer du foie, du lymphome et du mélanome. Pour les deux premiers, le miR était administré dans des liposomes, mais des effets secondaires indésirables trop importants ont été mis en évidence (essai NCT01829971). Le même problème s'est révélé pour le mélanome (essai NCT02862145).

Des TargomiR sont à l'étude dans le cancer du poumon et le mésothéliome pleural malin et montrent pour le moment moins d'effets indésirables. Ce sont des miR-mimétiques empaquetés dans des EnGeneIC's bacterially-derived EDV™ nanocell, portant à leur surface

un anticorps anti-EGFR permettant de cibler préférentiellement les cellules tumorales [344]. Il est possible que la cytotoxicité dépende du mode d'administration plus que du miRNA en lui-même.

Dans d'autres types de cancers, le miR pourrait être administré directement dans la tumeur, mais dans le cadre du GBM cette méthode n'est pas envisageable. C'est pourquoi plusieurs méthodes d'administration ont été spécifiquement développées pour des tumeurs difficilement accessibles. Par exemple, des disques chargés de carmustine, une chimiothérapie, en lieu et place de la tumeur après résection, le Gliadel.

Comme l'addition du miR-370-3p au traitement TMZ augmente son efficacité, cela pourrait permettre, au-delà d'induire une meilleure survie des patients, de diminuer la dose de TMZ utilisée et donc de limiter la cytotoxicité. En plus de l'impact direct du miR-370-3p sur la tumeur, son impact indirect sur l'état de santé global du patient, connu pour être lui aussi impliqué dans la survie, est intéressant.

Dans le cas du miR-370-3p, étant donné que dans le cas du glioblastome il fonctionne en ciblant MGMT, il sera inefficace dans les cellules qui n'expriment pas cette enzyme de réparation de l'ADN. Il faudrait dans ce cas cibler les patients qui possèdent cette enzyme. Cette étape est indispensable en amont de l'administration du traitement mais ne représente pas de gros défi technique, la méthylation de son promoteur pour l'administration de folate étant déjà étudiée en routine (essai FOLAGLI, RECF1898).

De plus, le miR-370-3p peut avoir un rôle versatile selon les cancers, certainement lié à l'ARNm ciblé. En effet, ce miR est connu pour cibler MGMT et FOXM1, oncogènes, mais aussi FOXO1 et TGFβR1, gènes suppresseurs de tumeurs [188, 197, 345, 346]. Avant l'administration du miR en traitement il semble pertinent de s'intéresser à la présence des cibles recherchées. Cela permettrait aussi de limiter les problèmes de spécificité associés à l'utilisation des miRNA en thérapie.

Même si le miR-370-3p n'est pas pronostique au moment du diagnostic, des études sont à mener au cours du traitement du patient. En effet, il est possible que l'évolution du miR au

cours du traitement permette d'évaluer la réponse du patient, et donc potentiellement d'adapter la thérapie.

Nous nous sommes ici concentrés sur l'utilisation de d'un miRNA pour le traitement du GBM, mais notre article reste pour le moment au début du cheminement indispensable au développement d'un nouveau traitement.

4.4.2) Avec un miRNA adénosine méthylé

Si les miRNA sont des cibles et outils thérapeutiques prometteurs, le même problème qu'avec la chimiothérapie se pose : comment éviter un effet off-target des miRNA utilisés ? La modification chimique de ces miR est une piste prometteuse : ainsi, seules les cellules tumorales seraient capables de restaurer leur activité de répresseurs transcriptionnels. Dans cet article, nous avons confirmé l'existence de l'adénosine méthylation des miRNA matures et décrit son effet sur l'activité de répression transcriptionnelle des miRNA ainsi que le potentiel thérapeutique de cette découverte (Figure 43).

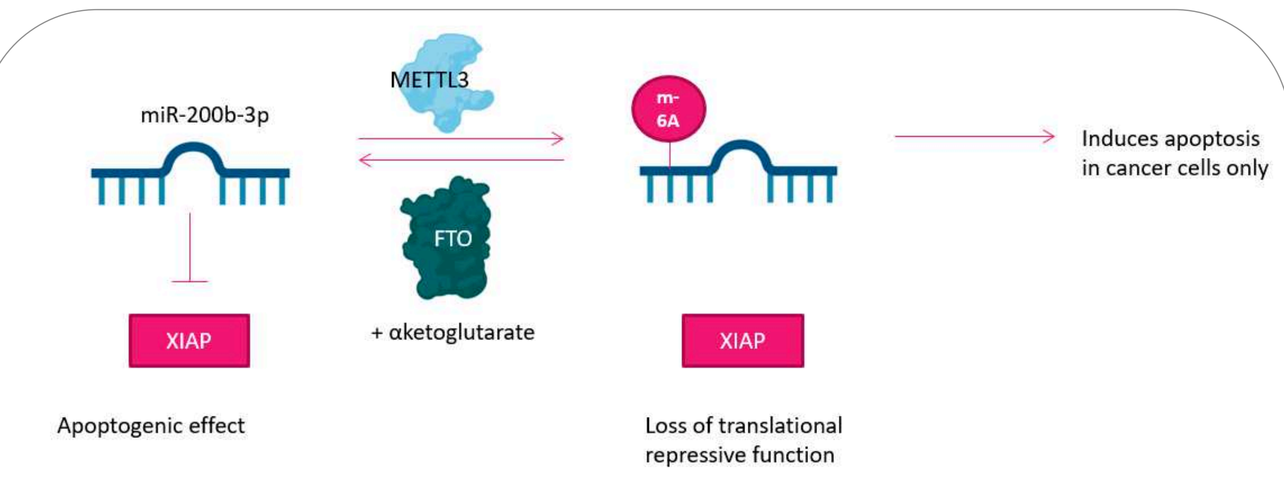


Figure 43 : Abstract graphique de l'article "N6-adenosine methylation of mature miRNA-200b-3p influences its funtionality and appears as a theranostic tool"

Mais quelles adénosines sont méthylées exactement ? Existe-t-il des dinucléotides type, comme les CpG pour la cytosine méthylation ? La séquence consensus semble être RRACH, avec R=A ou G et H=A, C, U [125, 347], et le miR-200b-3p étudié dans cet article contient cette séquence, à un nucléotide près.

Est-ce que la position des bases dans la séquence du miR a un impact sur la méthylation et sur son rôle biologique ? Un même miR méthylé différemment aura-t-il un rôle biologique différent ? À ce jour, il n'y a pas de réponses claires à ces différents questionnements.

Des articles semblent aller dans le sens où la m6A participerait à la maturation de primary miR en pre-miR [133], augmenterait la stabilité des miRNA [348], et nos données montrent qu'en étant adénosine méthylés les miRNA perdent leur activité de répresseur transcriptionnel.

Un éventail de rôles très large s'ouvre aux scientifiques, avec la découverte de ces modifications de miR : les phénomènes de «RNA directed methylation/demethylation» sont toujours discutés, mais personne n'a pris en compte la méthylation des miR dans ces études. Il est possible que la méthylation soit indispensable à ce phénomène.

Il est aussi possible que la méthylation des miR participent à leur export, et favorisent la liaison à des RNA binding proteins. En effet, il a été démontré que HNRNPA2B1 était capable de reconnaitre la m6A des miRNA [349], et cette protéine est impliquée dans l'export des ARN vers les exosomes [151].

Ou encore il a été démontré que des ARN circulaires étaient capables de jouer un rôle d'éponge et de se lier aux miRNA, les empêchant de jouer leur rôle de répresseur transcriptionnel [350]. La méthylation des miR pourraient favoriser ou empêcher ce phénomène.

Enfin, il est important de garder à l'esprit qu'à l'image des protéines MBD recrutées sur l'ADN méthylé, il existe des « readers » de la 6mA contenant un YT521-B homology (YTH) domain [134]. Ces protéines sont recrutées sur les sites méthylés, et que ce sont elles plus que la simple modification chimique qui participe à l'inhibition de l'effet de répresseur transcriptionnel. En effet, au laboratoire il a été démontré qu'en système acellulaire un miR méthylé se lie aussi bien à l'ARNm qu'un miR non méthylé.

Le fait que le miR-200b-3p ne soit adénosine déméthylé que dans les cellules tumorales non IDH mutées est un vrai atout thérapeutique car cela permet de limiter la toxicité à l'organisme entier, mais les raisons de cette spécificité restent à explorer. Les cellules

tumorales présentent peut-être une surexpression de FTO, une sous expression de METTL3 ou encore un métabolisme modifié qui induit une plus grande production d'αcétoglutarate. En effet, les cellules tumorales présentent un métabolisme dérégulé par rapport aux cellules normales, notamment au niveau du cycle de Krebs, lors duquel l'αcétoglutarate est produit [351, 352]. Les cellules IDH muté quant à elles possèdent plus de miR m6A méthylés, ce qui s'explique par le fait que la mutation d'IDH induit la production du 2-HG au lieu de l'αKG et donc une diminution de l'activité de FTO. La mutation d'IDH, au-delà d'être un évènement oncogénique de la gliomagenèse (mais présent dans seulement 3% des GBM primaires) [353] peut donc avoir un double rôle : diminuer l'efficacité des TET mais aussi de FTO, et donc augmenter le taux d'adénosine méthylation globale de l'ADN et des miRNA.

Plusieurs essais cliniques en cours testent l'utilisation du birinipant, un inhibiteur chimique des XIAP (NCT01940172). Mais dans un des essais le recrutement a été stoppé au vu d'un manque d'effet clinique observable (NCT01681368). Le miR-200b-3p adénosine méthylé présente une spécificité supérieure à cette molécule en ne ciblant que les cellules tumorales.

Les résultats de l'article sur le miR-370-3p sont à corréler avec celui sur l'adénosine méthylation des miR. En effet, même si le miR-370-3p montre un potentiel thérapeutique intéressant, la méthylation des miR pourrait amener un nouveau niveau de régulation et améliorer la spécificité. Cet argument est d'autant plus pertinent que le miR-370-3p possède la séquence consensus RRACH (GCCUGCUGGGGUGGAACCUGGU).

Au-delà de l'aspect thérapeutique de la méthylation des miR, Konno *et al.* ont démontré que le niveau de méthylation de deux miR était plus élevé dans le sérum de patients atteints de cancers colorectaux et pancréatiques et sont diminués après la chirurgie [130]. Afin de déterminer des biomarqueurs précoces du développement tumoral, il semble donc crucial d'aller au-delà de l'étude de l'expression des miR et de s'intéresser à leurs modifications chimiques.

Dans un article en cours de publication, nous démontrons que les miRNA pouvaient être cytosine méthylés, et que cette modification induit une perte de l'effet biologique. La question qui se pose maintenant est : existe-t-il un cross talk entre les différents types de méthylations ? Le fait qu'un miR soit cytosine méthylé favorise-t-il, ou au contraire empêche-t-il son adénosine méthylation ? La même problématique se pose en ajoutant la m7G dans l'équation.

Évidemment, cet article étant un des premiers à s'intéresser à la méthylation des miR, il soulève énormément de questions bien qu'il soit novateur et démontre des mécanismes prometteurs, notamment dans l'utilisation comme pro-drogue dans le traitement de cancer ou comme biomarqueur dans la détection précoce.

4.5) Discussion générale

Dans le domaine de la recherche en cancérologie, de nombreux axes sont envisageables et envisagés, mais il est à mon sens crucial de garder en tête le caractère global de la pathologie.

Dans ce travail de thèse, j'ai essayé de balayer les différents stades par lesquels passent les patients, mais il y en a deux que je n'ai pas abordé : la détection précoce, et l'après traitement.

À propos de la prédiction de l'apparition de cancers, le NGS liquide n'est-il pas l'avenir du diagnostic précoce ? En effet, si des signatures de développement d'un cancer sont connues en amont, il serait possible à partir d'une simple prise de sang de détecter au plus tôt l'apparition d'un cancer, pour soit essayer de l'empêcher soit prendre au mieux la pathologie en charge. Comme pour toute intervention médicale, qu'elle soit médicamenteuse ou de diagnostic, une notion de coût entre en jeu. Bien évidemment, ce coût est à mettre en regard des bénéfices sociétaux liés à la diminution d'apparition de cancers et à l'amélioration de la survie.

À propos de l'après traitement, lorsque le patient survit, mettre en place un suivi d'un biomarqueur pourrait permettre de prédire au plus tôt une rechute et ainsi d'agir en conséquence. Certains biomarqueurs épigénétiques et non épigénétiques ont déjà été

identifiés pour prévenir une rechute : la protéine amyloïde A dans le cancer nasopharyngé [354] ou le miR-29c dans le cancer colorectal [355].

Ce travail de thèse confirme le fait que la médecine de précision et personnalisée est l'avenir du traitement du cancer. De plus, la combinaison de différents types de thérapie permettra d'agir sur les différents leviers impliqués dans le développement tumoral. Un suivi du patient du diagnostic jusqu'à la rémission est un levier thérapeutique à ne pas négliger. La notion de prévention et de détection précoce reste aussi un point important, puisque limiter l'apparition d'une tumeur ou la détecter le plus précocement possible est le meilleur moyen d'augmenter l'efficacité des traitements et de réduire la mortalité.

Des progrès techniques et un développement clinique poussé seront néanmoins nécessaires à la démocratisation des outils épigénétiques, mais l'émergence des travaux à ce sujet nourrissent cet espoir.

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Glioblastome multiforme et épigénétique : de la prévention au développement de nouveaux traitements

Depuis de nombreuses années, la lutte contre le cancer est un enjeu de santé publique majeur. Le glioblastome multiforme est une tumeur primitive du cerveau particulièrement agressive, avec une survie à 5 ans inférieure à 5%. Lors de ce travail de thèse, je me suis intéressée à la lutte contre cette pathologie sous différentes formes, en me concentrant sur l'aspect épigénétique. Dans un premier temps, je me suis penchée sur les causes moléculaires à l'origine de l'apparition d'un glioblastome, afin d'améliorer sa prise en charge. Nous avons mis en évidence la capacité du diuron, un herbicide couramment utilisé jusqu'en 2008 à induire la gliomagenèse lorsque son exposition est couplée à la surexpression d'AKT dans des progéniteurs astrocytaires. Le diuron a aussi un impact sur la cytotoxicité des pDC et diminue leur capacité à détruire des cellules tumorales. Ensuite, nous avons démontré que TET2, une enzyme participant à la déméthylation de l'ADN n'était pas facteur pronostic au moment du diagnostic, mais que son augmentation était liée à un temps plus court entre deux résections, c'est-à-dire au moment de la rechute. La troisième partie de mon travail consistait à m'intéresser à la question du suivi de l'évolution de la réponse au traitement. Pour cela, nous avons démontré que la surexpression d'un exomiR était corrélée à la diminution du granzyme B dans le sang des patients. Enfin, nous avons étudié la mise au point de nouveaux traitements, basés sur les microARN, que ce soit tel quel ou sous forme d'une prodrogue méthylée. Derrière ce travail l'idée était de s'intéresser aux différentes phases par lesquelles le patient passe, pour essayer de répondre aux nombreux enjeux thérapeutiques et de participer à une meilleure prise en charge du patient à chaque stade.

Mots clés : *glioblastome multiforme, méthylation de l'ADN, microARN, épigénétique, épimarques circulantes*

Glioblastoma multiforme and epigenetics: from prevention to new therapies development

Since many years, fight against cancer is a major public health issue. Glioblastoma multiforme is a particularly aggressive primary brain tumor, with a 5 years survival inferior to 5%. During my thesis, I focused on struggling this pathology under several forms, concentrated on epigenetics. First, I tried to find a way to anticipate glioblastoma emergence, in order to improve its care. We brought to light diuron capacity, an herbicide often used until 2008, to induce gliomagenesis when its exposure is coupled to AKT overexpression in astrocytary progenitors. Diuron also has a negative impact on NK cells cytotoxicity. Secondly, we demonstrated that TET2, an enzyme implicated in DNA demethylation was not a prognosis factor at diagnosis time, but its increase is correlated to a shorter time between two resections. Third part of my work consisted in interesting in monitoring the evolution to patient response to treatment. To this end, we demonstrated that an exomiR overexpression was correlated to granzyme B decrease in patients' blood. Finally, we studied development of new treatments, based on microRNA, unaltered or under a methylated prodrug form. With this work, the idea was to be concerned by several phases which patients undergo, in order to try to answer to numerous therapeutic issue and to participate to a better care of patients to every level : before, during and after disease.

Keywords: *glioblastoma multiform, DNA methylation, microRNA, epigenetics, circulating epimarks*