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INTERET DE L'ETUDE DES HSP70-EXOSOMES DANS LE DIAGNOSTIC ET LE SUIVI DU CANCER

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Résumé

Le cancer est aujourd'hui une maladie qui fait malheureusement partie de notre quotidien, et bien qu'il soit de mieux en mieux traité, de plus en plus de gens en sont atteints. La recherche s'oriente sur deux pans : la thérapie et le diagnostic. Les nouvelles thérapies présentent des résultats spectaculaires jamais atteints à ce jour, mais l'amélioration du diagnostic peut aussi sauver des vies. Il est établi que plus un cancer est diagnostiqué tôt, plus le patient a de chances de guérir. Idem pour le suivi de la maladie. Dans ce contexte de précision et de précocité, la biopsie liquide émerge et possède un avenir très prometteur. Elle consiste en l'étude d'analytes présents dans les fluides corporels, particulièrement la circulation sanguine. On y retrouve principalement, mais pas exclusivement, l'ADN tumoral circulant, les cellules tumorales circulantes (CTCs) et les exosomes.

Ce manuscrit a pour objectif de replacer mon travail de recherche, qui porte sur les exosomes, dans le contexte de la biopsie liquide, afin de laisser libre court aux comparaisons et à la compréhension de leur potentiel diagnostic. Les exosomes sont des nanovésicules libérées par les cellules dans le sang. Elles contiennent du matériel génétique, des lipides et des protéines. Une étape clé pour leur utilisation en tant que biomarqueur est de différencier les exosomes dérivés de tumeur de ceux dérivés d'autres cellules de l'organisme.

La protéine de stress Heat shock protein-70 (HSP70) a été décrite comme étant surexprimée dans les cellules cancéreuses et associée à un mauvais pronostic. Nous avons précédemment démontré que seuls les exosomes dérivés de cellules cancéreuses portaient HSP70 à la membrane. Dans ce travail, nous avons ouvert une étude clinique pilote prospective incluant des patients atteints d'un cancer du sein et du poumon afin de déterminer s'il était possible de détecter et quantifier les HSP70-exosomes dans le sang de patients atteints de tumeurs solides malignes.

Nous avons montré que le taux de HSP70 dans les exosomes, contrairement à la forme soluble, reflétait le contenu en HSP70 dans la biopsie tumorale. Le taux de HSP70-exosomes circulants est augmenté chez les patients atteints d'un cancer à un stade métastatique comparé aux non-métastatique et aux donneurs sains. Nous avons ensuite démontré que les niveaux de HSP70-exosomes étaient corrélés au statut de la maladie, et étaient potentiellement de meilleurs marqueurs que les CTCs. Enfin, nous avons indiqué que le taux de HSP70-exosomes étaient inversement corrélés à la réponse au traitement, et, par conséquent, que le suivi du taux de HSP70 dans les exosomes pourrait être utile dans la prédiction de la réponse au traitement.

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Liste des abréviations

ABD	ATP Binding Domain
ALIX	ALG-2 interacting protein X
ATP	Adénosine Tri-Phosphate
ADNcf	ADN cell-free
ADNtc	ADN tumoral circulant
AR	Androgen Receptor
ARF6	ADP-ribosylation factor 6
ARNcf	ARN cell-free
ARv7	Androgen Receptor Variant 7
BCL-2	B-cell lymphoma 2
CAD	Caspase Activated DNase
circARN	ARN circulaire
CK	Cytokératine
CMH	Complexe Majeur d'Histocompatibilité
CMV	Corps Multivésiculaire
CNV	Copy Number Variation
CPA	Cellule Présentatrice d'Antigène
CRPC	Castration-Resistant Prostate Cancer
CTC	Cellule Tumorale Circulante
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
DAPI	4',6-diamidino-2-phénylindole
ddPCR	Droplet Digital PCR
DIABLO	Direct IAP Binding protein with Low pI
DISC	Death-Inducing Signaling Complex
dsDNA	Double Stranded DNA
EGFR	Epidermal Growth Factor Receptor
EPHB2	Ephrin type-B receptor 2
EPCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
ESCRT	Endosomal Sorting Complex Responsible for Transport
FAC	Fibroblaste associé au cancer
FADD	Fas Associated Death Domain
FAS-L	Fas ligand
FDA	Food and Drug Administration
GAPDH	Glycéraldéhyde-3-phosphate déshydrogénase
GiS1P	inhibitory G protein (Gi)-coupled sphingosine 1-phosphate (S1P)
GPC1	Glypican-1
GTPase	Guanosine Tri-Phosphatase
HER2	Human Epidermal Growth Factor Receptor-2
HSE	Heat Shock Element
HSF	Heat Shock Factor
HSP	Heat Shock Protein
ICAM	Intercellular Adhesion Molecule
IHC	Immunohistochemistry
ISEV	International Society of Extracellular Vesicle
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
LFA-1	Lymphocyte function-associated antigen 1

lncARN	Long ARN non codant
MCF-7	Michigan Cancer Foundation-7
MEC	Matrice Extracellulaire
MISEV	Minimal information for studies of extracellular vesicles
MDSC	Myeloid Derived Supressor Cell
MMP	Matrix Metallo-Proteinase
NGS	Next Generation Sequencing
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NK	Natural Killer
NKG2D	Natural Killer Group 2D
PARP	Poly (ADP-ribose) polymerase
PBD	Peptide Binding Domain
PCR	Polymerase Chain Reaction
PD1	Programmed cell Death-1
PD-L1	Programmed death-ligand 1
piARN	Piwi-interacting ARN
PI3KCA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha
PLD2	Phospholipase D2
PSA	Prostate Specific Antigen
PSMA	Prostate-Specific Membrane Antigen
SIGLEC	Sialic Acid-Binding Immunoglobulin-like Lectin
SMAC	Second Mitochondria-derived Activator of Caspases
SNAP23	Synaptosomal-Associated Protein 23
SNARE	Soluble N-éthylmaleimide-sensitive-factor Attachment protein REceptor
snARN	small nuclear ARN
snoARN	small nucleolar ARN
STAT3	Signal Transducer and Activator of Transcription 3
TGF	Tumor Growth Factor
TLR	Toll-Like receptor
TNF	Tumor Necrosis Factor
TRAIL	Tumor necosis factor-Related Apoptosis-Inducing Ligand
TRPC-5	short Transient Receptor Potential Channel 5
tsARN	tRNA-derived small ARN
TSG101	Tumor Susceptibility Gene 101
TYRP-2	Tyrosinase-Related Protein-2
VAMP7	Vesicle Associated Membrane Protein 7
VE	Vésicule Extracellulaire
VEGF	Vascular Endothelial Growth Factor
VIL	Vésicule Intra-Luminale
VLA-4	Very Late Antigen-4

ETAT DE L'ART

1. Epidémiologie des cancers :

Dans le monde, en 2018, on estime à 18,1 millions le nombre de nouveaux cas et 9,6 millions le nombre de décès par cancer (Figure 1)[1]. En France métropolitaine, on estime à 382 000 le nombre de nouveaux cas de cancer (204 600 hommes et 177 400 femmes) avec un âge médian au diagnostic de 68 ans chez l'homme et de 67 ans chez la femme. Le nombre de décès par cancer est quant à lui estimé à 157 400, parmi lesquels 89 600 hommes et 67 800 femmes, en légère hausse par rapport à 2017 (84 041 hommes et 66 000 femmes), avec un âge médian au décès de 73 ans chez l'homme et de 77 ans chez la femme[2,3].

Chez l'homme, le cancer de la prostate reste de loin le plus fréquent (50 430 nouveaux cas estimés en 2015), devant le cancer du poumon (31 231 cas estimés en 2018) et le cancer colorectal (23 216 cas estimés en 2018). Le cancer le plus meurtrier est le cancer du poumon avec 22 761 décès en 2018, devant le cancer colorectal (9 209 décès) et le cancer de la prostate (8 115 décès)[2,3].

Chez la femme, le cancer du sein se situe au premier rang avec 58 459 cas estimés en 2018, devant le cancer colorectal (20 120 cas) et le cancer du poumon (15 132 cas). Le cancer du sein est la première cause de décès par cancer (12 146 décès), devant le cancer du poumon (10 356 décès) et le cancer colorectal (7 908 décès)[2,3].

Il est désormais acquis que plus un cancer est diagnostiqué à un stade précoce, plus les chances de guérison sont importantes. Certains cancers peuvent être repérés par des tests simples permettant d'identifier dans une population les sujets qui ont un cancer mais n'en présentent pas les symptômes. En France des programmes nationaux de dépistage ont été mis en place pour trois cancers : sein (2004), colorectal (2008-2009) et col de l'utérus (2018-2019). Ainsi, toutes les femmes de 50 à 74 ans ne présentant aucun symptôme sont invitées à réaliser tous les deux ans une mammographie de dépistage, et pour les hommes et femmes du même profil, un test de recherche de sang occulte dans les selles. Pour le cancer du col de l'utérus il est recommandé de réaliser un dépistage tous les trois ans chez les femmes de 25 à 65 ans y compris chez les femmes vaccinées contre différents papillomavirus humains (HPV) car la vaccination ne protège pas contre tous les HPV liés au cancer du col de l'utérus.

Néanmoins, le dépistage ne constitue pas un diagnostic, le seul moyen étant l'analyse sur des prélèvements tissulaires : une biopsie.

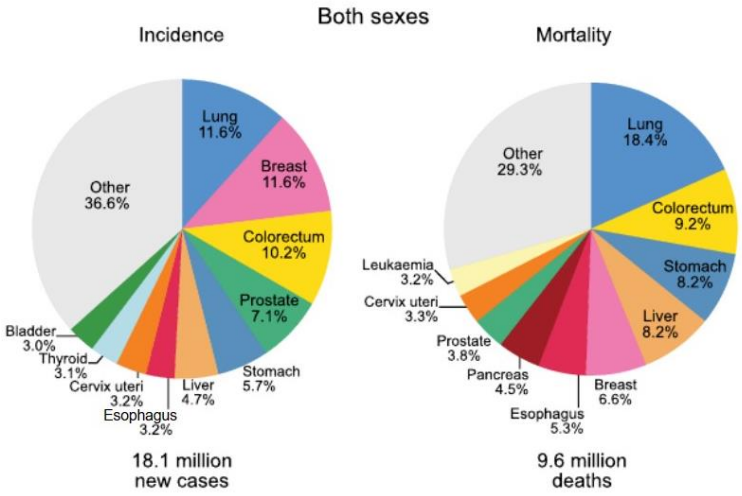

Figure 1 : Incidence et mortalité des 10 cancers les plus fréquents dans le monde, en 2018. *Lung = poumon, Breast = sein, Colorectum = colorectal, Prostate = prostate, Stomach = estomac, Liver = foie, Eosphagus = œsophage, Cervix uteri = utérus, Thyroid = thyroïde, Bladder = vessie, Leukaemia = leucémie, Pancreas = pancréas, other = autre. D'après Bray et al, CA cancer J Clin, 2018.*

2. Diagnostic :

2.1.La biopsie tumorale :

A ce jour, la technique de référence utilisée pour le diagnostic des cancers reste la biopsie tumorale, malgré l'apparition d'alternatives à fort potentiel. En effet, l'analyse anatomo-pathologique permet de préciser le stade de la maladie, son type histologique, et définit certaines caractéristiques biologiques, le tout permettant d'adopter le régime thérapeutique approprié ou encore de fournir des estimations pronostiques. L'apparition de technologies de pointe a permis de révéler les caractéristiques moléculaires des tumeurs et d'évoluer vers une médecine de précision, toujours plus personnalisée. Les traitements actuels consistent ainsi de plus en plus en des thérapies ciblant des altérations biologiques propres au cancer telles que la surexpression ou la mutation d'une protéine, l'amplification ou la mutation de gènes spécifiques ou encore sur la modulation du système immunitaire et du microenvironnement tumoral[4]. Ces altérations peuvent alors être identifiées par l'intermédiaire d'analyses anatomo-pathologiques ou de séquençage nouvelle génération (NGS) à partir du matériel issu de la biopsie tumorale.

Cependant, et bien que la biopsie tumorale soit toujours la technique privilégiée pour un diagnostic, elle présente plusieurs inconvénients :

- Le premier provient du fait que c'est un acte chirurgical invasif pour le patient, qui en plus n'est pas toujours réalisable. En effet, certaines tumeurs peuvent être localisées dans des endroits difficilement accessibles. De plus, le risque est parfois trop important lorsque l'acte doit être

réalisé à proximité d'un vaisseau sanguin majeur ou lorsque le patient possède d'autres comorbidités.

- Le deuxième réside dans l'hétérogénéité tumorale. La tumeur est ainsi composée de différents clones exprimant certaines protéines ou certaines mutations dans des endroits différents. Ce processus est dynamique et peut évoluer au cours de la croissance de la tumeur avec la dominance de certains clones dans certaines régions. De plus, chaque patient possède une hétérogénéité tumorale propre, y compris ceux porteurs d'une tumeur de même nature. Les métastases peuvent également présenter un profil génétique différent de celui de la tumeur primitive. Enfin, le stress thérapeutique, et plus particulièrement les thérapies ciblées, entraine une pression de sélection clonale qui modifient le paysage génétique de la tumeur[5–9] (Figure 2). Par conséquent, l'étude des caractéristiques de la tumeur via la biopsie ne reflète pas toute l'hétérogénéité tumorale, voire du corps entier s'il y a des métastases, et peut donc fausser la prise de décision thérapeutique.

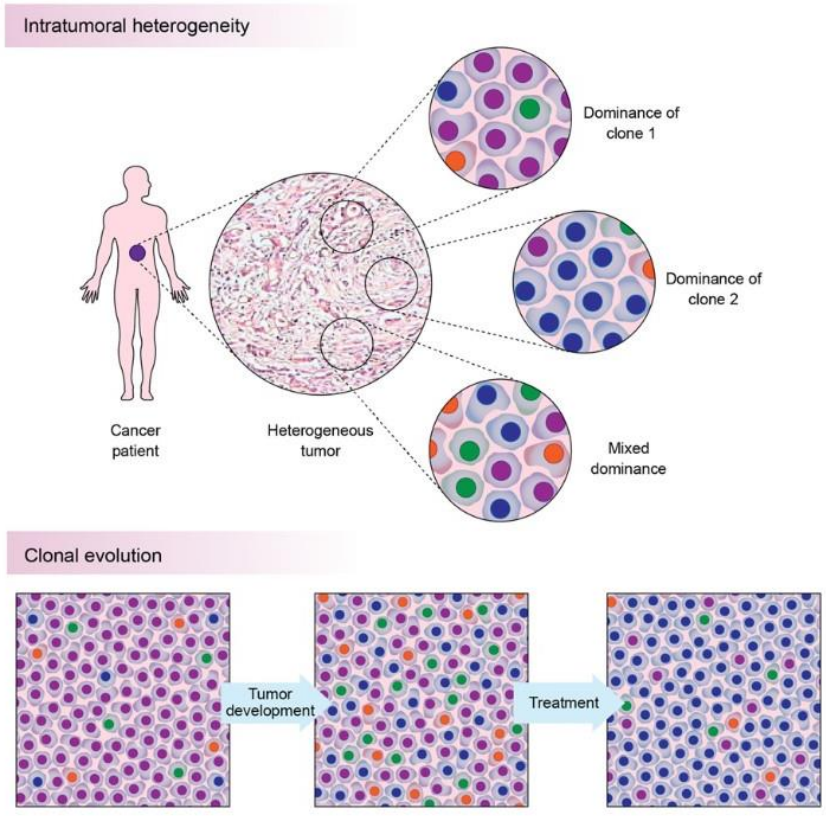

Figure 2 : Hétérogénéité tumorale et évolution clonale*. Puisque les tumeurs cancéreuses présentent des variations au sein des cellules qui la constituent, certains types de clones peuvent dominer un site spécifique de la tumeur (dominance of clone) ou être retrouvé de façon relativement équitable (mixed dominance). L'évolution clonale a lieu au cours de la progression tumorale (tumor development) ou à la suite de traitements spécifiques (treatment). Intratumoral heterogeneity = hétérogénéité intratumorale, heterogeneous tumor = tumeur hétérogène, cancer patient = patient cancéreux, clonal evolution = évolution clonale. D'après Oliveira et al, Molecular cancer research, 2020.*

- La troisième raison est sous-jacente aux deux premières : elle ne permet pas d'étudier la dynamique de la tumeur au cours de la ligne de traitement.

Ainsi, ces dernières années, la médecine de précision et notamment la recherche en cancérologie s'est focalisée sur la biopsie liquide puisqu'elle permet des prélèvements répétés de façon peu invasive. Elle offre également la possibilité d'une détection précoce contrairement à la biopsie tumorale[5–7].

2.2.La biopsie liquide :

Le terme « biopsie liquide » a été utilisé pour la 1e fois en 2010 par Klaus Pantel et Catherine Alix-Panabières, pontes en la matière, pour décrire une méthode basée sur une prise de sang et capable de fournir les mêmes informations que la biopsie tumorale[8]. Il se réfère au prélèvement et à l'analyse de tous les fluides biologiques corporels, principalement le sang, mais aussi l'urine, le liquide cérébro-spinal, la salive ou encore les effusions pleurales[9,10]. Le sang périphérique constitue la principale source d'analytes pour la biopsie liquide. Il contient notamment des cellules tumorales circulantes (CTCs), de l'ADN circulant libre (*ADN cell-free,* ADNcf) ou tumoral circulant (ADNtc), de l'ARN circulant libre (*ARN cell-free*, ARNcf), principalement des petits ARNs non codants mais aussi de l'ARN messager, des vésicules extracellulaires, majoritairement des exosomes, mais aussi des protéines et de nombreux métabolites[5,6,9–13]. Ainsi, ensemble, ces analytes ont le potentiel de refléter les caractéristiques de la tumeur, habituellement fournies par la biopsie tumorale. Par exemple, les données sur les altérations génomiques peuvent être fournies par les CTCs ou l'ADNtc, tandis que d'autres analytes circulants fournissent des informations sur la transcriptomique[14], l'épigénomique[15], la protéomique[16] ou encore la métabolomique[17] des tumeurs (Figure 3).

Ainsi, la biopsie liquide, au travers de ses composants, émerge comme un outil prometteur pour la médecine de précision grâce à sa capacité à refléter de façon globale l'hétérogénéité tumorale.

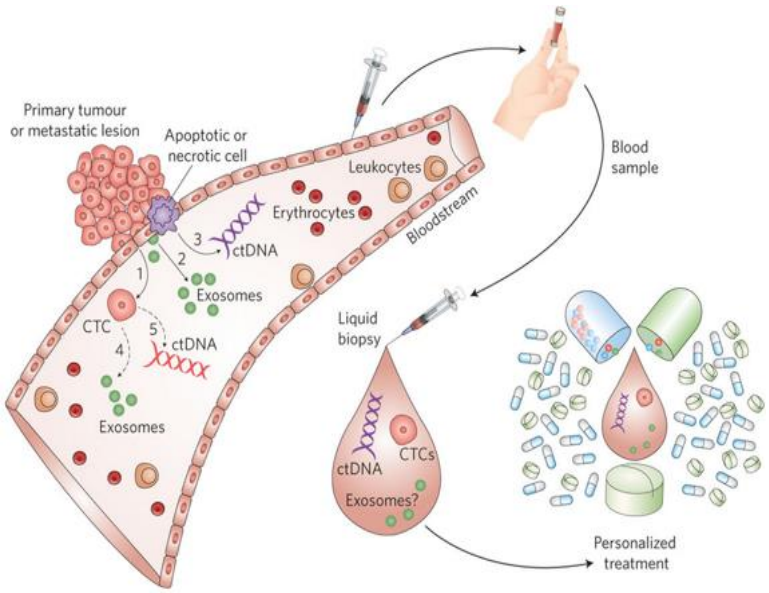

Figure 3 : Principe de la biopsie liquide. *1) Des cellules sont capables de se détacher de la tumeur primitive ou des lésions métastatiques (primary tumour or metastatic lesion) et d'entrer dans la circulation sanguine (bloodstream) : les cellules tumorales circulantes (CTC). 2) les exosomes sont libérés par les cellules tumorales et saines. 3) Les cellules apoptotiques ou nécrotiques (apoptotic or necrotic cell) libèrent de l'ADNtc (ctDNA) dans la circulation sanguine. Les CTCs sont ensuite capables de contribuer au pool d'exosomes (4) et d'ADNtc (5). Des prises de sang (blood sample) répétées peuvent être réalisées pour stratifier les patients, évaluer l'efficacité thérapeutique, identifier des cibles thérapeutiques, et détecter l'émergence de mécanismes de résistance du cancer. Personalized treatment = traitement personnalisé. D'après Alix-Panabières et al, Nature Biomedical Engineering, 2017.*

3. L'ADN tumoral circulant :

3.1.Généralités :

On appelle ADN tumoral circulant (ADNtc), l'ADN dérivé de tumeur primaires ou métastatiques qui se retrouve dans les fluides biologiques et notamment la circulation sanguine. Il provient des cellules tumorales apoptotiques[18,19] et nécrotiques[20] mais également de la sécrétion de cellules intactes sous forme libre[21] ou associées à des vésicules extracellulaires[22,23] (Figure 4). L'ADNtc se distingue de l'ADN dit « cell-free » (ADNcf) qui, lui, correspond à l'ADN libéré dans le milieu extracellulaire mais qui ne provient pas de cellules tumorales. Il provient des cellules non malignes qui meurent, mais aussi des tissus sains affectés par certaines thérapies chez les patients atteints de cancer telles que la chimiothérapie ou la radiothérapie, ce qui a pour effet de diluer l'ADNtc. Il est caractérisé par des altérations tumorales telles que des mutations ponctuelles, des réarrangements chromosomaux, des « copy-number variation » (CNV), et une méthylation propre[24–26] (Figure 4).

De façon intéressante, l'ADNtc possède une faible demi-vie dans la circulation sanguine, environs 2h, ce qui permet de suivre en temps réel la dynamique de la tumeur tout au long du traitement[24]. L'avancée des technologies de pointe a permis de montrer que l'ADNtc reflète le génome tumoral puisque l'ADN libéré provient de différentes régions de la tumeur[27–29] ou des foyers tumoraux[30]. En

effet, des études ont révélé la présence de mutations dans le sang qui n'étaient pas présentes dans les tissus correspondants mettant ainsi en lumière l'hétérogénéité tumorale[31] ainsi que la détection de mutations sous-clonales[32–35]. Elles ont aussi révélé la présence de sous-types moléculaires spécifiques avec des signatures génomiques distinctes[36,37]. La découverte de ces nouvelles données a donc très vite suggéré leur utilisation potentielle en tant que biomarqueur ; il s'en est suivi une explosion des études démontrant un fort intérêt dans le diagnostic, le pronostic et le suivi de la réponse au traitement des patients atteints de tumeurs solides[11,24,38]. L'analyse de l'ADNtc est aujourd'hui fréquemment utilisée en clinique puisqu'il constitue une alternative sérieuse à la biopsie tissulaire, en particulier dans les cas où cette dernière est impossible à réaliser, ou lorsque le risque est trop important. Il permet également un suivi constant, presque en temps réel de la réponse au traitement, ce qui est impossible avec une biopsie classique.

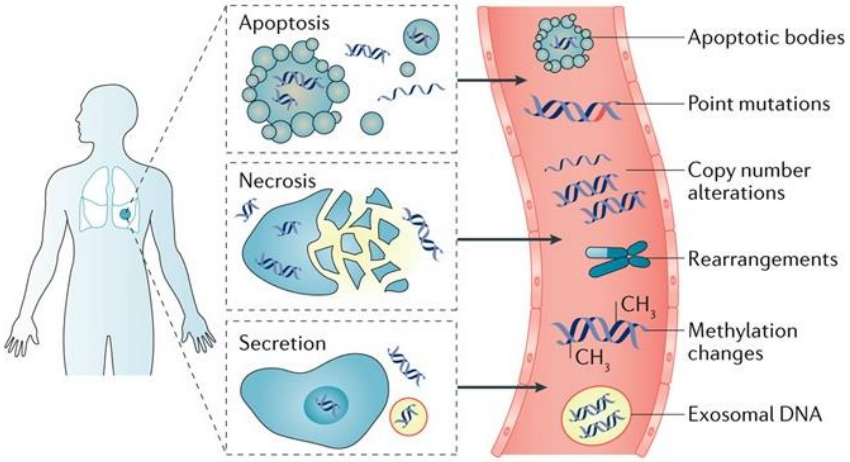

Figure 4 : Origine et types d'altérations de l'ADNtc. *Les cellules libèrent de l'ADN qui provient d'une combinaison de sécrétion (secretion), d'apoptose (apoptosis) et de nécrose (necrosis). Dans la circulation sanguine, l'ADNtc circulant peut être libre ou dans les exosomes (exosomal DNA). On retrouve différents types d'altérations génétiques : des mutations ponctuelles (point mutations), des variations du nombre de copies (copy number alterations), des réarrangements (rearrangements), ou encore des méthylations particulières (methylation changes). D'après Wan et al, Nature reviews cancer, 2017.*

3.2. Détection de l'ADNtc :

L'ADNtc est présent à faible concentration dans la circulation et se retrouve noyé dans l'ADNcf. La capacité de détection des mutations est ainsi directement corrélée à la charge tumorale ; plus la tumeur est grosse, plus le taux d'ADNtc libéré dans la circulation sanguine est important[39]. Il y a néanmoins une variabilité inter-individuelle puisqu'il existe des patients avec un stade avancé et peu d'ADNtc dans le sang, y compris des patients atteints d'une même tumeur[33,40,40]. Il est donc nécessaire d'avoir des technologies suffisamment sensibles et spécifiques pour détecter des mutants pouvant être présent à une fréquence de 0,01%[25,39] (Figure 5).

Il existe aujourd'hui une multitude de technologies de pointe qui font preuve d'une innovation constante afin d'en améliorer la sensibilité et la spécificité ; elles reposent soit sur le principe de la PCR (*Polymerase Chain Reaction*), ou du NGS (*Next Generation Sequencing*)[11,25,41]. Pour la détection de mutations ponctuelles, des techniques spécifiques de l'analyse de mutations basée sur la PCR ont été développées telles que le BEAMing (beads, emulsion, amplification, and magnetics)[42] ou la ddPCR (droplet digital PCR)[43]. Elles sont capables d'identifier et quantifier avec une grande précision les altérations génétiques circulantes, mais ne permettent pas ou peu l'analyse de plusieurs cibles en parallèle. Des techniques de NGS ont également été adaptées à l'analyse de l'ADNtc, en passant du séquençage du génome entier ou de l'exome[44], au séquençage ciblé[45] sur un panel de gènes plus restreint[46,47]. Néanmoins, bien que ces techniques de séquençage permettent une analyse multiplexée, elles sont moins sensibles et moins spécifiques que les techniques basées sur la PCR, du fait du taux d'erreur de l'ADN polymérase et du séquençage. Ainsi, afin de contourner ces inconvénients, des approches nouvelles ont intégré des méthodes de séquençage ultrasensibles, des code-barres moléculaires ou encore des algorithmes de suppression d'erreurs afin d'en améliorer la limite de détection[35,48,49]. Il existe donc aujourd'hui une grande variété de techniques permettant d'analyser très précisément les altérations génétiques circulantes, chacune ayant des caractéristiques différentes au niveau de la sensibilité de la spécificité et du multiplexage. Il est donc nécessaire d'adapter le choix de l'analyse à réaliser en fonction de la nature de l'altération, de sa fréquence dans la circulation et de l'information que l'on souhaite en tirer (Figure 5).

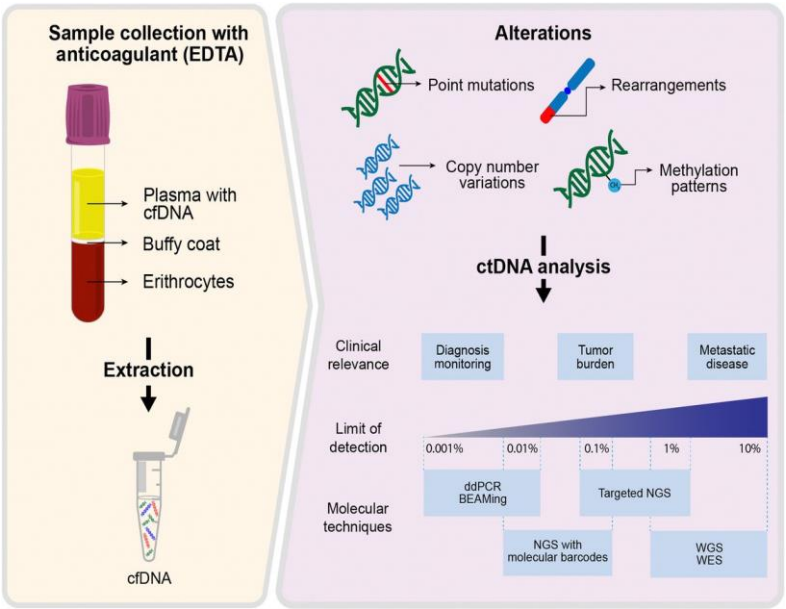

Figure 5 : Analyse de l'ADNtc. *Une fois extrait du plasma, l'ADNtc est analysé pour détection des altérations associées au cancer. Les différentes méthodes de détection ont chacune une limite de sensibilité (limit of detection), qui régit son utilité clinique (clinical relevance). Molecular techniques = techniques moléculaires, diagnosis = diagnostic, monitoring = suivi, tumor burden = volume tumoral, metastatic disease = maladie métastatique. D'après Oliveira et al, Molecular cancer research, 2020.*

3.3.Intérêt clinique de l'ADNtc :

3.3.1. Diagnostic et profilage moléculaire :

La connaissance précise de l'hétérogénéité tumorale est aujourd'hui fondamentale dans la médecine oncologique puisqu'elle permet notamment de sélectionner les thérapies à administrer aux patients. La potentielle utilisation de la biopsie liquide afin de réaliser le profilage moléculaire de la tumeur de façon peu invasive à fait de l'ADNtc un outil attractif.

Une grande majorité des études réalisées sur des cohortes importantes montrent une grande concordance entre la tumeur et le plasma (80 à 90%)[44,50–52]. Ainsi, plusieurs équipes se sont intéressées à la pertinence de l'analyse de l'ADNtc dans la détection précoce des cancers. Les résultats sont encourageants pour les tumeurs avancées mais moins pour les cancers primaires, notamment parce que l'ADNtc y est extrêmement rare et que l'on atteint la limite de détection des technologies d'analyse[29,33].

Malgré ces données encourageantes, la biopsie tumorale reste à ce jour l'option majoritaire pour plusieurs raisons ; la première réside dans le fait qu'elle permet une analyse histologique et d'autres marqueurs, en particulier protéiques, très utiles pour le diagnostic, la stratification des patients ainsi que la prise de décision thérapeutique. Ensuite, plusieurs études ont démontré que certains patients ne possédaient pas suffisamment d'ADNtc en dépit de la nature de la tumeur et du stade de la maladie[33,52].

Néanmoins, l'analyse de l'ADNtc peut être une alternative pertinente lorsque que la biopsie tumorale n'est pas possible ou trop dangereuse à réaliser, c'est pourquoi elle est de plus en plus utilisée en clinique pour la sélection des traitements de ces patients[51,53]. D'un point de vue du suivi, la possibilité de suivre en temps réel l'évolution de la tumeur via des prélèvements réguliers au cours de la ligne de traitement permet, par exemple, de détecter l'apparition d'altérations génétiques responsables de la résistance aux thérapies (Figure 6)[54–60]. Le meilleur exemple d'utilisation de l'analyse de l'ADNtc en clinique concerne la détection de l'apparition de la mutation EGFR-T790M chez les patients atteints d'un cancer du poumon non à petites cellules EGFR muté, traités par des anti-EGFR. Des études clés ont démontré une très grande concordance dans la détection de la mutation T790M circulante et dans la tumeur, qui répondent à l'osimertinib, un inhibiteur de EGFR de troisième génération[61,62]. Ainsi, le « cobas EGFR Mutation Test » a été approuvé par la FDA en tant que test diagnostic de complément pour le choix des thérapies EGFR[63].

3.3.2. Suivi du cancer :

3.3.2.1. Suivi de la réponse au traitement :

De nombreuses études ont montré la corrélation entre le volume tumoral et le taux d'ADNtc, qui chute après un traitement tel qu'une chirurgie ou une thérapie à base de drogues suggérant ainsi la potentielle utilisation en tant que biomarqueur[24,42,45,57]. Le gros avantage de l'ADNtc est son court temps de demi-vie, approximativement 2h, qui permet une analyse en temps réel de la tumeur comparativement aux marqueurs tumoraux classiques, qui ont une demi-vie de plusieurs jours à plusieurs heures, et à l'imagerie médicale. En effet, des études ont montré une pauvre sensibilité et spécificité des marqueurs tumoraux comparé à l'ADNtc[57,64], qui, lui, permet de détecter une progression tumorale de façon plus précoce que la radiographie[32,57–59].

3.3.2.2. Détection de la résistance au traitement et de l'hétérogénéité tumorale :

La résistance au traitement se définit par une inefficacité de la thérapie. Celle-ci peut être « primaire » lorsque le patient, d'emblée, ne répond pas au traitement, ou « secondaire » lorsque le patient répond dans un premier temps jusqu'à ce que la drogue devienne inefficace à un moment donné de la ligne de traitement. Ceci s'explique par la création de nouvelles mutations induites par les drogues dans certains clones qui prolifèrent et qui permettent à la tumeur de croître via d'autres voies de signalisations[54,56,59,60,65]. Sachant qu'il existe une grande hétérogénéité au sein de la tumeur chez un même individu, y compris dans les sites métastatiques, la biopsie solide, qui ne permet d'analyser qu'une partie de la tumeur primitive, peut potentiellement fausser le diagnostic. De plus, on sait dorénavant que l'ADNtc reflète complètement l'hétérogénéité tumorale du corps entier[54,56,66]. Ces données permettent donc aux cliniciens de déterminer avec précision le moment à partir duquel le patient devient réfractaire à la thérapie, mais aussi le type de thérapie à adopter pour contrer cette résistance secondaire (Figure 6). Une étude menée sur des patients atteints d'un cancer colorectal et traités aux anti-EGFR a par exemple détecté pas moins de 13 mutations de résistance différentes via l'ADNtc, avec moins d'un dixième des patients seulement présentant une seule mutation de résistance[52].

Ainsi, en plus de la détection de l'émergence de la résistance au traitement, l'ADNtc peut être utilisé pour son suivi. Un exemple marquant est celui discuté précédemment de la mutation EGFRT790M dans le cancer du poumon non à petites cellules[61,62,65]. D'autres études sur le cancer colorectal ont démontré que l'apparition de mutations du gène KRAS, KRASG12R et KRASG13D notamment, engendraient une résistance aux anti-EGFR[32,58,59]. L'arrêt de ce type de traitement entraine quant à lui une chute du taux de mutations du gène RAS circulant, ce qui suggère au clinicien la possibilité de réintroduire les anti-EGFR dans la ligne de traitement[32]. Des recherches similaires ont été menées dans différents cancers tels que le cancer du sein[47] ou encore le cancer de la prostate[67,68].

Ces études indiquent donc que l'évolution clonale durant les thérapies peut être suivie via l'analyse de l'ADNtc.

3.3.2.3. Détection du risque de rechute :

L'ADNtc concentre aussi de nombreux espoirs dans la détection précoce du risque de rechute. Le traitement initial pour une majorité de tumeurs est la chirurgie, qui est suivie ou non d'une thérapie adjuvante afin d'éliminer les éventuelles cellules cancéreuses restantes et donc de diminuer le risque de rechute. Néanmoins, il est difficile pour les cliniciens de discriminer les patients avec un grand risque de rechute.

Ainsi, dans certains cas bien particuliers comme le cancer colorectal de stade II, les patients avec un faible risque clinique ne se voient pas tous administrer une thérapie adjuvante alors que 15% environ rechuterons[24]. Une étude menée par Diehl et ses collègues a montré que la détection d'ADNtc chez les patients atteints d'un cancer colorectal quelques semaines après une chirurgie rechutaient dans les 1an[69]. Une autre étude également réalisée dans ce même cancer a montré que de grandes concentrations d'ADNtc, et notamment des mutants KRAS, étaient un indicateur précis du risque de rechute[70]. Des résultats similaires ont été publiés dans le cancer du sein[71,72], du poumon[73] et du pancréas[74] soulignant ainsi toute l'importance que pourrait avoir l'ADNtc dans le suivi du cancer (Figure 7). Des études cliniques sont actuellement en cours pour démontrer sa valeur clinique.

L'analyse de l'ADNtc a donc connu un bond en avant conséquent ces dernières années, et ce de manière extrêmement rapide au vu des applications cliniques potentielles extrêmement prometteuses. Il faut néanmoins un consensus méthodologique afin de l'introduire complètement en clinique. Bien qu'elle soit déjà utilisée pour certaines applications, l'analyse de l'ADNtc possède le potentiel de transformer plus encore la prise en charge des patients atteints de cancer.

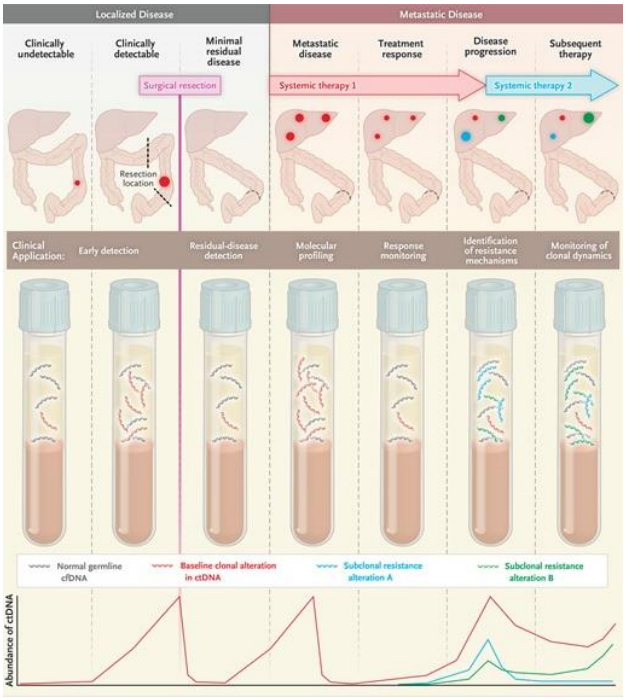

Figure 6 : Applications cliniques de l'analyse de l'ADNtc*. L'analyse de l'ADNtc à de potentielles applications cliniques à différentes étapes cliniques du cancer. Des méthodes de détection précoce (early detection) de tumeurs naissantes sont en cours de développement. La biopsie liquide est capable d'identifier des cancers à un stade pécoce, lorsqu'il est cliniquement détectable (clinically detectable). Après la chirurgie (surgical resection), l'ADNtc peut être analysé pendant des mois afin de détecter la présence éventuelle d'altérations présentes dans la tumeur réséquée, c'est-à-dire détecter la maladie résiduelle (minimal residual disease). Puisque la demi-vie de l'ADNtc est très courte, la détection de mutations de plasma post-chirurgical peut fournir une preuve directe d'une possible rechute. De plus, la détection d'ADNtc rapidement après la chirurgie peut permettre de stratifier les patients selon le risque de récurrence et peut offrir une opportunité pour une intervention précoce. Dans le contexte d'un cancer métastatique (metastatic disease), le profilage moléculaire (molecular profiling) par séquençage de l'ADNtc peut identifier de potentielles altérations génétiques qui peuvent être ciblées par des thérapies de précision. Des études ont montré que le taux d'ADNtc corrélait avec le volume tumoral et peut être utilisé comme un moyen précis de suivre la réponse au traitement (response monitoring) et l'apparition d'une résistance (resistance mechanisms). Lorsque la maladie progresse, l'analyse de l'ADNtc a prouvé son efficacité pour identifier des altérations génétiques émergentes qui entrainent une résistance à la thérapie, et peut donc guider la prise de décision thérapeutique. Enfin, l'analyse de l'ADNtc a été utilisée pour suivre la dynamique d'évolution clonale (monitoring of clonal dynamics) de différents sous clones au cours de la thérapie. D'après Corcoran et al, NEJM, 2018.*

4. Les cellules tumorales circulantes (CTCs) :

4.1.Généralités :

Les cellules tumorales circulantes sont des cellules émergeant de carcinomes, c'est-à-dire de cancers d'origine épithéliale, qui ont la capacité de migrer de la tumeur primaire et des sites métastatiques dans la circulation sanguine afin de coloniser un autre organe[75]. Lors de leur intravasation ces cellules sont soumises à des conditions de sélection difficiles. En effet, leur temps de demi-vie est estimé de 1 à 2,4 heures[76]. De plus, des CTCs apoptotiques ou fragmentées sont fréquemment retrouvées dans le sang périphérique de patients atteints de cancer ; ainsi la clairance des cellules cancéreuses circulantes survivantes intervient au moment de l'extravasation dans les organes secondaires[77].

La détection et l'analyse des CTCs ont été corrélées à différents paramètres cliniques et démontrées comme étant utile dans le diagnostic et le suivi de nombreuses tumeurs solides, notamment le cancer du sein, le cancer colorectal et de la prostate dont la technique de détection CellSearch® est approuvée par l'administration fédérale de l'alimentation et de médicaments américaine (*Food and Drug Administration*, FDA)[11,78–80]. Il y a cependant quelques inconvénients à la détection des CTCs, le principal étant que ces cellules sont rares ; on estime qu'il y a 1 à 10 cellules pour dix millilitre de sang, soit une cellule pour $10^{6\text{-}7}$ leucocytes[81,82]. Il faut donc avoir des technologies capables de détecter avec suffisamment de sensibilité et de spécificité une CTC parmi les millions d'autres cellules présentes dans le sang. L'autre obstacle majeur provient du phénotype même de ces cellules ; elles sont caractérisées biologiquement par la présence du marqueur d'adhésion cellulaire épithélial EPCAM ainsi que des membres de la famille des cytokératines tels que CK8, CK18 et CK19, et physiquement par leur taille plus importante, leur forme irrégulière ainsi que leur morphologie subcellulaire particulière[75,83]. Cependant, ce n'est pas toujours le cas ; en effet, les CTCs sont capables de procéder à un mécanisme appelé transition épithélio-mésenchymateuse qui consiste en la perte de ses caractéristiques épithéliales au profit d'un phénotype mésenchymateux[84]. Il en résulte une augmentation de la plasticité ainsi que de la capacité de migration et d'invasion, de résistance à l'anoïkis et à l'apoptose ; autant de paramètres qui font de ces CTCs les plus à même de parvenir à coloniser un organe secondaire[84,85]. Enfin ces cellules sont capables de former des clusters avec un potentiel métastatique 25 à 50 fois supérieur[86].

Il apparaît donc que ces cellules existent avec différents phénotypes ce qui rend leur utilisation en tant que biomarqueur plus complexe. Les progrès technologiques des dernières années permettent de mieux étudier la biologie des CTCs, notamment les approches « single cell », qui ont révélé de nouvelles approches possibles pour leur utilisation en tant que biomarqueur circulant[11,87,88].

4.2. Détection des CTCs :

Les CTCs sont essentielles pour la compréhension de la biologie des métastases et représentent un fort potentiel en tant que biomarqueur non invasif pour évaluer la progression tumorale et la réponse au traitement. Néanmoins, cette méthode qui présente certains avantages tels que la robustesse et la reproductibilité comporte aussi de nombreuses limites[84,89]. La première difficulté réside dans le fait de pouvoir différencier ces cellules extrêmement rares au milieu des millions d'autres cellules présentes dans un échantillon sanguin. Il existe ainsi deux grands types d'approches qui tirent parti de l'aspect biologique de ces cellules, ou de leur aspect physique mais à ce jour une seule est approuvée par la FDA : le système CellSearch® (Figure 7)[84]. Cette approche basée sur la biologie des CTCs consiste en premier lieu en une étape d'enrichissement par sélection positive des cellules exprimant EPCAM. Le prélèvement de sang périphérique (7,5mL) est suivi d'une étape de capture immunomagnétique avec des nanoparticules de fer recouverte d'un polymère de biotine, couplé avec des anticorps anti-EPCAM. Des réactifs fluorescents sont ensuite ajoutés pour la détection des CTCs : le DAPI pour la coloration du noyau cellulaire, des anticorps dirigés contre les cytokératines 8, 18 et 19 (marqueurs spécifiques des cellules épithéliales) et contre CD45 (un marqueur spécifique des leucocytes). Le mélange réactifs/échantillon est ensuite déposé à l'intérieur d'une cartouche qui est insérée dans un dispositif comprenant un champ magnétique attirant les cellules épithéliales marquées magnétiquement à la surface de la cartouche. Un microscope fluorescent couplé à un logiciel permet ensuite la lecture automatique et le décompte des cellules tumorales circulantes, c'est-à-dire les cellules positives pour le marquage au DAPI et aux cytokératines et négatives pour le marquage CD45. Des kits de réactifs de phénotypage HER2 et EGFR sont également commercialisés et permettent de mettre en évidence les CTC surexprimant ces récepteurs.

Il existe actuellement d'autres techniques de détection des CTCs en cours de développement ou de validation clinique qui tirent parti de leurs différences phénotypiques et moléculaires, qui pourraient accélérer leur utilisation en clinique [84](Figure 7)[11,84].

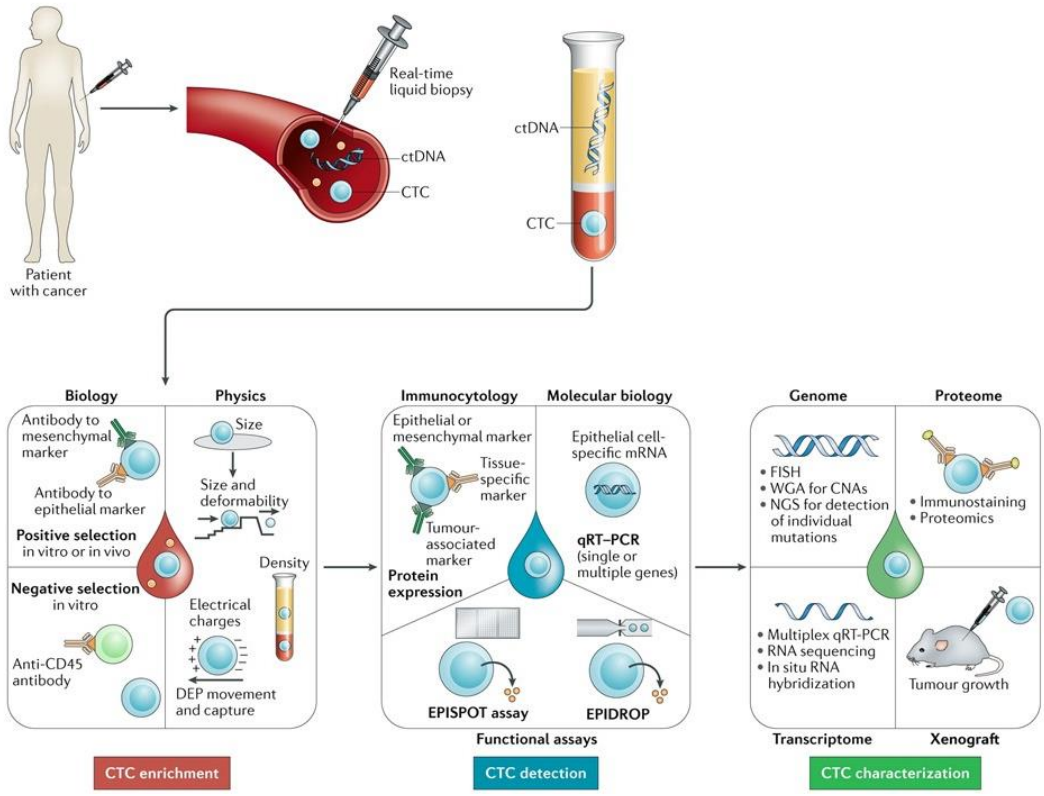

Figure 7 : Analyse des CTCs. *Les CTCs peuvent être enrichies à partir du sang grâce à des techniques utilisant des marqueurs biologiques (enrichment) : sélection positive in vitro ou in vivo en utilisant des anticorps dirigés contre des protéines épithéliales et/ou mésenchymateuses (antibody to epithelial or mesenchymal marker), ou négativement à travers la déplétion de leucocytes avec des anticorps anti-CD45 (anti-CD45 antibody). Un enrichissement positif peut aussi être réalisé in vitro grâce à des méthodes basées sur les caractéristiques physiques des CTCs tels que la taille (size), la défromabilité (deformability), la densité (density) et la charge électrique (electric charge). Ensuite, les CTCs peuvent être détectées (CTC detection) par des techniques qui reposent sur l'immunocytologie (immunocytology), la biologie moléculaire (molecular biology) ou des essais fonctionnels (functionnal assays). Concernant l'immunocytologie, les CTCs sont identifiées par des anticorps dirigés contre des marqueurs épithéliaux, mésenchymateux, spécifiques de la tumeur (tumour-associated marker) ou des tissus (tissu-specific marker). Les méthodes moléculaires utilisent des essais basés sur l'analyse de l'ARN tels que la RT-qPCR, le NGS ou l'hybridation ARN in situ. Les essais fonctionnels sont eux principalement des tests EPISPOT ou EPIDROP. Enfin, la caractérisation des CTCs (CTC characterization) est réalisée en étudiant son génome, son protéome et son transcriptome ainsi qu'en testant ses propriétés in vivo, par xénogreffe (xenograft). D'après Pantel et al, Nature Review clinical oncology, 2019 .*

4.3.Intérêt clinique des CTCs :

4.3.1. Valeur pronostique :

De nombreuses études démontrent l'intérêt pronostique des CTCs chez des patients atteints de différentes tumeurs solides, particulièrement dans le cancer du sein[80,86,90,91]. Une étude de Rack et ses collègues réalisée sur 2026 patients avec un cancer du sein à un stade précoce avant chimiothérapie et chez 1492 patients après chimiothérapie a démontré que la présence des CTCs était associée à une faible survie sans maladie (DFS : *Disease Free Survival*) et une survie globale (OS : *Overall Survival*)[91]. De plus, les chercheurs ont remarqué que le pronostic des patients avec au moins 5 CTCs/30mL de sang était plus mauvais et que la persistance de ces cellules au cours du traitement

chimiothérapeutique avait une influence pronostique négative. Ainsi, la détection des CTCs à la fois avant et après la chimiothérapie adjuvante est liée à un risque plus élevé de rechute du cancer du sein primaire. Une autre étude très complète publiée par Zhang et ses collègues portant sur la méta-analyse de 49 études incluant 6825 patients a démontré à grande échelle que la présence des CTCs était significativement associée à une survie plus courte à la fois dans les cancers du sein précoces et métastatiques[90].

D'autres travaux prometteurs ont démontré des corrélations significatives entre le nombre de CTCs, le pronostic, et la rechute métastatique dans différents types de tumeurs tels que le cancer de la prostate[79,82,92–94], le cancer colorectal[82,95], le cancer du rein[96], du foie[97], de l'œsophage[98] ou encore de la tête et du coup[99]. Néanmoins, ces études se doivent d'être confirmées à plus large échelle avec des tests plus sensibles afin d'explorer la pertinence clinique réelle des CTCs, particulièrement dans le suivi des patients où l'on ne connait pas pour l'instant la cinétique d'expression de ceux qui rechutent.

4.3.2. Un outil pour guider la thérapie :

Actuellement, les prises de décisions thérapeutiques sont majoritairement basées sur l'analyse de la tumeur primitive tandis que les prélèvements des lésions métastatiques restent compliqués cliniquement car elles sont invasives pour les patients. Ainsi, les CTCs apparaissent comme un bon moyen de contourner la biopsie solide, tout en permettant un suivi de la réponse au traitement en temps réel. Le développement de technologies sophistiquées du type « single cell » ont permis une meilleure compréhension des mécanismes de résistance aux thérapies et d'ouvrir des pistes dans le cadre d'une médecine personnalisée.

4.3.2.1. Analyse de l'ADN :

Des études d'analyse génomique comparative de CTCs, de tumeurs primaires ainsi que de métastases chez des patients atteints d'un cancer colorectal ou de la prostate ont montré que des mutations présentes dans les CTCs étaient les mêmes que ceux détectées dans la tumeur primaire et les métastases ouvrant ainsi de nouvelles perspectives d'utilisation en tant que biomarqueur liquide, notamment pour la réponse au traitement[25,93]. En effet, des mutations dans des protéines cibles (ou en aval de la voie de signalisation) affectent l'efficacité des molécules dirigées contre ces protéines. Par exemple, des mutations du gène KRAS sont fréquemment retrouvées dans les cancers colorectaux et conduisent généralement à une activation de la voie de signalisation de l'EGFR. Deux études séparées sur le cancer colorectal ont révélé une grande hétérogénéité des mutations KRAS dans les CTCs des patients, suggérant ainsi que la détection précoce des CTCs porteuses de la mutation KRAS pourrait aider les cliniciens dans le choix d'administrer ou non une thérapie à base d'anti-EGFR[100,101]. De la même manière, dans le cancer de la prostate des mutations du récepteur aux androgènes (AR :

Androgen Receptor), qui confèrent aux cellules tumorales une résistance aux thérapies de blocage des récepteurs androgéniques, ont été retrouvées dans les CTCs de patients[102]. Autres exemples, la détection des mutations PI3KCA et BRAF dans les CTCs des patients atteints d'un cancer du sein HER2+ et du mélanome, respectivement[103,104].

4.3.2.2. Analyse de l'ARN :

Au niveau de l'ARNm, des études se focalisant sur le cancer de la prostate ont révélé que l'analyse de l'ARNm des CTCs pouvait aussi révéler des informations sur la résistance aux traitements. Le cancer de la prostate résistant à la castration (CRPC : *Castration-Resistant Prostate Cancer*) se définit par une croissance tumorale continue malgré la prise d'anti-androgènes. Une étude de Antonarakis et ses collègues ont mis en lumière la présence d'une forme tronquée de l'ARNm du récepteur aux androgènes dans les CTCs, appelée ARv7 (*androgen-receptor variant 7*), qui pourrait prédire l'échec d'une thérapie aux anti-androgènes suggérant ainsi la potentielle utilisation en tant que marqueur de sélection des patients pour ce type de traitement[105].

4.3.2.3. Analyse protéique :

Un autre moyen d'utiliser les CTCs pour guider la décision thérapeutique consiste à combiner la détection de ces cellules avec l'analyse de certaines protéines qu'elles contiennent. Par exemple, dans le cancer du sein le récepteur aux oestrogene (ER : *Estrogen Receptor*) est une cible clé. On estime les patients comme étant ER+ lorsque au moins 1% des cellules tumorales expriment le récepteur ; ils vont alors être traités par thérapie hormonale. Cependant 1% représente un seuil faible, ainsi les tumeurs du sein ER+ peuvent présenter des CTCs ER- et donc engendrer une résistance au traitement anti-hormonal[106]. Une équipe de chercheur a mis au point un test multiparamétrique, le CTC-ETI (*CTC-Endocrine Therapy Index*), qui pourrait être capable de prédire la résistance à la thérapie hormonale chez les patients atteints d'un cancer du sein ER+ métastatique[107]. Ce test combine le comptage des CTCs avec l'expression dans ces cellules de quatre marqueurs : ER, HER2, BCL2 et KI67. Il est actuellement utilisé dans le cadre d'un essai clinique dont les résultats sont attendus très prochainement (COMETI P2, NCT 01701050). De la même manière, d'autres recherches se sont concentrées sur une autre cible importante dans le cancer du sein à savoir l'oncogène HER2[108,109].

Plus récemment, les inhibiteurs des points de contrôles immunitaires tels que ceux de la voie PD1/PD-L1 ont révolutionné la prise en charge des patients atteints d'un cancer avec des résultats spectaculaires, le taux de réponse objective aux anti-PD1 étant d'environ 28 % tous cancers confondus. Fort de ces résultats encourageants, ces traitements sont prescrits à tous les patients, sans sélection. Au vu des effets secondaires importants et du coup pour la société, il est urgent de trouver des biomarqueurs. Dans cette optique, Mazel et ses collègues ont fourni la première preuve de

l'expression fréquente du ligand PD-L1 à la surface des CTCs de patients atteints d'un cancer du sein, ce qui suggère une potentielle utilisation dans la réponse à l'immunothérapie[110,111]. Depuis, deux études ont démontré leur valeur pronostique dans les cancers de la tête et du coup ainsi que du poumon non à petites cellules[99,112].

Dans le CRCP, la prise inefficace d'anti-androgènes est souvent suivie d'autres thérapies hormonales ciblant la voie d'activation du récepteur aux androgènes, qui a cependant des effets variables. Myiamoto et ses collègues ont mis au point une technologie à base de microfluidique afin de détecter et analyser l'activation de cette voie de signalisation dans les CTCs via l'analyse de la PSA et PSMA (*prostate-specific membrane antigen*). Les chercheurs ont démontré que les patients traités possédaient des CTCs avec des signaux d'activation mixtes suggérant ainsi l'intérêt de leur technologie dans l'aide à la prise de décision thérapeutique pour ce genre de traitement hormonal[113].

Ainsi, l'ensemble de ces données démontre que l'analyse des CTCs et de son contenu en ADN, ARN et en protéines pourrait avoir un impact important sur la compréhension des mécanismes de résistance à la thérapie des patients atteints d'un cancer et donc dans leur utilisation en tant que biomarqueur.

5. Les vésicules extracellulaires :

L'étude des vésicules extracellulaires (VE) a connu une croissance impressionnante ces 10 dernières années, notamment au vu de leur potentielles applications cliniques pour de nombreuses pathologies telle que le cancer. Cet engouement a abouti à une incroyable quantité de publications sur le sujet, avec parfois un manque clair de consensus que ce soit au niveau de la nomenclature, de la caractérisation ou encore des technologies d'analyse. Pour contourner ce manque d'homogénéité, la communauté de chercheurs s'est réunie pour encadrer le domaine de l'étude des VEs et créer « *l'International Society of Extracellular Vesicle* » (ISEV). Elle édifie les règles à suivre pour l'étude rigoureuse des VEs : les « *Minimal information for studies of extracellular vesicles* » (MISEV), qui sont mises à jour tous les 4 ans[114]. L'engouement est tel qu'il a abouti à la fois à la création d'un journal spécialisé, le « *Journal of Extracellular Vesicle* » dont le facteur d'impact est passé de 4 à 11 en 2020, ainsi que d'un congrès internationalement renommé. Ceci a notamment permis de mieux connaître la biologie et la fonction des VEs. Ainsi, leur sécrétion par les cellules semble être bien plus qu'un simple mécanisme de recyclage des protéines, tel que cela a été décrit pour la 1^{e} fois[115,116]. On sait dorénavant que les VEs sont capables d'échanger des composants entre cellules, aussi bien des protéines que des acides nucléiques ou des lipides, et d'agir comme un moyen de communication à part entière[117,118]. De

plus, elles sont retrouvées dans la grande majorité des fluides biologiques c'est pourquoi elles concentrent beaucoup d'intérêts dans le domaine de la biopsie liquide.

La classification des vésicules extracellulaires évolue constamment[114]. Néanmoins, on peut les diviser en deux catégories avec d'un côté les ectosomes (ou microvésicules), et de l'autre les exosomes[119]. Les ectosomes sont formés par bourgeonnement de la membrane plasmique pour former des microvésicules, des microparticules ou encore des vésicules de grande taille, qui mesurent de 50nm jusqu'à 1µM de diamètre. Au contraire, les exosomes ont une origine endosomale et ont une taille comprise entre 40 et 160nm. Les différentes VEs se distinguent par leur origine subcellulaire, leurs propriétés physico-chimiques et leur composition spécifique[117–119].

Nous nous intéresserons ici plus particulièrement aux exosomes qui concentrent l'essentiel des recherches sur les VEs.

6. Les exosomes :

6.1. Généralités :

Le terme « exosomes » a été utilisé pour la première fois en 1981 par Trams et ses collègues pour désigner de petites vésicules d'origine inconnue sécrétées par différents types de cellules[120]. En 1983, Pan et Johnstone découvrent que ces vésicules ont une origine endosomale[121]. A l'époque, les chercheurs ont pensé que c'était un moyen pour les cellules d'éliminer les protéines obsolètes, c'est pourquoi pendant une longue période les exosomes n'ont généré que peu d'intérêt. Ce n'est qu'à la fin des années 1990 qu'ils ont suscité l'attention des chercheurs, après la découverte de la sécrétion d'exosomes par les lymphocytes B, par Raposo et ses collègues en 1996[122], puis par les cellules dendritiques en 1998 par Zitvogel et al[123], qui étaient capables d'induire une réponse immunitaire[124]. Dès lors, il s'en est suivi une multitude de travaux portant sur de nombreux types cellulaires différents, et leur implication dans les mécanismes de communication intercellulaire en condition pathologiques[117,118,125,126]. Il est ainsi décrit que la grande majorité des types cellulaires sont capable de sécréter des exosomes tels que les cellules épithéliales[127], les neurones[128] et les cellules tumorales[124], les cellules immunitaires[117,129], la quantité et la composition des exosomes étant dépendante de l'état physiologique de la cellule[118]. Ils peuvent être isolés à partir de surnageant de culture cellulaire et ont été retrouvés dans de nombreux fluides biologiques tels que le sang[130], l'urine[131], la salive[132], le liquide bronchoalvéolaire[133], ou encore le liquide cerebro-spinal[134].

6.2. Biogenèse des exosomes :

La biogenèse des exosomes débute par une étape d'endocytose au cours de laquelle les cellules internalisent diverses molécules. Ensuite, une deuxième invagination de la membrane va avoir lieu dans les endosomes, avant d'être libérés par un processus d'exocytose.

La première étape consiste en l'invagination de la membrane plasmique qui forme une structure contenant des protéines membranaires et des protéines solubles du milieu extracellulaire. Cette structure va aboutir à la formation d'un endosome précoce qui, dans certains cas, va fusionner avec d'autres endosomes précoces. Il est important de noter que le réticulum endoplasmique ainsi que le système de Golgi peuvent, ici, participer à la formation et au contenu de ces endosomes précoces[135–137]. Ensuite, ces endosomes précoces vont maturer et former des endosomes tardifs, puis des corps multivésiculaires (CMVs). Ces CMVs sont formés par invagination de la membrane de l'endosome tardif et former des petites vésicules appelées vésicules intraluminales (VILs) ; les futurs exosomes. Enfin, les CMVs, contenant les VILs, peuvent soit fusionner avec les autophagosomes et/ou les lysosomes et être recyclés et/ou dégradés, soit fusionner avec la membrane plasmique et libérer les exosomes (Figure 8)[136,138–140].

Les mécanismes précis de formation des exosomes sont encore mal connus. Néanmoins plusieurs études suggèrent l'implication de différentes molécules laissant à penser qu'il n'y a pas de mécanisme universel, mais plutôt différents systèmes dont l'activité dépend de plusieurs paramètres tels que le type cellulaire et son état physiologique.

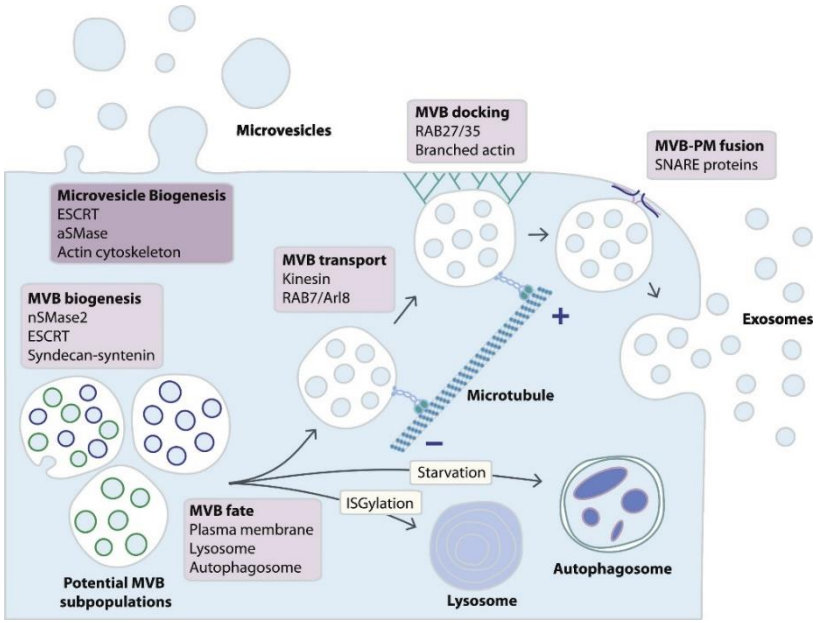

Figure 8 : Mécanismes de la biogenèse des exosomes. *Les exosomes sont générés à l'intérieur des CMVs (MVB), qui, lors de leur maturation, vont fusionner avec la membrane plasmique. Ce mécanisme multi-étape est étroitement régulé et inclue un acheminement via les microtubules, une fixation à la membrane (MVB docking) et la fusion médiée par les protéines SNAREs. D'après Bebelman et al, Pharmacology & Therapeutics, 2018.*

6.2.1. Formation des vésicules intraluminales :

6.2.1.1. Le complexe protéique ESCRT (Endosomal Sorting Complex Responsible for Transport) :

Le complexe ESCRT est un acteur majeur de la plasticité des membranes biologiques, en particulier dans la formation des CMVs et des VILs[141]. Cette machinerie moléculaire agit en plusieurs étape : les sous-unité ESCRT-0 et ESCRT-1 vont d'abord rassembler des protéines transmembranaires ubiquitinylées de la membrane des CMVs, puis recruter via les sous-unités ESCRT-II et ESCRT-III des complexes moléculaires qui vont entrainer le bourgeonnement et la fission du microdomaine néoformé pour créer les VILs[141–144]. Parmi ces protéines, on retrouve notamment TSG101 (*Tumor Susceptibility Gene 101*) qui est présent dans les exosomes et très régulièrement utilisé comme marqueur.

Néanmoins, il existe d'autres mécanismes de formation des exosomes qui sont indépendants du complexe ESCRT[145].

6.2.1.2. Les céramides :

Les céramides sont une classe de lipides en forme de cônes, générés par le clivage de la sphingomyéline par les sphingomyélinases. Cette forme particulière leur permet notamment d'être les principaux acteurs de la courbure des membranes. Par conséquent les céramides jouent un rôle important dans la formation des VILs. Une étude a par exemple montré que l'inhibition de la nSMAse-2 (*neutral Sphingomyélinase 2*) empêchait le bourgeonnement des VILs dans les CMVs ainsi que la libération des exosomes, via un mécanisme indépendant de la machinerie ESCRT[146]. De plus, les céramides peuvent être métabolisées en sphingosine-1 phosphate qui activent les récepteurs GiS1P [(*inhibitory G protein (Gi)-coupled sphingosine 1-phosphate (S1P*)] à la membrane des CMVs, régulant ainsi la formation et le tri dans les VILs[147].

6.2.1.3. L'axe Syndécane-Synténine-ALIX :

Le tri sélectif de protéines transmembranaires dans les VILs des CMVs peut également avoir lieu via des interactions protéiques. C'est notamment le cas des protéines Syndécane, Synténine et ALIX. En effet, la syndécane se fixe à la synténine-1 et forme un complexe qui recrute la protéine ALIX (*ALG-2 interacting protein X*) qui, ensemble, vont permettre l'invagination de la membrane des CMVs pour former les VILs (Figure 8)[148]. Elles sont donc présentes au sein des exosomes. Ces mécanismes sont régulés par d'autres protéines telles que SRC[149,150], ARF6 et PLD2[151].

6.2.1.4. Les tétraspanines :

Les tétraspanines sont une famille de glycoprotéines transmembranaires possédant une forme conique, avec une cavité qui leur confèrent la capacité de fixer des molécules de cholestérol[152]. Ceci

leur permet notamment de se regrouper pour former des « tetraspanin-enriched microdomains », sorte de plateforme de signalisation au niveau de la membrane plasmique. Ainsi, les tétraspanines jouent un rôle dans différent processus tels que l'adhésion cellulaire, la modulation du système immunitaire ou encore le cancer[153]. Parmi elles, CD63 est particulièrement enrichie à a surface des exosomes. Des études ont montré que cette protéine était capable de réguler le tri des protéines dans les exosomes de mélanocytes[154] et de cellules de mélanome[155]. Une autre publication révèle un rôle de CD63 dans la biogenèse de ces nanovésicules dans les fibroblastes de patients atteints du syndrome de Down[156]. Les tétraspanines CD9 et CD81, également enrichies dans les exosomes, sont aussi impliquées directement dans le tri de leur contenu[157,158].

6.2.2. Traffic intracellulaire des corps multivésiculaires jusqu'à l'exocytose :

6.2.2.1. Les Rab GTPases :

Les Rab GTPases sont des petites protéines G appartenant à la superfamille des protéines Rab et qui possèdent une activité GTPase. Elles régulent plusieurs processus tels que le trafic membranaire, notamment la formation de vésicules, et leur mouvement sur les réseaux du cytosquelette que sont l'actine et la tubuline[159]. Enfin, elles jouent également un rôle dans la fusion des membranes et par conséquent dans la libération des exosomes dans le milieu extracellulaire[160,161]. Il a par exemple été démontré que Rab27a et Rab27b jouent un rôle dans la morphologie des CMVs ainsi qu'à sa fixation à la membrane, en vue d'une libération des exosomes (Figure 8)[160]. De fait, l'utilisation d'ARN interférant dirigé contre ces protéines a été utilisé dans différentes études afin de démontrer le rôle spécifique des exosomes[162,163]. D'autres Rab GTPases sont également capable de réguler la sécrétion des exosomes tels que Rab7, Rab11 et Rab35[148,159,161].

6.2.2.2. Les protéines SNAREs :

Les SNAREs (*Soluble N-éthylmaleimide-sensitive-factor Attachment protein REceptor*) sont des protéines majoritairement transmembranaires responsables de la fusion et du trafic membranaire au sein des cellules eucaryotes. Elles vont ainsi agir sous forme de complexe au niveau des CMVs fixés à la membrane pour permettre la libération des exosomes (Figure 8). Plusieurs protéines SNAREs ont été décrit comme étant impliquées dans ce mécanisme telles que YKT6[164], VAMP7[165], SNAP23[166] ou encore la syntaxin-1a[167]. Cette activité est régulée par différents mécanismes parmi lesquels la concentration en Ca^{2+} ou encore leur état de phosphorylation[168–170].

Il est important de noter ici que toutes ces protéines n'ont pas un rôle exclusif à la biogenèse des exosomes. En effet, elles sont pour la plupart engagées dans de nombreux processus moléculaires responsables du trafic intravésiculaire et membranaire. Il semblerait donc que les exosomes soient

produits via différentes voies de signalisation interconnectées qui sont activées en fonction de différents paramètres tels que le type cellulaire ou les conditions physiologiques, et en régisse ainsi la composition.

6.3.Composition :

Les exosomes ont une composition particulière qui les distingue des autres VEs (Figure 9)[171,172]. Cette composition spécifique leur confère des fonctions propres qui sont régulées par la dynamique de libération de ces nanovésicules. Le contenu des exosomes est référencé sur le site internet Exocarta (http://www.exocarta.org/).

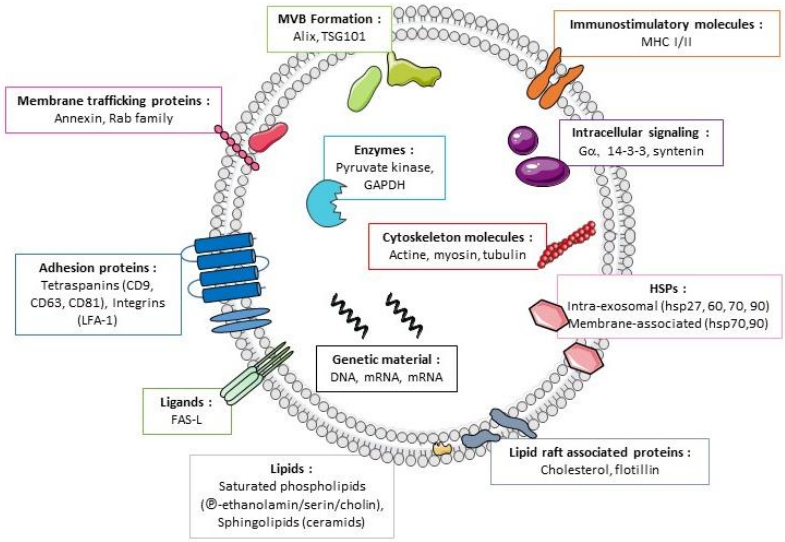

Figure 9 : Composition des exosomes. *Ces nanovésicules sont constituées d'un bicouche lipidique enrichie en cholestérol. Des récepteurs et des ligands sont incorporés dans cette membrane. A l'intérieur, on retrouve également des protéines diverses et variées et du matériel génétique. MVB formation = Formation des CMV, immunostimulatory molecules = molécules immunostimulantes, intracellular signaling = signalisation intracellulaire, HSPs = Protéines de choc thermique, Lipid raft assoiated proteins = protéines associées aux radeaux lipidiques, Lipids = lipides, Ligands = ligands, Adhesion proteins = protéines d'adhésion, membrane trafficking proteins = protéine du trafic membranaire, cytoskeleton molecules = molécules du cytosquelette, genetic material = matériel génétique, enzymes = enzymes. Adapté de Cordonnier & Chanteloup et al, Cell Adh Migr, 2017.*

6.3.1. Contenu protéique :

Des études montrent qu'une des voies possibles pour le chargement des protéines à l'intérieur des exosomes est via l'interaction avec les composants de la machinerie de biogenèse des exosomes[131,172]. On y retrouve donc des protéines du trafic membranaire telles que Rab 5 et l'annexine, des protéines impliquées dans la formation des CMVs comme Alix, TSG101 ou la synténin-1 ou encore des molécules du cytosquelette avec par exemple l'actine ou la tubuline (Figure 9)[172].

De la même manière, l'association des protéines membranaires avec les tétraspanines facilitent leur incorporation dans les exosomes et plus précisément CD9, CD63 et CD81. On peut aussi noter la

présence d'intégrines telles que LFA-1 ou des ligands de récepteurs comme FAS-L ou PD-L1. On y retrouve différents récepteurs capables d'induire une réponse physiologique comme le CMH I/II, ou encore des enzymes telles que la pyruvate kinase ou la GAPDH (Figure 9)[172].

Enfin, plusieurs protéines de choc thermique (HSPs : *Heat Shock Protein*) sont présentes dans la lumière des exosomes parmi lesquelles HSP27, HSP60, HSP70, HSP90, dont certaines sont retrouvées à leur surface (HSP70, HSP60 et HSP90)[126] (Figure 9).

Cette composition particulière permet d'identifier les VEs comme exosomes, en plus de leur taille. Ainsi, les MISEVs préconisent de révéler la présence d'au moins 3 marqueurs des exosomes parmi lesquels 2 tétraspanines et 1 molécule impliquée dans la biogenèse telle que TSG101 ou ALIX, et l'absence d'un marqueur négatif comme une protéine du réticulum endoplasmique (Grp94)[114].

6.3.2. Contenu lipidique :

Les lipides sont des composants essentiels de la membrane des exosomes. Il est désormais bien décrit dans la littérature que ces nanovésicules possèdent une double membrane lipidique dont la composition est identique à celle de la cellule mère, mais avec certains enrichissements[173]. Les exosomes sont donc enrichis en phospholipides saturés (phosphatidyl-éthanolamine, phosphatidyl-sérine, phosphatidyl-coline), en sphingolipides (céramides) et en cholestérol (Figure 9). Cette composition particulière leur confère une grande rigidité qui leur permet de protéger leur contenu de la dégradation et d'être plus stable dans le milieu extracellulaire.

6.3.3. Contenu en acide nucléiques :

6.3.3.1. Les ARNs :

En 2007, Valadi et ses collègues ont démontré pour la première fois que les cellules étaient capables de procéder à un transfert horizontal d'ARN fonctionnel[174]. Cette étude pionnière a été à l'origne d'intenses efforts de la part des chercheurs pour étudier l'expression et le rôle de différents types d'ARN présents dans ces nanovésicules.

Outre l'ARNm, les exosomes sont hautement enrichis en petits ARNs non codants, ce qui suggère un rôle important dans la régulation des gènes. On y retrouve ainsi majoritairement des micro-ARNs sous leur forme de précurseur ou mature, mais aussi des snARN (*small nuclear* ARN) et snoARN (*small nucleolar* ARN), des Y-ARNs, des piARN (*piwi-interacting* ARN), ou encore des tsARN (*tRNA-derived small* ARN). On retrouve également des longs ARNs non codants (lncARN, *long non coding* ARN) et des ARNs circulaires (cirARN). Enfin, de l'ARN mitochondrial est présent au sein des exosomes[139,171,175,176]. Tous ces ARNs ont été identifiés comme des ARNs non codants capables de modifier la physiologie des cellules, notamment dans le cadre du cancer. Leur chargement au sein des exosomes est lié à un

processus sélectif ce qui entraine une différence d'expression entre la cellule mère et l'exosome[176]. Leur détection au sein des exosomes circulants représente ainsi un grand espoir dans le diagnostic non invasif de cette pathologie[177–179].

6.3.3.2. L'ADN :

La présence d'ADN au sein des exosomes a longtemps été mise en doute mais il est aujourd'hui prouvé qu'ils contiennent bien de l'ADN. Kahlert et ses collègues ont montré pour la première fois que les exosomes dérivés de sérum de patients atteints d'un cancer du pancréas contenaient de longs fragments d'ADN double brin (dsADN, *double stranded* ADN), en l'occurrence les mutations KRAS et p53[22]. Cette étude a été confirmée quelques années plus tard lorsque Thakur et ses collègues ont affirmé que la majorité de l'ADN associé aux exosomes étaient double brin[23]. D'autres types d'ADN sont également retrouvés au sein des exosomes tels que de l'ADN simple brin, de l'ADN viral ou encore de l'ADN mitochondrial[180]. Cet ADN est constitué de fragments plus longs que le ADNcf ce qui permet de les différencier[180].

6.4. Hétérogénéité des exosomes :

La diversité des voies de signalisation et de régulation de la biogenèse des exosomes, ainsi que l'impact de l'environnement cellulaire, entrainent une hétérogénéité phénotypique des exosomes. En effet, il existe des sous-populations de tailles différentes, provenant de sources différentes, au contenu différent et donc qui exercent une fonction différente[139,181] (Figure 10). Cette hétérogénéité a notamment été révélée par des analyses de protéomique qui ont permis d'identifier des signatures protéiques spécifiques d'un type de cellule par exemple, tandis que des études de séquençage ont soulignées les différences d'expression avec la cellule mère, suggérant ainsi un mécanisme de tri spécifique[172,176,182].

Les fonctions des exosomes sur les cellules réceptrices peuvent donc varier en fonction de leur taille, du type de récepteurs exprimés à leur surface, de la nature et de la quantité du matériel qu'ils contiennent et enfin de la façon dont ils vont interagir avec la cellule réceptrice (Figure 10).

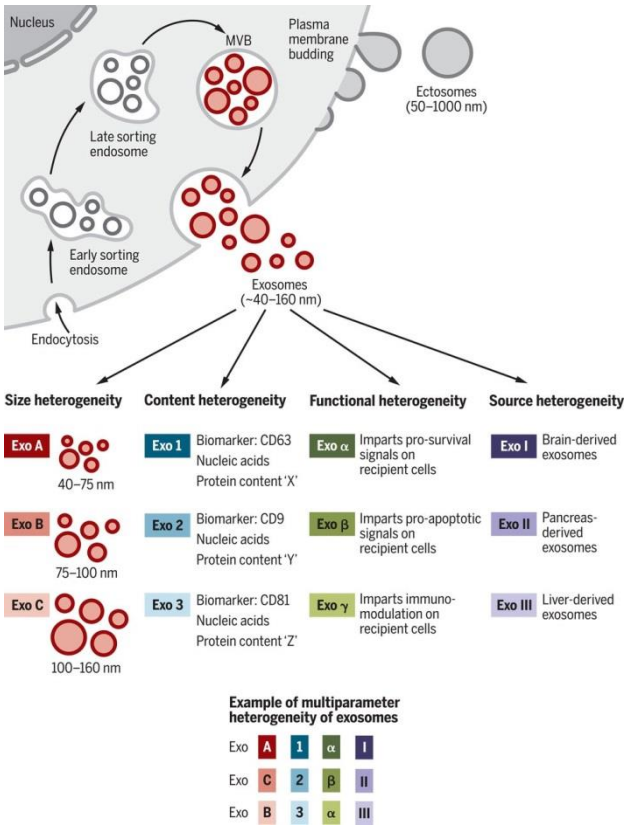

Figure 10 : Hétérogénéité des exosomes. *Ces nanovésicules peuvent être très hétérogènes et ainsi engendrer des réponses biologiques complexes. Cette hétérogénéité (heterogeneity) des exosomes peut être conceptualisée sur la base de leur taille (size), leur contenu (content), leur fonction sur les cellules réceptrices (functional), et la cellule d'origine (source). Des combinaisons distinctes de ces caractéristiques soulignent toute la complexité de l'hétérogénité des exosomes. Daprès Kalluri et al, Science, 2020.*

6.5.Interaction avec la cellule réceptrice :

Une fois libérés dans le milieu extracellulaire, les exosomes peuvent atteindre les cellules réceptrices et y délivrer leur contenu, qui va engendrer une réponse fonctionnelle et modifier leur état physiologique. Ils peuvent interagir différemment à la suite de la fixation à la membrane de la cellule réceptrice, mais ces mécanismes sont encore mal connus. On ne sait pas, par exemple, si un mode d'interaction différent agit sur le devenir de l'exosome.

6.5.1. Fixation à la surface des cellules cibles :

Le ciblage des cellules semble avoir lieu via des interactions spécifiques entre des protéines enrichies à la surface des exosomes et des récepteurs au niveau de la membrane plasmique de la cellule. Bien que ces processus ne soient pas clairement décrits, plusieurs acteurs majeurs sont identifiés parmi lesquels les tétraspanines, les lipides, les lectines, les protéoglycanes ou encore les composants de la matrice extracellulaire (MEC). Ainsi, les intégrines présentes dans la membrane des exosomes peuvent par exemple à la fois interagir avec les molécules d'adhésion à la surface de la cellule réceptrice telle que les ICAMs (*intercellular adhesion molecules*)[183], et les composants de la MEC pour effectuer une

fixation[184]. Hoshino et ses collègues ont également montré que la composition spécifique des exosomes en certaines intégrines définissait le lieu de formation des métastases dans le cancer, induites par ces mêmes exosomes[185]. Les tétraspanines ont également été identifiées comme partenaires d'interaction des intégrines de façon à promouvoir la fixation des exosomes sur les cellules cibles[186,187]. Enfin, d'autres molécules ont un rôle dans ce processus, telle que les lectines[188] ou la composition lipidique des exosomes, notamment la phophatidyl-sérine[189] (Figure 11).

Cependant, on ne sait pas à ce jour si le ciblage des cellules par un sous-type particulier d'exosomes résulte d'un processus spécifique ou non, la littérature décrivant les 2 hypothèses[137].

6.5.2. Signalisation intercellulaire médiée par les exosomes :

Une fois fixés à la cellule réceptrice, les exosomes peuvent soit (i) rester à la membrane et engendrer une signalisation[157], (ii) être internalisés par différents processus de dynamique des membranes à savoir l'endocytose dépendante de la clathrine ou non, la macropinocytose[190] et la phagocytose[191] ou l'endocytose via la caveole et les radeaux lipidiques[192], (iii) ou encore par fusion des membranes[193] (Figure 11).

Les exosomes sont donc capables de se fixer puis d'activer des récepteurs présents à la surface des cellules. Les premiers exemples de ce processus ont été décrit auparavant et concernent les travaux de Raposo et Zitvogel qui démontrent que les exosomes dérivés de lymphocytes B et de cellules dendritiques, respectivement, sont capables d'induire une réponse immunitaire spécifique[122,123]. Dès lors, de nombreuses études ont révélé un mécanisme similaire et seront détaillées dans le chapitre suivant.

Une fois fixés, les exosomes peuvent également pénétrer dans la cellule par l'intermédiaire des processus évoqués ci-dessus. Ils vont ensuite pénétrer dans un endosome précoce puis un CMV, qui contient possiblement des exosomes néoformés. Il y a alors plusieurs possibilité ; (i) les exosomes vont être dirigés vers le lysosome où ils vont être dégradés[194], (ii) fusionner avec la membrane du CMV et libérer leur contenu dans le cytoplasme ou vers le réticulum endoplasmique[195], (iii) ou être resécrétés via la fusion du CMV avec la membrane plasmique[196] (Figure 11).

Enfin, la fusion des membranes entraine le transfert des composants de la membrane de l'exosome dans celle de la cellule réceptrice, et libère son contenu à l'intérieur (Figure 11).

Ainsi, l'hétérogénéité phénotypique des exosomes impacte la manière dont ils interagissent avec la cellule réceptrice qui, à son tour, régit sa fonction.

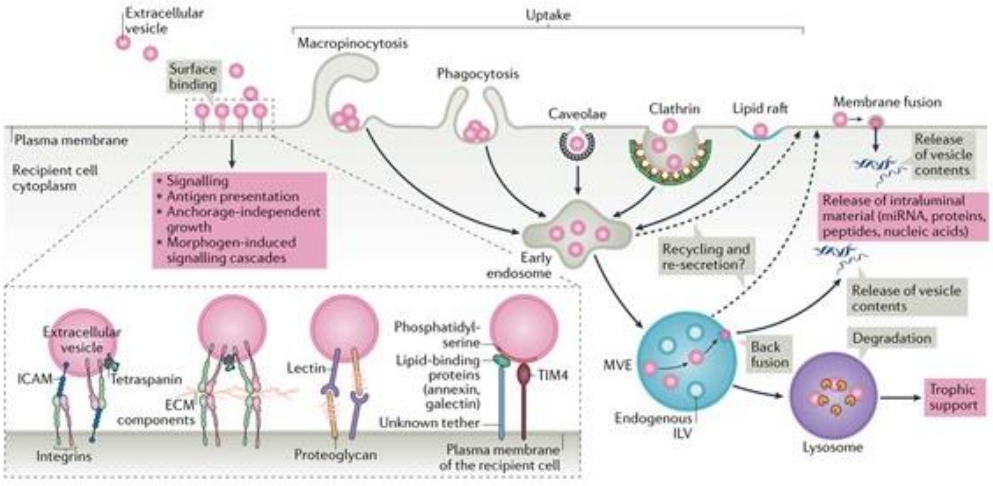

Figure 11 : Interactions des exosomes avec la cellule réceptrice. *En fonction du type cellulaire, les exosomes peuvent rester fixés à la membrane (surface binding) et initier une signalisation intracellulaire. Les exosomes peuvent également être internalisés via différentes voies (uptake). L'internalisation de ces nanovésicules mène à l'endosome précoce (early endosome) qui va se différencier en CMV dans lequel les vésicules internalisées vont être mixées avec les VILs (ILVs). La fusion des CMVs avec le lysosome entraine la dégradation des exosomes et le recyclage du contenu pour alimenter le métabolisme cellulaire. Les exosomes peuvent également libérer leur contenu (release of vesicle content) dans le cytoplasme de la cellule réceptrice via fusion avec la membrane plasmique (membrane fusion) ou des CMVs (back fusion). Enfin, les exosomes peuvent être re-sécrétés (re-secretion). D'après Niel et al, Nat Rev Mol Cell Biol, 2018.*

6.6. Fonction des exosomes dans le cancer :

Dans le contexte tumoral, la communication cellulaire est un processus dynamique majeur. Il est désormais acquis que les exosomes en font partie intégrante. Les conditions physiologiques particulières dans le microenvironnement modifient l'état des cellules cancéreuses qui, de fait, libèrent plus d'exosomes que les cellules saines[197]. De plus, leur contenu est également différent de la cellule mère ce qui suggère un processus de tri spécifique qui permet de promouvoir la croissance tumorale.

6.6.1. Induction de lésions néoplasiques :

Différentes études ont démontré que les exosomes étaient capables d'induire la formation d'un cancer. Ainsi, il a récemment été démontré que les exosomes dérivés de cancer du pancréas possèdent la capacité de transformer les cellules NIH/3T3 en y induisant des mutations[198]. De la même manière, les exosomes dérivés de cancer du sein et de la prostate semblent être capable d'induire des lésions néoplasiques via le transfert de leur contenu en micro-ARNs[199,200]. Plus précisément, les miR-125b, miR-130 et mir-155, ainsi que l'ARNm HRAS et KRAS des exosomes de cellules de cancer de la prostate semblent pouvoir initier la reprogrammation néoplasique et la formation d'une tumeur des cellules souches adipocytaires[200].

6.6.2. Rôle dans la progression tumorale :

Une des premières études démontrant le fait que les exosomes dérivés de tumeur étaient capables de promouvoir la croissance tumorale fût celle de Liu et ses collègues en 2006. Les auteurs ont démontré

que l'injection d'exosomes dérivés de lignées de cancer du sein murin 4T1 et TS/A *in vivo* entraînait une augmentation de la croissance cellulaire[201]. Depuis, de nombreuses études portent sur l'étude de la fonction des exosomes dérivés de tumeur sur le stroma, notamment les composants de la MEC, les fibroblastes et les cellules immunitaires.

6.6.2.1. Effets sur la matrice extracellulaire :

Les exosomes dérivés de tumeurs sont capables de remodeler la MEC afin d'augmenter la capacité migratoire des cellules cancéreuses[202] (Figure 12). En effet, ils interagissent avec les composants de cette matrice via des molécules d'adhésion et libèrent des protéines qui vont la dégrader telles que des métalloprotéinases (MMPs) ou des cathepsines[202]. Ce remodelage de la MEC permet la libération de cytokines et de facteurs de croissance qui vont affecter d'autres composants du microenvironnement tumoral afin de favoriser la croissance tumorale, comme les fibroblastes. Ainsi, il a été démontré *in vivo* que les exosomes tumoraux, via la sécrétion de TGF-β peuvent induire la différenciation de ces derniers en fibroblastes associés au cancer (FAC), qui possèdent un phénotype mésenchymateux[203,163]. Enfin, les exosomes semblent être capable de libérer des signaux chimiotactiques[184,204].

6.6.2.2. Effets sur le système immunitaire :

Des études précoces s'intéressant aux fonctions biologiques des exosomes tumoraux ont révélé un rôle sur le système immunitaire[117,205,206] (Figure 12). Depuis, de nombreux mécanismes ont été identifiés, notamment dans la suppression de l'immunité anti-tumorale. Ainsi, les exosomes sont capables d'inhiber l'activité des lymphocytes T via l'expression à leur surface de points de contrôles du système immunitaire tels que CTLA-4 et PD-L1, qui, une fois liés à leur récepteurs sur les lymphocytes T, les rendent anergiques[207–209]. Poggio et ses collègues ont récemment démontré que PD-L1 exosomal était capable d'induire une immunosuppression à lui seul, renforçant un peu plus le rôle des exosomes dans le cancer[210]. Ces nanovésicules sont aussi capables d'induire l'apoptose des cellules T, notamment via l'expression de ligands tel que Fas[211,212] mais aussi via leur contenu en micro-ARNs[213]. Ils sont également capables de recruter d'autres acteurs du système immunitaire comme les cellules T régulatrices, via l'expression de la chemokine CCL20, puis d'induire leur expansion, ce qui augmente leurs fonctions immunosuppressives[214]. Notre équipe a aussi démontré que les exosomes dérivés de tumeurs pouvaient activer les cellules myéloïdes suppressives (MDSCs) via la forme membranaire de HSP70, et de promouvoir la croissance tumorale[215]. Un autre effet immunosuppresseur des exosomes réside dans leur capacité à inhiber les cellules NK, via l'inhibition de l'expression de son récepteur NKG2D[216,217]. Enfin, les exosomes peuvent affecter la fonction et la différenciation des cellules dendritiques et des macrophages[218]. Ainsi, il existe une large diversité de mécanismes qui soulignent les différents effets des exosomes sur le système immunitaire dans la littérature[129,218].

6.6.2.3. Effets sur l'angiogenèse et l'hypoxie :

La néoangiogenèse accompagne la croissance tumorale ; elle permet d'acheminer les nutriments et autres métabolites nécessaire à la croissance des cellules cancéreuses. Elle constitue également une voie privilégiée de la formation des métastases. Des études ont montré que les exosomes dérivés de tumeurs favorisaient la vascularisation de la tumeur (Figure 12). Par exemple, la tétraspanine 8 contribue au recrutement sélectif de protéines et d'ARNm dans les exosomes tumoraux qui, une fois absorbés par les cellules endothéliales, induit une augmentation de la prolifération, de la migration et de la maturation des progéniteurs endothéliaux, favorisant ainsi la néoangiogenèse au niveau de la tumeur[186]. De la même manière, dans le mélanome, la protéine WNT5A entraine la libération d'exosomes contenant des facteurs proangiogéniques tels que le VEGF ou MMP2[219], tandis que dans le cancer de la tête et du coup, les exosomes contiennent la protéine EPHB2 et contribuent à l'angiogenèse tumorale[220].

L'hypoxie est un phénomène particulièrement présent au cœur des tumeurs solides, de même que la néoangiogenèse. Ainsi, les exosomes dérivés de glioblastome sont enrichis en facteurs de régulation de l'hypoxie et reprogramment les cellules endothéliales afin de stimuler la formation de nouveaux vaisseaux sanguins[221]. De la même manière, les exosomes dérivés de tumeur du poumon hypoxique libèrent le miR-23a qu'ils contiennent, dans les cellules endothéliales afin de favoriser l'angiogenèse[222].

Les cellules cancéreuses sont donc capables d'utiliser les exosomes pour stimuler l'angiogenèse et favoriser la croissance tumorale.

6.6.2.4. Effets sur la formation des métastases :

Plusieurs études ont également démontré un rôle des exosomes dérivés de tumeurs au niveau de différentes étapes de la formation des métastases (Figure 12). Ainsi, dans un modèle de cancer mammaire murin, Tominaga et ses collègues ont révélé que le miR-181c exosomal entrainait la dégradation de la barrière hémato-encéphalique et promouvait ainsi la formation de métastases cérébrales[223]. De façon similaire, le miR-105 contenu dans les exosomes dérivés de cancer du sein entraine la formation de métastases pulmonaires et cérébrales via la dégradation de l'ARNm codant pour la protéine ZO-1, constituant important de la perméabilité vasculaire[224].

Dans le mélanome, deux études ont souligné le rôle des exosomes contenant la protéine MET dans la formation des métastases[225,226]. Ces derniers sont capables de recruter et d'éduquer des progéniteurs de la moelle osseuse vers un phénotype pro-métastatique via le transfert du récepteur de tyrosine kinase MET, et ainsi d'initier la formation d'une niche pré-métastatique[226]. Dans le cancer du pancréas, les exosomes dérivés de tumeur sont enrichis en la protéine MIF qui, une fois assimilés par les cellules de Kupffer du foie, induit une libération de TGF-β qui créé un environnement favorable à la formation

de métastases dans ce même organe[227]. De plus, Hoshino et ses collègues ont démontré que le profil en intégrines des exosomes dictait l'organotropisme, c'est-à-dire l'endroit où les métastases vont préférentiellement se développer[185].

Ainsi, les exosomes semblent être une voie de communication importante dans l'établissement de foyer métastatiques. Il est néanmoins nécessaire de poursuivre les recherches afin de déterminer dans quelles mesures les exosomes sont importants dans ce processus. Des techniques d'imageries innovantes ont été développées dans cette optique[228,229].

6.6.2.5. Rôles dans la résistance à la thérapie :

Plusieurs études ont indiqué que les exosomes peuvent médier la résistance à la thérapie, notamment au travers de l'export des drogues chimiothérapeutiques en dehors de la cellule (Figure 12). Par exemple, les cellules de carcinome ovarien humain résistantes au cisplatine possèdent un dérèglement du fonctionnement lysosomal qui aboutit à une sécrétion accrue d'exosomes contenant du cisplatine[230]. Ensuite, les exosomes tumoraux sont capables de transférer des protéines associées à la résistance aux drogues. Parmi ces protéines, TRPC5, présente dans les exosomes dérivés de cellules MCF-7 résistantes à la doxorubicine, est capable de transférer la résistance à la chimiothérapie dans les cellules réceptrices[231]. Mais les exosomes peuvent aussi agir via le transfert de matériel génétique. Ainsi, des chercheurs ont montré que les exosomes étaient capables de transférer la capacité de résistance aux drogues via le transfert du miR-100-5p dans le cancer du poumon[232], ou le long ARN non codant ARSR dans le cancer du rein[233]. De plus, ces nanovésicules dérivées de tumeur peuvent transférer des molécules anti-apoptotiques ou qui favorisent la survie cellulaire. C'est par exemple le cas des exosomes dérivés de cancer du foie qui inhibent l'apoptose induite par le Sorafenib[234]. Enfin, les exosomes sont capables d'agir comme des leurres afin de tromper les thérapies ; dans le cancer du sein, les exosomes porteurs de la protéine HER2 sont capables de fixer le trastuzumab et par conséquent d'empêcher son action contre les cellules cancéreuses[235]. De la même manière, dans le lymphome B malin les exosomes porteurs de CD20 protègent les cellules cancéreuses des anticorps dirigés contre cette protéine[236].

Ces études soulignent ainsi l'importance de l'étude des exosomes dans les mécanismes de résistance aux thérapies et constituent, de fait, des cibles thérapeutiques.

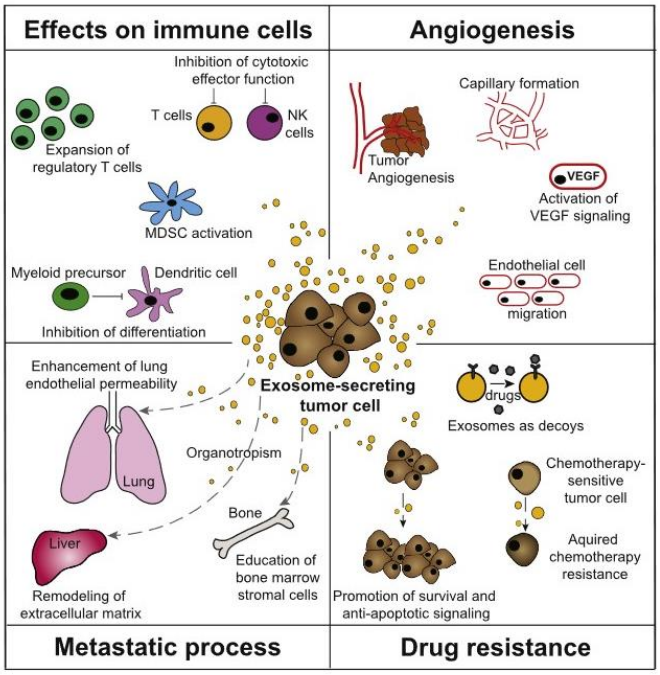

Figure 12 : Rôle des exosomes dérivés de tumeur*. Les exosomes dérivés de cellules tumorales peuvent influencer le microenvironnement local mais aussi systémique. Ils peuvent interagir avec le microenvironnement tumoral pour promouvoir l'angiogenèse (angiogenesis), en activant les cellules endothéliales (endothelial cell) et en augmentant la formation de capillaires (capillary formation) via une sécrétion accrue de VEGF. Les exosomes sont également capables d'induire un environnement immunosuppressif via l'activation des MDSCs et des Treg, qui aboutit à l'inhibition de la fonction cytotoxique des cellules T (inhibition of cytotoxic effector function). De plus, les exosomes modulent la formation de niche pré-métastatique et détermine l'organotropisme en y altérant le comportement des cellules. Enfin, via le transfert de transporteurs de drogues ou via son action en tant que leurre (decoy), les exosomes favorisent la progression tumorale, la survie (promotion of survival) et la résistance aux drogues (drug resistance). D'après Dassler-Plenker, Biochim Biophys Acta Rev Cancer, 2020.*

6.7.Utilisation des exosomes en tant que biomarqueur :

La biologie des exosomes dans le cancer est encore un sujet émergent et le nombre de travaux sur leur utilisation en tant que biomarqueur ne cesse de croitre. Sachant de plus en plus que ces nanovésicules jouent un rôle important dans le cancer, les chercheurs tentent de tirer parti du contenu biologique des exosomes afin d'établir des biomarqueurs circulants robustes, notamment via des analyses multiparamétriques. Il existe ainsi pléthore de publications suggérant l'utilisation d'une ou plusieurs molécules particulières présentes dans les exosomes pour le diagnostic, le pronostic ou le suivi du cancer[139,237–239]. Le but de cette partie n'est pas d'être exhaustif mais d'illustrer les potentielles applications cliniques des exosomes à l'aide de quelques exemples marquants.

6.7.1. Le contenu protéique des exosomes en tant que biomarqueur :

De nombreuses équipes de recherche ont révélé la surexpression d'une ou plusieurs protéines au sein des exosomes et leur corrélation avec la présence ou le stade de la maladie. Par exemple, en 2012, Peinado et ses collègues ont identifié une signature protéique spécifique dans les exosomes circulants de patients atteints de mélanome métastatique, composée de MET, VLA-4 et TYRP-2[226]. Une autre étude suggère l'utilisation des protéines MIA et S100B pour le diagnostic et le pronostique du mélanome[240]. Moon *et al* ont quant à eux identifié dans deux études différentes l'utilité potentielle des protéines Del-1 et de la fibronectine dans les exosomes pour la détection précoce du cancer du sein[241,242]. Des techniques à haut débit ont également permis de révéler une utilité potentielle en tant que biomarqueur, notamment la spectrométrie de masse. Ainsi, Arbelaiz et ses collègues ont identifié une combinaison de protéines dans les exosomes capable de diagnostiquer un cancer du foie avec une précision correcte[243]. De la même manière, une équipe a démontré que le phosphoprotéome des exosomes pouvait avoir une utilité diagnostique dans le cancer du sein[244].

Mais les exosomes semblent aussi utiles dans le suivi du cancer. Ainsi, des chercheurs ont montré que les patients atteints d'un cancer des ovaires qui possèdent un taux élevé de E-cadhérine dans les exosomes circulants, avaient une probabilité de survie statistiquement inférieure[245]. Mais une des protéines exosomales qui concentre le plus d'espoir reste PD-L1, dont le ciblage par des anticorps monoclonaux a révolutionné la prise en charge des patients atteints de cancer. L'intérêt est d'autant plus grand que la détection de la présence de cette protéine par IHC présente plusieurs inconvénients. Ainsi, Chen et ses collègues ont récemment démontré que le dosage de PD-L1 dans les exosomes circulants de patients atteints de mélanome était capable de prédire la réponse à l'immunothérapie[246]. Notre équipe a également obtenu les mêmes résultats et démontré son utilité en tant que facteur pronostique puisque le taux de PD-L1 dans les exosomes corrèle également avec la probabilité de survie globale et sans progression[207]. D'autres études ont aussi mis en valeur l'utilité de cette protéine en tant que marqueur circulant dans d'autres cancers[208,209,247]. Enfin, une autre possibilité réside dans l'étude du profil des intégrines présentes dans les exosomes afin d'identifier précocement l'organe où les métastases vont préférentiellement se développer[185].

La recherche sur les exosomes s'orientent cependant vers l'étude de sous-populations d'exosomes particulières, en vue d'une médecine toujours plus personnalisée[144,181]. Melo et ses collègue ont par exemple démontré l'utilité des exosomes porteurs de la protéine glypican-1 (GPC1) dans le diagnostic précoce et le suivi du cancer du pancréas[248]. En effet, les auteurs ont révélé que la détection d'exosomes GPC1+ circulants permettait non seulement de diagnostiquer un cancer du pancréas avec une parfaite précision, mais également de prédire la survie globale et sans progression des patients[248]. Cette étude a également été réalisée par des laboratoires indépendants dans le cancer du sein et du

colon[249–251]. D'autres chercheurs ont identifié des sous-populations particulières d'exosomes au potentiel biomarqueur ; Yioshioka et ses collègues ont par exemple mis au point une technologie basée sur l'immunocapture des exosomes et identifié que les exosomes CD147+ pouvaient être utiles pour diagnostiquer un cancer colorectal[252].

L'étude des protéines exosomales, qu'elles soient dans la lumière ou membranaires, présente donc un fort potentiel en tant que biomarqueur. Néanmoins, les exosomes contiennent également du matériel génétique qui possède aussi une valeur clinique potentielle.

6.7.2. Le contenu en acide nucléiques des exosomes en tant que biomarqueur :

Les exosomes tumoraux contiennent des acides nucléiques fonctionnels dont l'expression est altérée vis-à-vis de la cellule hôte. On y retrouve ainsi différents types d'ARN et d'ADN[23,171,238].

En 2016, Yuan et ses collègues ont réalisé une analyse transcriptomique des exosomes qui a révélé la composition en ARN des exosomes. On retrouve ainsi majoritairement des micro-ARNs (40,4%), des piARNs (40%), des pseudo-gènes (3,7%), des lncARNs (2,4%), des ARNt (2,1%) et de l'ARNm (2,1), chacun présentant un potentiel biomarqueur[253]. D'autres types d'ARNs sont également présents comme les ARNs circulaires[254].

La très grande majorité des études sur le matériel génétique dans les exosomes concerne les micro-ARNs ; on trouve ainsi une grande quantité de publications traitant de la potentielle utilisation d'un ou plusieurs micro-ARNs en tant que biomarqueur de diagnostic, de pronostic ou de suivi du cancer[255,256]. Un des micro-ARNs des exosomes circulants les plus décrits dans la littérature est le miR-21, dont la surexpression a été identifiée dans de nombreux cancers tels que le glioblastome, le pancréas, le cancer colorectal, du foie, du sein, des ovaires ou encore les cancers oraux, mais aussi dans les exosomes dérivés d'urine de patient atteints d'un cancer de la vessie et de la prostate[255–257]. La surexpression du miR-1246 a été quant à elle corrélée aux différents stades et à la probabilité de survie globale des patients atteints d'un cancer de la prostate[258]. Il a également été décrit comme étant surexprimé dans d'autres cancers[259,260]. Les miR-155 et 17-92 ont eux aussi été identifiés comme des oncomirs, c'est-à-dire des micro-ARNs qui favorisent la croissance tumorale, et associés avec différents paramètres cliniques dans plusieurs types de cancers[256]. Mais il est également possible de cibler l'expression de micro-ARNs suppresseurs de tumeurs comme le miR-143 dont la sous-expression a été associée aux cancers du foie, du sein, du colon ou encore du pancréas[256]. Une autre stratégie utilisée par certaines équipes de recherche vise à utiliser une combinaison de micro-ARNs afin d'améliorer la précision clinique de ces biomarqueurs. Différentes études ont ainsi été publiées sur le potentiel clinique d'une signature particulière de micro-ARNs des exosomes dans différents cancers, notamment pour le diagnostic et le pronostic[261–263].

La détection de mutations au niveau de l'ARNm semble également être pertinente en clinique. Ainsi, McKiernan et ses collègues ont mis au point un test d'expression génique des exosomes dérivés d'urine capable de prédire un cancer de la prostate[264–266]. Enfin, l'avancée des technologies a récemment permis aux chercheurs d'étudier l'expression d'autres types d'ARN dans les exosomes tels que les lncARNs ou les ARNs circulants et leur potentielle utilisation en tant que biomarqueur du cancer[178,267–270].

Des études ont également suggéré que de petites quantités d'ADN pouvaient être détectées dans les exosomes et permettait d'identifier des mutations associées au cancer[22,23,271]. Ainsi, plusieurs équipes sont parvenues à détecter des mutations des gènes KRAS et TP53 dans les exosomes, dont la fréquence permettrait de diagnostiquer un cancer du pancréas[22,271–274].

Les exosomes, au travers de leur composition, présentent donc de nombreuses applications potentielles en tant que biomarqueur dans le diagnostic et le suivi du cancer. La recherche s'oriente désormais vers une approche combinatoire qui vise à analyser des marqueurs de nature différente afin d'améliorer la spécificité et la sensibilité des tests. C'est par exemple le cas de l'étude de Melo et ses collègues qui a détecté la mutation $KRAS^{G12D}$ dans les exosomes GPC1+ uniquement, et dont la combinaison permet de détecter des lésions intra-épithéliales pancréatiques avant l'imagerie[248]. Une autre équipe a démontré que la détection des mutations du gène EGFR à partir de l'ARN et de l'ADN des exosomes, combiné à l'ADNtc permettait d'améliorer la sensibilité du test diagnostic[275]. Cependant, malgré les nombreuses recherches menées à ce jour, les exosomes ne sont pas encore utilisés en clinique, faute d'études cliniques démontrant un gain réel chez un grand nombre de patients. C'est ce vers quoi doit désormais se tourner la recherche sur les exosomes ; il y a ainsi actuellement 32 essais cliniques en cours dans le monde qui visent à étudier l'utilité des exosomes en tant que biomarqueur du cancer (https://clinicaltrials.gov/).

7. Les protéines de choc thermique (HSPs) :

7.1. La réponse au stress cellulaire :

L'homéostasie se définit par la capacité que peut avoir un système quelconque à conserver son équilibre de fonctionnement en dépit des contraintes qui lui sont extérieures. Ainsi, l'homéostasie cellulaire consiste en un mécanisme d'autorégulation dynamique, mis en place par les cellules afin de répondre à tout type de stress. Ces stimuli peuvent être de nature environnementale (stress oxydatif, hypoxie, température élevée), chimique (chimiothérapies, alcool) ou encore physiologique (inflammation). Les cellules sont donc capables de s'accommoder à ces types de stress afin de résister et de survivre.

Le concept de réponse cellulaire au stress a été décrit pour la première fois en 1962 par Ritossa et ses collègues[276]. Les chercheurs ont en effet observé une boursouflure des chromosomes géants de glandes salivaires de drosophile suite à un choc thermique ou à un agent chimique, qui se traduisait par une synthèse d'ARN très rapidement augmentée[276,277]. C'est la naissance du concept de réponse au choc thermique. Douze ans après ces premières observations, Tissières et ses collègues ont démontré que ces boursouflures des chromosomes en réponse à un choc thermique s'accompagnait d'une plus grande production de protéines, qu'ils vont appeler les « protéines de choc thermique » (Heat Schock Proteins, HSPs)[278]. Depuis, on a découvert que ces protéines sont très conservées et présentes dans la grande majorité des espèces[279,280].

La réponse au stress se caractérise par la surexpression des HSPs en réponse à un stress aigu ou chronique[280]. Cette augmentation de l'expression protéique a lieu grâce à un facteur de transcription appelé « Heat shock factor » (HSF) qui, une fois activé, se lie sur une région particulière de l'ADN appelée « Heat shock element » (HSE). Cette fixation va déclencher la transcription des gènes des HSPs inductibles, puis entrainer leur traduction dans la cellule[280]. Chez les vertébrés, il existe plusieurs membres de la famille des HSF (HSF1-4). De façon générale, HSF1 est le facteur de transcription majoritaire chez les vertébrés. HSF1 et HSF3 sont activés en réponse à différents types de stress, alors que HSF2 est induit lors de processus de différenciation[279,281,282] et HSF4 comme un modulateur dans le développement et la maintenance des organes sensoriels[283,284].

HSF1 reste donc l'acteur majeur de la réponse au stress chez l'Homme. En réponse à un stimuli, HSF1 va passer de sa forme monomérique inactive dans le cytoplasme, à une forme trimérique active qui va subir de nombreuses modifications post-traductionnelles régulant son activité et sa stabilité. Cette trimérisation est suivie d'une translocation dans le noyau dans lequel les trimères de HSF1 vont se lier aux HSE, dans le promoteur des gènes des HSPs[285]. Cette fixation déclenche alors la synthèse des HSPs

qui vont avoir au sein de la cellule un rôle de chaperon moléculaire qui permet de maintenir l'homéostasie cellulaire, favoriser la survie et réparer les dommages protéiques[279]. Le retour à une situation normale se fait via l'acétylation du domaine de liaison à l'ADN de HSF1 ainsi que par les protéines de choc thermique HSP90 et HSP70 qui vont maintenir HSF1 sous forme de monomère inactif (Figure 13). Un stress trop sévère ou trop long entraine quant à lui des dommages trop importants pour les capacités de réparation de la cellule, qui entrera alors dans un processus de mort par apoptose ou par nécrose.

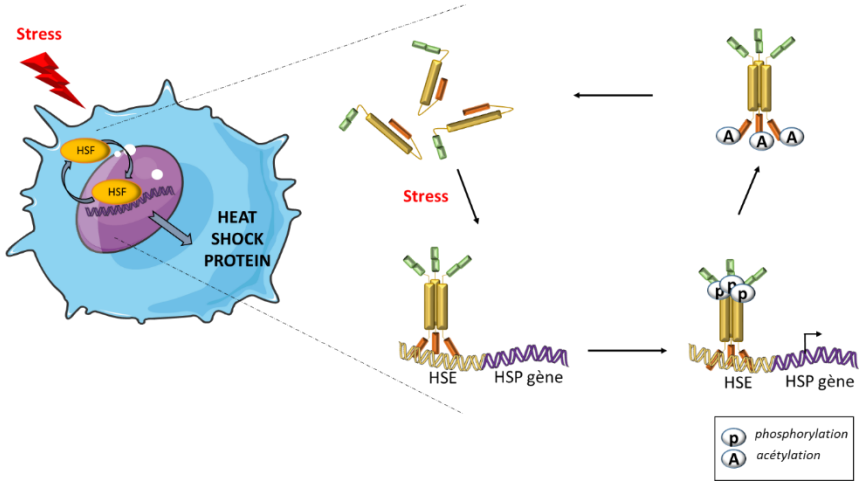

Figure 13 : Représentation schématique de la réponse au stress par HSF1 chez les vertébrées. *Le stress cellulaire induit la trimérisation de HSF1 puis sa relocalisation dans le noyau cellulaire. La phosphorylation stimule son activité transcriptionnelle, permettant ainsi la synthèse des gènes des HSPs. D'après la thèse de Margaux Sevin, Rôle des protéines de choc thermique dans les néoplasies myéloprolifératives : implication de HSP27 dans la myélofibrose, 2017.*

7.2.Classification des HSPs :

Les HSPs sont classées en fonction de leur poids moléculaires. On distingue ainsi 6 grandes familles de HSPs chez les mammifères : HSP110, HSP90, HSP70, HSP60, HSP47 et les petites HSPs ou small heat shock proteins (sHSP) (Tableau 1)[286]. Bien que les HSPs partagent des propriétés communes, chaque classe possède des caractéristiques particulières du fait de leur localisation cellulaire, leur dépendance ou non à l'ATP ou encore leur mécanisme d'action. Les HSPs peuvent être exprimé de façon constitutionnelle ou induite, et son retrouvées dans différents compartiments cellulaires : le cytoplasme, le noyau, la mitochondrie, le réticulum endoplasmique ou encore dans les membranes. Il existe une liste non exhaustive des HSPs, certaines étant plus étudiées que d'autres. Quatre familles nous intéressent particulièrement ici.

Tableau 1 : Nomenclature des principales familles des HSPs chez l'Homme. *D'après Kampinga et al, Cell stress and chaperones, 2009.*

	Gene name	Protein name	Old names	Human gene ID	Mouse ortholog ID
HSP A					
1	*HSPA1A*	HSPA1A	HSP70-1; HSP72; HSPA1	3303	193740
2	*HSPA1B*	HSPA1B	HSP70-2	3304	15511
3	*HSPA1L*	HSPA1L	hum70t; hum70t; Hsp-hom	3305	15482
4	*HSPA2*	HSPA2	Heat-shock 70kD protein-2	3306	15512
5	*HSPA5*	HSPA5	BIP; GRP78; MIF2	3309	14828
6	*HSPA6*	HSPA6	Heat shock 70kD protein 6 (HSP70B')	3310	X
7	*HSPA7*[a]	HSPA7	Heat shock 70kD protein 7	3311	X
8	*HSPA8*	HSPA8	HSC70; HSC71; HSP71; HSP73	3312	15481
9	*HSPA9*	HSPA9	GRP75; HSPA9B; MOT; MOT2; PBP74; mot-2	3313	15526
10	*HSPA12A*	HSPA12A	FLJ13874; KIAA0417	259217	73442
11	*HSPA12B*	HSPA12B	RP23-32L15.1; 2700081N06Rik	116835	72630
12	*HSPA13*[b]	HSPA13	Stch	6782	110920
13	*HSPA14*	HSPA14	HSP70-4; HSP70L1; MGC131990	51182	50497
HSP H					
1	*HSPH1*	HSPH1	HSP105	10808	15505
2	*HSPH2*[b]	HSPH2	HSPA4; APG-2; HSP110	3308	15525
3	*HSPH3*[b]	HSPH3	HSPA4L; APG-1	22824	18415
4	*HSPH4*[b]	HSPH4	HYOU1/Grp170; ORP150; HSP12A	10525	12282

The HSP90/HSPC family

	Gene name	Protein name	Old names	Human gene ID	Mouse ortholog ID
1	*HSPC1*[a]	HSPC1	HSP90AA1; HSPN; LAP2; HSP86; HSPC1; HSPCA; HSP89; HSP90; HSP90A; HSP90N; HSPCAL1; HSPCAL4; FLJ31884	3320	15519
2	*HSPC2*[a]	HSPC2	HSP90AA2; HSPCA; HSPCAL3; HSP90ALPHA;	3324	X
3	*HSPC3*[a]	HSPC3	HSP90AB1; HSPC2; HSPCB; D6S182; HSP90B; FLJ26984; HSP90-BETA	3326	15516
4	*HSPC4*[a]	HSPC4	HSP90B1;ECGP; GP96; TRA1; GRP94; endoplasmin	7184	22027
5	*HSPC5*[a]	HSPC5	TRAP1; HSP75; HSP90L	10131	68015

The HSPB family (small heat shock proteins)

	Gene name	Protein name	Old names	Human gene ID	Mouse ortholog ID
1	*HSPB1*	HSPB1	CMT2F; HMN2B; HSP27; HSP28; HSP25; HS.76067; DKFZp586P1322	3315	15507
2	*HSPB2*	HSPB2	MKBP; HSP27; Hs.78846; LOH11CR1K; MGC133245	3316	69253
3	*HSPB3*	HSPB3	HSPL27	8988	56534
4	*HSPB4*[a]	HSPB4	crystallin alpha A; CRYAA, CRYA1	1409	12954
5	*HSPB5*[a]	HSPB5	crystallin alpha B, CRYAB; CRYA2	1410	12955
6	*HSPB6*	HSPB6	HSP20; FLJ32389	126393	243912
7	*HSPB7*	HSPB7	cvHSP; FLJ32733; DKFZp779D0968	27129	29818
8	*HSPB8*	HSPB8	H11; HMN2; CMT2L; DHMN2; E2IG1; HMN2A; HSP22	26353	80888
9	*HSPB9*	HSPB9	FLJ27437	94086	75482
10	*HSPB10*[a]	HSPB10	ODF1; ODF; RT7; ODF2; ODFP; SODF; ODF27; ODFPG; ODFPGA; ODFPGB; MGC129928; MGC129929	4956	18285
11	*HSPB11*	HSPB11	HSP16.2; C1orf41; PP25	51668	72938

7.3.Structure des principales HSPs :

7.3.1. HSP27 :

La famille des petites HSPs est composée de 10 membres partageant tous un domaine commun appelé α-crystallin et diffèrent par leurs région N-terminale et C-terminale (Tableau 1, Figure 14). Le membre le plus étudié des petites HSPs est HSP27. HSP27 une protéine chaperon de 27 kDa exprimée dans tous les tissus humains. Elle possède des sites de phosphorylation en N-terminal qui vont réguler sa forme tridimensionnelle : à l'état phosphorylé, HSP27 se trouve sous forme de dimère tandis que sous une forme non phosphorylée, HSP27 est capable de former des oligomères allant jusqu'à 800 kDa[287] (Figure 14). Ainsi, HSP27 agit tel un senseur cellulaire qui modifie son activité via des interactions moléculaires en fonction de l'environnement physiologique[287,288].

7.3.2. HSP90 :

La famille HSP90 est fortement conservée au cours de l'évolution. On distingue 5 sous-familles classées en fonction de leur localisation subcellulaire (Tableau 1)[286]. Ce sont également des chaperons moléculaires dépendants de l'ATP qui sont exprimés à des niveaux très élevés dans la cellule.

Les formes les plus étudiées sont HSP90α, dont l'expression dans le cytosol et le noyau est inductible, et HSP90β qui, elle, est constitutivement exprimée dans le cytoplasme. Tous les membres de la famille possèdent une structure conservée, divisée en 3 parties : un domaine C-terminal responsable de la dimérisation, un domaine médian qui constitue le PBD, et un domaine N-terminal contenant un site de liaison à l'ATP (ABD)[289,290] (Figure 14). HSP90 agit le plus souvent sous forme d'homodimère mais peut, dans certains cas, agir sous forme d'hétérodimère ou de monomère.

7.3.3. HSP110 :

La famille HSP110 est constituée de 4 membres, chacun étant encore mal connu[286]. La plus étudiée dans la littérature est HSP105. C'est aussi un chaperon moléculaire de 105 kDa qui existe sous 2 isoformes : HSP105α, constitutivement exprimée dans le cytoplasme et le noyau des cellules, et HSP105β, exprimée de façon inductible suite à un épissage alternatif de la forme α, dans le noyau exclusivement. Leur structure sont également très proches et sont constituées de 4 principaux domaines : un domaine N-terminal qui forme l'ABD, un domaine qui forme le PBD, un domaine « Loop » qui contient signal de localisation nucléaire (NLS, *Nuclear Localization Signal*) et un domaine C-terminal qui contient un signal d'export nucléaire (NES, *Nuclear Export Signal*)[291,292] (Figure 14).

7.3.4. HSP70 :

La famille HSP70 est la plus conservée au cours de l'évolution. C'est également la plus connue et la plus étudiée. Cette famille est constituée de 13 isoformes qui diffèrent par leur séquence en acide-aminés, leur niveau d'expression et leur localisation subcellulaire (Tableau 1)[286]. Certaines sont exprimés constitutionnellement comme HSC70, tandis que d'autres sont inductibles telle que HSP70-1 (HSPA1)[293].

Néanmoins, la structure des isoformes de HSP70 est très conservée. HSP70-1 (HSPA1) est le membre le mieux décrit dans la littérature. C'est un chaperon moléculaire de 70 kDa, dépendant de l'ATP[294]. Sa structure se divise en deux domaines principaux : un domaine N-terminal qui forme l'« *ATP Binding Domain* » (ABD), capable de fixer l'ATP et de l'hydrolyser, et un domaine C-terminal qui forme le « *Peptide Binding Domain* » (PBD) permettant d'interagir avec ses partenaires[295]. Ce dernier inclue notamment un motif EEVD, nécessaire à la liaison et au repliement du substrat, ainsi qu'à la fixation d'autres HSPs ou co-chaperons (Figure 14). Contrairement à HSP27, HSP70 est présent sous forme de

monomère dans la cellule, et peut être localisée dans la cellule, à la membrane, ou dans le milieu extracellulaire. Dans ce travail, nous nous intéresserons plus particulièrement à HSP70.

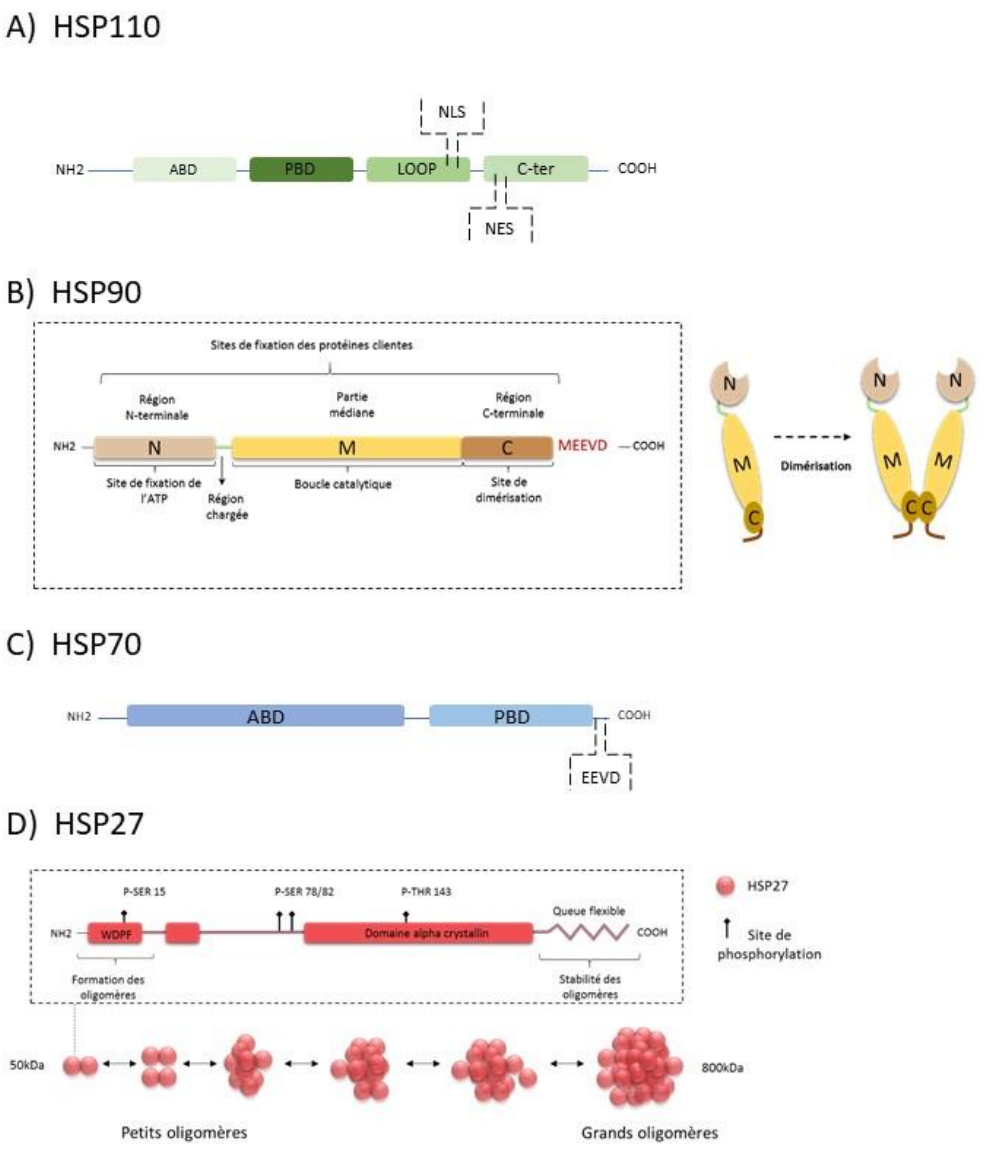

Figure 14 : Structure des principales HSPs. *A) Structure de HSP110 qui est composée de 4 domaines distincts. B) Structure d'un monomère de HSP90 : N représentant le domaine amino-terminal capable de lier l'ATP, M représentant le domaine médian, C représentant le domaine c-terminal responsable de la dimérisation de HSP90 et qui contient le motif MEEVD nécessaire à la fixation de co-chaperons. C) Structure de HSP70. D) Structure et organisation de HSP27 selon son statut de phosphorylation. Adaptée de la thèse de Margaux Sevin, Rôle des protéines de choc thermique dans les néoplasies myéloprolifératives : implication de HSP27 dans la myélofibrose, 2017.*

7.4.Fonctions de HSP70 :

7.4.1. Rôle de chaperon moléculaire :

Les protéines HSP70 sont des composants essentiels du réseau cellulaire des chaperons moléculaires. Elles participent à une large diversité de processus de repliement de protéines dans la cellule par leur association transitoire avec leur substrat. Les HSP70 participent à de nombreuses fonctions cellulaires en condition normales incluant le repliement des protéines néosynthétisées, la translocation des polypeptides dans les mitochondries et le réticulum endoplasmique, à l'assemblage et désassemblage de complexes protéiques, à la régulation de l'activité de certaines protéines ou encore en assistant le cycle d'autres HSPs ou co-chaperons (Figure 15)[296,297].

En réponse à un stress cellulaire, la cellule va rapidement synthétiser les formes inductibles de HSP70, notamment HSPA1, afin de permettre de maintenir l'homéostasie et la survie cellulaire. HSP70 va ainsi empêcher l'agrégation des protéines, solubiliser les agrégats protéiques, promouvoir le repliement des protéines incorrectement repliées ou non repliées ou encore favoriser la dégradation des protéines aberrantes ou des agrégats via des systèmes de dégradation tels que le protéasome ou l'autophagie médiée par les chaperonnes[294,296,297] (Figure 15).

Ces activités sont rendues possibles par l'intermédiaire de son cycle allostérique chaperon qui a lieu en trois étapes : (i) HSP40 prend en charge la protéine cliente dénaturée et viens la fixer à l'ABD de HSP70. A cette étape, l'ATP est déjà fixée à l'ABD de HSP70 et lui permet d'avoir une conformation dite « ouverte ». (ii) L'hydrolyse de l'ATP en ADP entraîne le transfert du substrat, en l'occurrence la protéine dénaturée, dans le PBD de HSP70 qui se referme. HSP40 quitte alors le complexe. (iii) Des facteurs d'échanges nucléotidiques (NEF) comme HSP110 viennent s'associer au complexe afin de stimuler l'échange de l'ADP par l'ATP, ce qui provoque le relargage de la protéine renaturée. Une fois le cycle terminé, si la protéine cliente relarguée est toujours défectueuse, elle est soit dirigée vers le protéasome ou l'autophagie médiée par les chaperonnes, soit dirigée vers HSP90 pour un repliement plus complexe[294,296–298] (Figure 16).

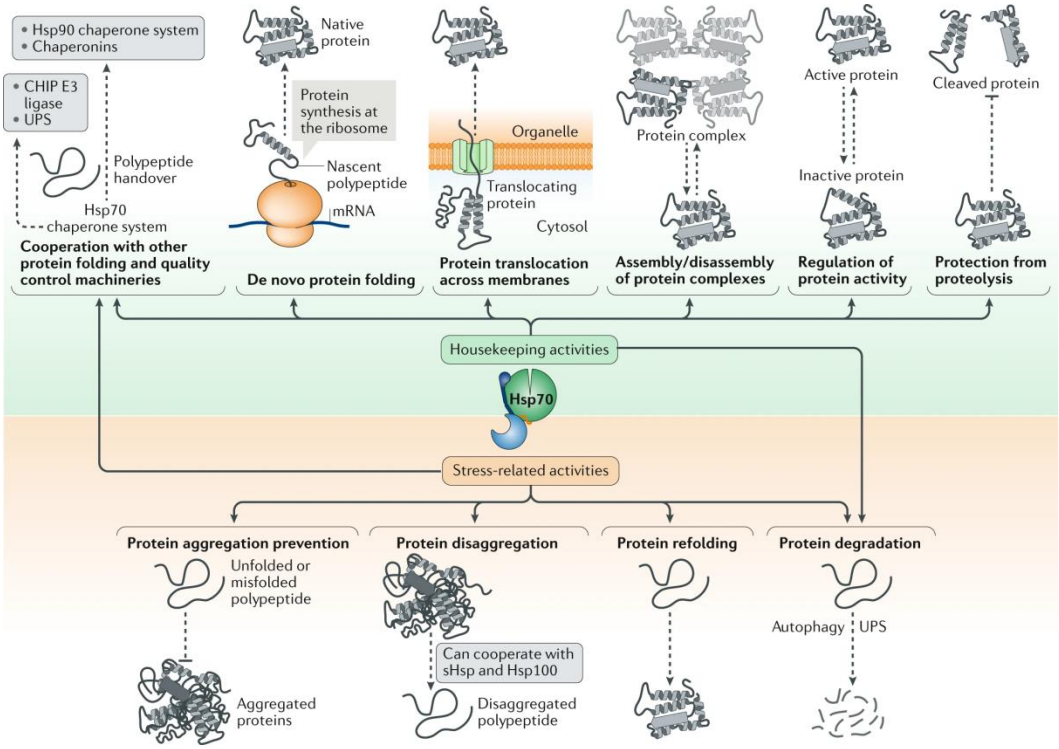

Figure 15 : Rôles de HSP70 dans la cellule*. HSP70 joue un rôle en conditions normales (housekeeping activities) et en condition de stress (stress-related activities). En conditions de stress, HSP70 est capable d'empêcher l'agrégation des protéines (protein agrégation prevention) ou de les désagréger (protein disagregation), de replier correctement les protéines (protein folding) ou de favoriser leur dégradation (protein degradation). D'après Rosenzweig et al, Nature Reviews Molecular Cell Biology, 2019.*

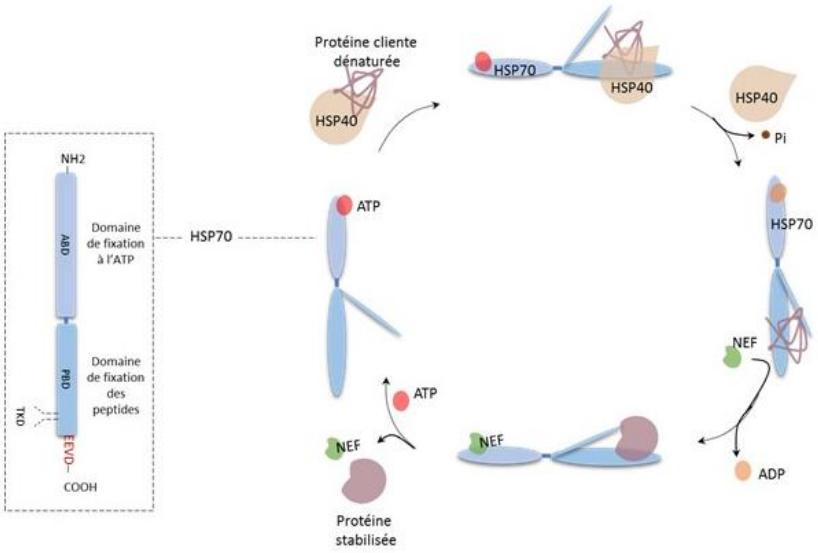

Figure 16 : Cycle allostérique chaperon de HSP70. *HSP40 vient fixer la protéine cliente dénaturée sur HSP70 déjà liée à l'ATP. L'hydrolyse de l'ATP entraîne un changement de conformation de HSP70 qui se « referme » sur son substrat pour le stabiliser. Des facteurs d'échanges nucléotidiques (NEF) viennent ensuite catalyser le remplacement de l'ADP par l'ATP menant ainsi au relargage du substrat. D'après la thèse Margaux Sevin, Rôle des protéines de choc thermique dans les néoplasies myéloprolifératives : implication de HSP27 dans la myélofibrose, 2017.*

7.4.2. Rôle anti-apoptotique :

HSP70 est une protéine hautement conservée dont l'expression augmente les capacités de la cellule à survivre en conditions de stress intense. Plusieurs études ont montré qu'une modification de l'expression de HSP70 jouait sur la mort cellulaire à différents niveaux[299–301].

Comme son nom l'indique, la voie extrinsèque de l'apoptose est déclenchée par un signal extérieur à la cellule. Cette voie est activée par liaison des ligands des récepteurs à domaines de mort tels que TNF (*Tumor Necrosis Factor*), FAS et TRAIL (*Tumor necosis factor-Related Apoptosis-Inducing Ligand*). Cette fixation entraine la formation à la membrane d'un complexe protéique appelé DISC (*Death-Inducing Signaling Complex*), qui est composé des molécules adaptatrices FADD (*Fas Associated Death Domain*) et de la procaspase 8, et déclenche ensuite la cascade des caspases. HSP70 est capable d'interagir avec différentes protéines de cette voie afin d'empêcher la cascade de signalisation aboutissant à l'apoptose. Ainsi, HSP70 se lie à JNK (*c-Jun N-terminal Kinase*) inhibant ainsi la mort cellulaire[302,303]. Ceci a également pour effet de bloquer l'activation de la protéine Bid[302]. Par ailleurs, HSP70 est également capable d'empêcher la formation du complexe DISC et la signalisation qui en découle[304]. Enfin, HSP70 chaperonne la protéine Akt, ce qui la stabilise, et promeut la voie de signalisation qui favorise la survie cellulaire[305] (Figure 17).

Contrairement à la voie extrinsèque, la voie intrinsèque de l'apoptose est déclenchée par des signaux internes à la cellule. En effet, elle repose principalement sur la rupture ou l'ouverture de la mitochondrie, ce qui permet la libération dans le cytosol de molécules pro-apoptotiques telles que le cytochrome c, SMAC/DIABLO, ou encore l'AIF (*Apoptosis Inducing Factor)*. Ces molécules vont ensuite chacune induire l'apoptose via différentes voies ; Le cytochrome c s'associe à APAF1 et la procaspase-9 pour former un complexe nommé apoptosome, qui est capable de déclencher la voie des caspases. SMAC/DIABLO inhibe les protéines de la famille des IAP (*Inhibitor of apoptosis*) et lève ainsi l'inhibition de l'activité des caspases tandis que AIF est capable d'induire à lui seul l'apoptose. Ici aussi, HSP70 est capable de prévenir la mort cellulaire via des interactions avec de nombreux membres de cette voie de signalisation. Par exemple, HSP70, couplé à HSP40, bloque la translocation de Bax, empêchant ainsi la perméabilisation de la membrane mitochondriale et donc la libération du cytochrome c et de l'AIF[299,306]. De plus, HSP70 est capable de se fixer directement à AIF et d'inhiber ainsi l'apoptose[307]. D'autre part, il a été démontré que HSP70 peut se lier à Apaf-1 et empêcher le recrutement de la procaspase-9 pour former l'apoptosome[308,309]. Enfin, HSP70 est capable d'inhiber des cibles de la caspase-3 telles que GATA-1 (*GATA binding protein 1*)[310], CAD (*Caspases-Activated DNase*)[311] et la protéine de réparation de l'ADN, PARP (*Poly ADP-ribose Polymerase*)[312] (Figure 17).

En agissant ainsi à différents niveaux de l'apoptose, HSP70 est un puissant agent cytoprotecteur qui protège efficacement les cellules de la mort cellulaire.

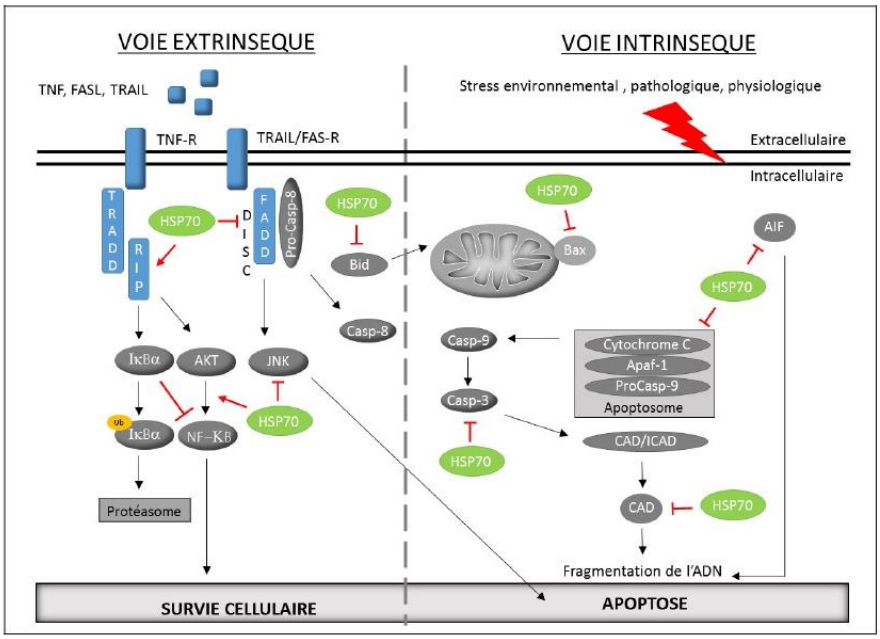

Figure 17 : Rôle anti-apoptotique de HSP70. *HSP70 agit sur de nombreux effecteurs de la voie apoptotique extrinsèque (à gauche) et intrinsèque (à droite) constituant ainsi une des protéines les plus importantes dans la survie cellulaire.*

8. HSP70 et cancer :

En conditions normales, HSP70 est faiblement exprimé. Cette expression peut augmenter fortement mais de façon transitoire en réponse à un stress. Au contraire, les cellules cancéreuses ont la propriété de surexprimer continuellement HSP70, qui est essentielle à la survie de nombreux cancers[299].

8.1.HSP70 : tumorigénicité et résistance des cellules cancéreuses :

Dans une grande majorité de tumeurs solides, HSP70 est surexprimé afin de promouvoir la survie des cellules cancéreuses, la tumorigénicité et la résistance à l'apoptose. Des expériences menées sur des modèles *in vivo* ont indiqué que la surexpression de HSP70 entrainait une augmentation de la capacité des cellules cancéreuses à former une tumeur[313], tandis que la diminution de son expression la réduisait fortement[314]. Ainsi, la forte expression de HSP70 a été corrélée avec une prolifération cellulaire élevée, le stade clinique et le mauvais pronostic de patients atteints de cancer du poumon non à petites cellules[315], de cancer du sein, de l'endomètre, de l'utérus[316], ou encore de leucémie[317], de cancer colorectal[318], de la prostate[319] ou du foie[320].

L'expression de HSP70 a également été associée à la réponse au traitement. Ainsi, il a été démontré que la surexpression de HSP70 dans la leucémie chronique myéloïde BCR-ABL était associée à la résistance à l'imatinib, une chimiothérapie efficace utilisée pour bloquer l'activité tyrosine kinase de ce récepteur[321,322]. De la même façon, Vargas-Roig et ses collègues ont observé que la surexpression de HSP70 était corrélée à la résistance à la chimiothérapie du cancer du sein[323]. Enfin, Une étude a montré que la résistance à la radiothérapie était liée à une augmentation de HSP70 dans des cellules humaines de glioblastome[324].

Outre ses rôles cytoprotecteurs dans le cytoplasme des cellules, HSP70 a été retrouvé dans le milieu extracellulaire, sous une forme libre (soluble), ou associée aux membranes, en particulier à la surface des exosomes[325,326]. Hightower and Guidon ont démontré que cette sécrétion était indépendante des mécanismes de mort cellulaire[327]. Il a été supposé que la surexpression de HSP70 dans les cellules cancéreuses entrainait une partie de ces protéines à être exposée à la surface (10 à 15%)[328], laissant seulement un petit fragment de 14 acides aminés exposé à l'extérieur de la cellule, que l'on appelle le « TKD »[329]. Cependant, les HSPs cytosoliques ne contiennent pas de peptide leader leur permettant d'être transportés à la membrane. Plusieurs mécanismes d'externalisation ont ainsi été suggérés tels que le chaperonnage de protéines transmembranaires ou encore via l'association avec la phosphatidyl-sérine[330,331]. Cependant ces mécanismes sont encore mal connus[330].

8.2.Rôle de HSP70 extracellulaire soluble :

Tandis que HSP70 intracellulaire est considéré comme un acteur majeur du maintien de l'homéostasie cellulaire, sa forme extracellulaire est décrite pour ses fonctions immunomodulatrices[332,333]. Il a été démontré que HSP70 est capable d'induire une réponse immunitaire anti-tumorale via le chaperonnage de peptides antigéniques. En effet, HSP70 a été décrit comme ayant un rôle important dans la cross-présentation de peptides antigéniques dérivés de tumeur qui sont internalisés par les cellules présentatrices d'antigènes (CPA)[334]. Cette captation par les CPA est suivi d'un « processing » de l'antigène, puis de sa présentation sur les molécules de CMH I, qui va induire une réponse T CD8 cytotoxique dirigée contre les cellules cancéreuses[332,333,335]. Les complexes peptide-HSP70 ont également été décrit comme améliorant l'activation des cellules T CD4 via la présentation sur le CMH II des CPA[336]. Ces données ont poussé les chercheurs à utiliser HSP70 en tant que vaccin dans de nombreuses études[337–342].

Cependant, HSP70 est aussi capable d'induire des réponses anti-inflammatoires qui vont favoriser la croissance tumorale[343]. Ainsi, Stocki et ses collègues ont révélé que HSP70 induisait un phénotype tolérogène dans les cellules dendritiques monocytaires, et contribue donc à la formation d'un microenvironnement immunosuppressif[344]. De la même manière, des chercheurs ont montré que

HSP70 augmentait l'activité des cellules T régulatrices et donc leur capacité à neutraliser le système immunitaire[345].

Plus récemment, Fong et ses collègues ont mené une série d'expériences qui ont abouti à la découverte de nouveaux récepteurs de HSP70 extracellulaire : les SIGLECs (*sialic acid-binding immunoglobulin-like lectin*)[346]. Les SIGLECs sont des immunoglobulines qui bloquent l'inflammation via leur interaction avec les TLRs. Ainsi, les chercheurs ont montré qu'une liaison de HSP70 avec les différents SIGLECs induisait des réponses immunitaires opposées, ce qui pourrait expliquer les effets contradictoires décrits dans la littérature[346,347].

Ainsi, de nombreuses études se sont intéressé à la présence de HSP70 soluble dans le sang des patients atteints de cancer[348]. Plusieurs publications relatent d'une corrélation du taux de HSP70 circulant avec la présence d'un cancer du foie ou du pancréas[349,350], ou avec le volume tumoral du cancer du poumon[348,351]. Enfin, l'équipe de Gabriele Multhoff a également lié la quantité de HSP70 circulant avec la réponse à la radiothérapie[349,349,352].

8.3. Rôle de HSP70 extracellulaire lié aux membranes :

En 1995, Multhoff et ses collègues ont démontré que les cellules tumorales humaines différaient des cellules normale de par leur capacité à exprimer HSP70 à leur surface[353]. En effet, une large proportion de cellules cancéreuses possède une forme membranaire de HSP70, dont seuls 14 acide aminés sont exposés au niveau extracellulaire ; ce domaine est appelé le « TKD »[329]. Cette découverte a mené à l'étude du rôle de HSP70 membranaire dans le cancer. Ainsi, Multhoff et son équipe ont montré que HSP70 membranaire est capable d'activer les cellules NK et d'induire une lyse tumorale[354,355]. Par la suite, l'équipe a montré que les cellules NK pré-stimulées avec HSP70 ou un dérivé peptidique en présence d'IL-2 ou d'autres cytokines induisait une réponse anti-tumorale conséquente, allant jusqu'à une étude clinique de phase II dans le cancer du poumon non à petites cellules[356–359].

L'équipe de Multhoff a ainsi réalisé une étude qui a permis de révéler qu'environ 50% des tumeurs présentent HSP70 à la membrane[360]. De plus, la densité membranaire de HSP70 sur les cellules cancéreuses est sélectivement augmentée en réponse à un traitement tel que la chimiothérapie ou la radiothérapie[361]. Elle est également augmentée considérablement dans les métastases[362], ce qui suggère un rôle de HSP70 membranaire dans leur formation. Ainsi, l'expression de HSP70 membranaire a été associée avec un pronostic défavorable et une survie globale diminuée chez les patients atteints d'un cancer rectal et du poumon[363]. Sur la base des résultats obtenus, la présence de HSP70 à la membrane peut donc être considérée comme un marqueur spécifique des tumeurs agressives.

Mais plusieurs études indiquent également que HSP70 est présent sous une forme membranaire dans la circulation, soit à la membrane des CTCs[364], soit des vésicules extracellulaires et notamment des exosomes[215,357,365,366]. Différentes recherches ont indiqué un rôle immunomodulateur des exosomes présentant HSP70 à la membrane. Ainsi, les « HSP70-exosomes » exercent des fonctions immunostimulantes via l'activation des cellules NK[357,366], la maturation des cellules dendritiques[365], et la surexpression du CMHII sur les CPA[367], ce qui aboutit à l'amélioration de la réponse anti-tumorale (Figure 18).

Cependant, d'autres études dont plusieurs de notre équipe, ont également démontré un rôle immunosuppresseur des HSP70-exosomes via l'activation des MDSCs[215,368,369]. Plus précisément, notre équipe a révélé que le domaine extracellulaire de HSP70 membranaire (TKD) est capable de se lier au récepteur TLR-2 à la surface des MDSCs, et d'activer la voie de signalisation NF-kB. Cette signalisation déclenche l'expression de la cytokine inflammatoire IL-6, qui se lie à son récepteur, l'IL-6R, de manière autocrine et conduit à la phosphorylation de STAT3 par la voie JAK2[215,368]. Une fois activées, les MDSCs vont inhiber l'activité d'autres effecteurs du système immunitaire et promouvoir la progression tumorale (Figure 18).

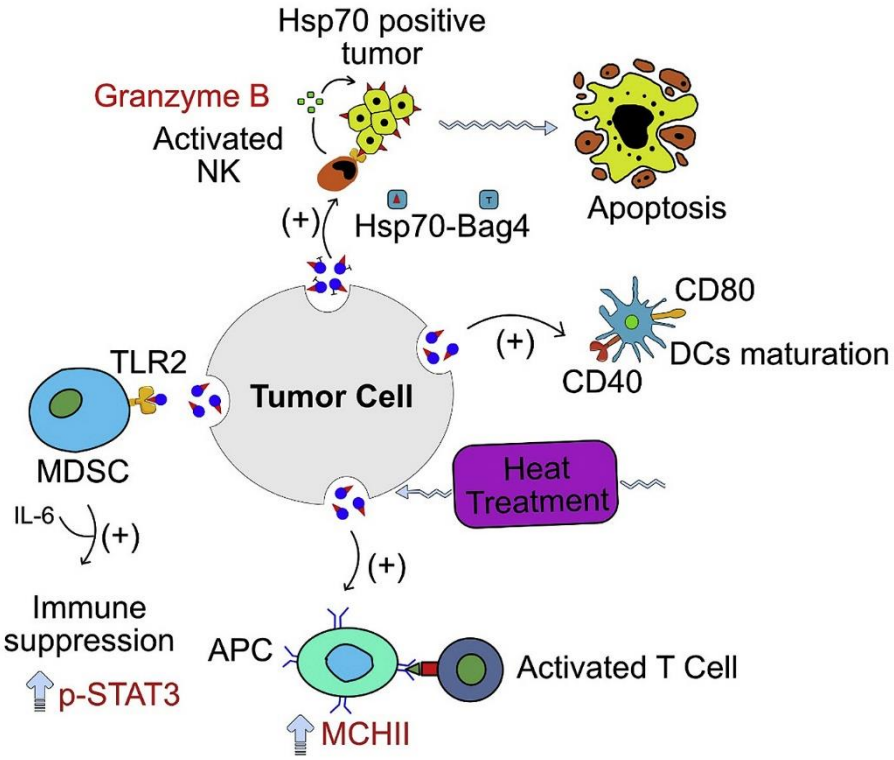

Figure 18 : Rôle des HSP70-exosomes sur le système immunitaire. *La présence de HSP70 à la membrane des exosomes dérivés de tumeur peut engendrer une réponse immunitaire anti-cancéreuse via l'activation des cellules NK, la cytotoxicité des cellule T médiée via la maturation des cellules dendritiques, et la surexpression du CMHII à la surface des CPA. Les HSP70-exosomes peuvent également favoriser la croissance tumorale via l'activation des MDSCs. D'après Elmallah et al, Cancer letters, 2020.*

PROJET DE RECHERCHE

Les exosomes concentrent énormément d'espoir dans le domaine de la biopsie liquide. Ces nanovésicules sont sécrétées par toutes les cellules et sont présentes dans tous les fluides biologiques, ce qui les rends très attractif en vue d'un prélèvement peu voire pas invasif, avec le potentiel de pouvoir suivre la maladie. La voie de formation des exosomes entraine le chargement spécifique de molécules qui peut permettre une analyse multiparamétrique, dans l'optique d'une utilisation en clinique. Néanmoins, l'hétérogénéité des exosomes aussi bien au niveau de leur contenu que de leur fonction, est un frein à son utilisation en clinique. Un des défis actuels du domaine des VEs réside dans la possibilité d'identifier et d'analyser une sous-population particulière d'exosomes.

Notre équipe travaille depuis de nombreuses années sur les HSPs et notamment HSP70 qui a un rôle majeur dans le cancer. HSP70, une des HSPs les étudiées, est un chaperon moléculaire aux rôles divers dans le cancer. Son expression, notamment à la membrane des cellules cancéreuses, a été corrélée avec le diagnostic, le pronostic et/ou la réponse au traitement[329–331]. Notre équipe a démontré que les exosomes possédant HSP70 à la membrane étaient capables d'activer les MDSCs via son interaction avec le TLR2, et ainsi d'inhiber la réponse immunitaire antitumorale[215,368]. Nous les avons appelés les « HSP70-exosomes ». Par la suite, notre équipe a mis au point une technologie basée sur l'interférométrie optique capable de capturer les HSP70-exosomes grâce à un aptamère peptidique qui cible spécifiquement la partie extracellulaire de HSP70. Ceci a permis de révéler qu'une large partie des exosomes dérivés de cellules cancéreuses présentaient HSP70 à la membrane, comparé aux cellules normales, suggérant que ce phénomène est universel dans le cancer (Figure 19)[368].

Mon projet de thèse s'inscrit dans le cadre d'une étude clinique pilote appelée ExoDiag, qui vise à déterminer si les HSP70-exosomes dérivés de tumeurs peuvent être utilisés dans le diagnostic et le suivi du cancer. Pour cela, nous avons isolé et analysé les exosomes dérivés de plasma de 20 patients atteints d'un cancer du sein et 20 atteints d'un cancer du poumon non à petites cellules, deux des cancers dont l'incidence est la plus importante en France et dans le monde. Nous les avons également suivis tout au long de leur ligne de traitement, pendant 1 an.

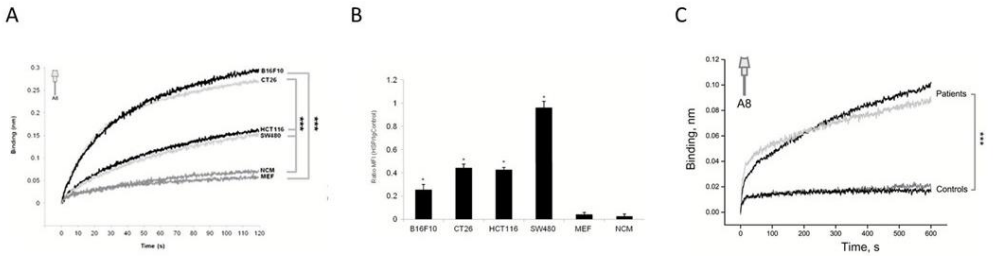

Figure 19 : Les HSP70-exosomes sont universel au cancer*. A. Liaison des exosomes dérivés de MEF, NCM, B16F10, CT26, HCT116 et SW480 immobilisés sur un biocapteur fonctionnalisé avec l'aptamère peptidique A8, déterminé par BLI. B. Analyse de l'expression de HSP70 à la membrane des exosomes dérivés de surnageant cellulaire, déterminé par cytométrie en flux. C. Courbes d'association des HSP70-exosomes circulants de patients atteints d'un cancer du sein (n=3) et du poumon (n=3), ou de contrôles (n=3). Mann-Whitney, p<0,001. D'après Gobbo et al, JNCI, 2016.*

RESULTATS

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Monitoring HSP70 exosomes in cancer patients' follow up: a clinical prospective pilot study

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Monitoring HSP70 exosomes in cancer patients' follow up: a clinical prospective pilot study

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ABSTRACT

Exosomes are nanovesicles released by all cells that can be found in the blood. A key point for their use as potential biomarkers in cancer is to differentiate tumour-derived exosomes from other circulating nanovesicles. Heat shock protein-70 (HSP70) has been shown to be abundantly expressed by cancer cells and to be associated with bad prognosis. We previously showed that exosomes derived from cancer cells carried HSP70 in the membrane while those from non-cancerous cells did not. In this work, we opened a prospective clinical pilot study including breast and lung cancer patients to determine whether it was possible to detect and quantify HSP70 exosomes in the blood of patients with solid cancers. We found that circulating exosomal HSP70 levels, but not soluble HSP70, reflected HSP70 content within the tumour biopsies. Circulating HSP70 exosomes increased in metastatic patients compared to non-metastatic patients or healthy volunteers. Further, we demonstrated that HSP70-exosome levels correlated with the disease status and, when compared with circulating tumour cells, were more sensitive tumour dissemination predictors. Finally, our case studies indicated that HSP70-exosome levels inversely correlated with response to the therapy and that, therefore, monitoring changes in circulating exosomal HSP70 might be useful to predict tumour response and clinical outcome.

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Introduction

A key issue to improve cancer patients' outcome relies on earlier cancer diagnosis. Metastases, as opposed to primary tumours, are responsible for most cancer deaths and patients' prognosis is closely linked to early cancer management. Today, cancer patient follow-up care relies mainly on imagery techniques that unfortunately are not sensitive enough to detect metastasis at an early stage. Thus, it is essential to develop new strategies for the early detection of recurrent or metastatic disease.

Although different circulating tumour markers have been characterised, only a few have demonstrated to be clinically useful for monitoring response to therapy and detecting early relapse. Therefore, there is an urgent need for development of biomarkers for the prediction of the effectiveness of the treatment or the chance of cancer recurrence. A major advance for metastasis diagnosis and monitoring has been the detection of circulating tumour cells (CTCs) in the blood. Current detection of CTCs from circulating blood is based on the epithelial cell adhesion molecule (EpCAM), frequently over-expressed in many cancers and seems to allow earlier detection of recurrence [1]. However, this approach suffers from important limitations, the main being that CTCs are rare events: only one single circulating tumour cell in a background of as many as 10^9 blood cells [2].

Exosomes constitute a heterogeneous population of small extracellular vesicles from 50 to 150 nm, present in all body fluids and involved in cell-cell communication [3]. Indeed, exosomes can be bioactive cargos of

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Supplemental data for this article can be accessed here.

proteins, lipids and nucleotides that will be introduced into the receptor cells and thereby modify their physiology [4]. A broad range of cells secrete exosomes, including tumour cells, which have specific biological functions as they are able to promote cancer growth, metastasis formation and modulation of the immune system [5–8]. These properties have promoted exosomes as new potential biomarkers for the diagnosis and monitoring of cancer. However, since most cells can secrete exosomes that will be found circulating in the blood, a key issue is to differentiate those coming from cancer cells.

We and others have recently shown that whereas exosomes released by tumour cells express in their membrane the stress protein HSP70 (heat shock protein-70), exosomes released by normal cells do not [7,9–11]. We have called this sub-population of tumour-derived nanovesicles "HSP70-exosomes" [11]. Among the different HSPs, HSP70 is the most strongly and widely induced by different stresses. HSP70 is over-expressed in many cancer types, about 70% of solid tumours, and HSP70 expression level has been shown to be useful for the diagnosis, monitoring and response to treatment [12]. Taking into consideration these results and knowing that compelling recent literature supports that exosome-based diagnostics provide higher sensitivity and specificity over conventional biopsy or liquid biomarkers due to their stability in biofluids, we hypothesise that HSP70 exosomes might be used as biomarkers in the monitoring of cancer. To this end, we opened a prospective clinical pilot study called ExoDiag that aimed at quantifying HSP70 exosomes in the blood of cancer patients for the monitoring of malignant solid tumours.

Patients and methods

Study population and specimen collection

40 adult patients with either non-metastatic or metastatic solid tumours were included in the study (NCT02662621). Briefly, 20 women with breast cancer, 10 men and 10 women with non-small cell lung cancer were included (Table 1). Eligibility criteria for cancer patients are listed in Table 1. Additionally, 14 healthy volunteers with no previous cancer history, negative serology for HIV, HCV and HBC and aged 50–70 years old were also included. Age group of healthy volunteers is in agreement with the average age of occurrence of the different solid tumours studied. Upon patient written consent, serology to HIV, HCV and HBV was tested. Only patients with negative serology for these infectious agents were included. During the study, 10 ml of blood, collected in an EDTA tube were necessary for each exosomal HSP70 analysis and additional 10 ml of blood, collected with preservative cell save (Cell Search®) were required for each CTCs analysis. *Initial sampling*. Blood were collected prior to any treatment (radiotherapy, hormone therapy, surgery or chemotherapy). *Follow-up*. Sample collection schedule were dependent from the standard treatment line. (i) Follow-up D1 C (x)-Day1CureX: upon a surgical treatment, initial follow-up blood samples for HSP70 analysis were collected during the first visit after surgery; (ii) upon hormone therapy, blood samples were collected at each follow-up oncology visit, in average every 6 months; (iii) upon chemotherapy samples

Table 1. Patient's characteristics.

	Patient
	N = 40
Age	
N	40
Mean (Std)	62.5 (10.2)
Median [min–max]	57.0 [36.0–83.0]
Sex	
Women	26 (65.0%)
Men	14 (35.0%)
OMS	
0	18 (45.0%)
1	17 (42.5%)
2	2 (5.0%)
3	2 (5.0%)
Missing	1 (2.5%)
Localisation	
Breast	17 (42.5%)
Lung	20 (50.0%)
Missing	3 (7.5%)
Breast hystologic type	
Lobular	4 (23.5%)
Ductal	13 (76.5%)
Lung histologic type	
Adenocarcinoma	16 (80.0%)
Squamous cell carcinoma	4 (20%)
Stagde M	
M0	20 (50.0%)
M1	14 (35.0%)
Mx	2 (5.0%)
Missing	4 (1.0%)
	Controls
	N = 14
Age	
N	14
Mean (Std)	55.0 (5.7)
Median [min–max]	53.0 [50.0–69.0]
Sex	
Women	7 (50.0%)
Men	7 (50.0%)
OMS	
0	14 (100.0%)

were collected every two cures. *Disease progression*. If disease progression was observed, blood was collected and the study was stopped.

Isolation, characterisation and quantification of exosomes

Exosomes were isolated from fresh plasma samples using an optimised protocol derived from Théry et al [13]. Briefly, plasma samples were differentially centrifuged 300 × *g* for 5 min at 4°C and then 17,000 × *g* for 10 min at 4°C. Next, supernatant obtained from the previous step were ultracentrifuged at 2,00,000 × *g* for 1 h at 4°C (Beckman Coulter, Optima XPN-100, Brea, California, the USA). The supernatants were carefully removed and the exosome pellets re-suspended in 100 µl of 1% RIPA lysis buffer or PBS and frozen at −80°C until further use.

Exosome presence was verified by transmission electron microscopy (TEM). The samples dissolved in PBS buffer were dropped into a carbon-coated copper grid and then were stained with 3% uranyl acetate. Images of the sample were captured using a Hitachi 7500 electron microscope (Hitachi high technologies, Tokyo, Japan).

Exosomes were evaluated for their size and concentration by nanoparticle tracking analysis (NTA) using a NS300 Instrument (Malvern Instruments, Malvern, the UK). Briefly, exosome preparations were homogenised by vortexing followed by dilution of 1:500 in filtered phosphate saline buffer and analysed by NanoSight NS300. Each sample analysis was conducted for 60 s. Data were analysed by Nanosight NTA 3.2 Analytical Software (Malvern Instruments, Malvern, the UK) with the detection threshold optimised for each sample and screen gain at 10 to track as many particles as possible with minimal background. A blank 0.1 µm-filtered 1× PBS was also run as a negative control. At least three analyses were done for each individual sample.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumour tissue samples were also obtained for correlation analysis of HSP70 (ADI-SPA-810, Enzo Life science) expression between tissue and circulating exosomes. All IHC procedures were performed using a Benchmark apparatus (Ventana).

Statistical analysis

Statistical analysis was performed using the Graphpad Prism 8 software. Data presented are from at least three independent experiments. Error bars shown in graphical data represent mean ± SEM. When data are presented as box plots, the bar indicates the median, the box shows the interquartile range (25–75%) and the whiskers extend to 1.5 the interquartile range. For normally distributed data, significance of mean differences was determined using two-tailed paired or unpaired student t-test or ANOVA. For data that were not normally distributed, non-parametric Wilcoxon tests were used for paired analysis. A Firth logistic regression was used to determine the association between HSP70-exosomes concentration and the presence of metastasis. Odds ratio was given with its 95% confidence interval. For information, the best cut-off maximising both sensitivity and specificity was determined using ROC curves and the Youden index. Tests were two sided and a p value less than 0.05 was considered significant. All analyses were performed using SAS version 9.4.

Results and discussion

The ExoDiag prospective clinical study includes 40 cancer patients suffering from a lung cancer (n = 20) or a breast cancer (n = 20). Inclusion of patients started in 2016 and ended in March 2019. Patients were followed for 1 year (Table 1). Three patients with breast cancer had to be removed from the study because no follow up was possible (pre-mature death). Samples were taken at diagnosis, after surgery and before and after each cure. In addition, baseline assays of the patients were compared with 14 healthy volunteers (with bilateral alpha of 5% and 40 patients, we will have a power of more than 95% to compare exosome HSP70 concentrations between cases and controls).

Exosomes were isolated from plasma samples and characterised by the nanovesicle size (50–150 nm), by the expression of the exosomal markers such as TSG101, CD9, CD63, by the absence of the endoplasmic reticulum marker Grp94, and by their typical cup-shaped appearance as observed by TEM (Supplementary Figure 1). When comparing HSP70 in lysates of circulating exosomes versus soluble in the plasma by ELISA, we found that whereas HSP70 within exosomes was detected in all patients, soluble HSP70 level was in most patients undetectable (Figure 1(a)). This could be explained by the fact that HSP70 is stabilised by the cholesterol-rich membrane of the exosome [2]. We used an already validated biolayer interferometry (BLI) protocol using a peptide aptamer that binds to the extracellular part of membrane-bound HSP70, to capture exosomes expressing HSP70 in their membrane (HSP70-exosomes) from total blood exosomes [11,14]. We found that the number of HSP70-exosomes in the plasma samples from patients was significant higher than in those from healthy volunteers, in which the level of HSP70 exosomes was barely detectable (Figure 1), thus confirming our previous reports [11].

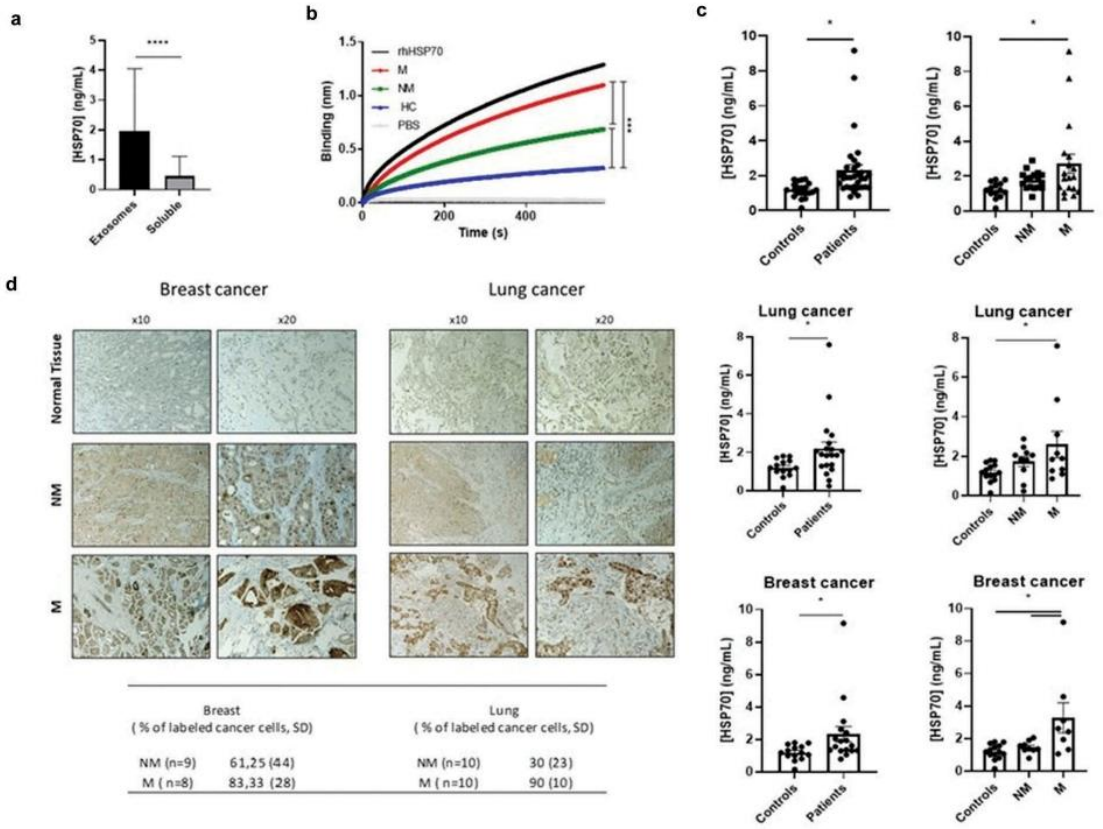

Figure 1. Exosomal HSP70 as a potential cancer biomarker. (a) Expression of circulating HSP70 within exosomes or soluble in paired samples at different time points (n = 68), Wilcoxon's test, $p < 0.0001$. (b) Capture of tumour-derived exosomes by BLI using the peptide aptamer A8 from metastatic (M, n = 18), non-metastatic patients (NM, n = 19) and healthy volunteers (HC, n = 14), one way ANOVA, $p < 0.0001$. Recombinant human HSP70 (rhHSP70) and PBS are used as controls. (c) Expression of HSP70 within HSP70 exosomes either in all cancer patients (n = 37) or in lung cancer (n = 20) or breast cancer patients (n = 17) and healthy volunteers (n = 14), student t-test and ANOVA, $p < 0.05$, mean ± SEM. (d) Immunohistochemical HSP70 staining within tumour biopsy slides. A semi-quantitative evaluation was performed by counting positive cells in 100 cells, from three randomly selected fields. A representative immunohistochemistry picture of HSP70 expression is shown. NM = Non-metastatic, M = Metastatic.

This result is all the more interesting given that in our cohort the total number of exosomes did not differ between patients and healthy volunteers (Supplementary Figure 2). HSP70-exosomes levels were increased in metastatic patients compared to non-metastatic patients and healthy volunteers, as quantified by BLI (Figure 1(b)) and ELISA (Figure 1(c,d)). Therefore, as happens in cells where the amount of HSP70 in the membrane is in general proportional to the intracellular expression of HSP70 [15], exosomes expressing HSP70 in the membrane also contained high amounts of this stress protein inside the vesicle.

As in plasma samples, the number of total exosomes in urine samples was measured by NTA and that of HSP70 exosomes by BLI (Supplementary Figure 3). In contrast to plasma samples, no differences were found in the number of HSP70 exosomes between metastatic and non-metastatic patients (Supplementary Figure 3). It should be mentioned that the cellular origin of the exosomes found in the blood and in the urines must be different.

Interestingly, when analysing HSP70 in tumour biopsies, we found that blood HSP70 exosomes matched HSP70 expression within the tumour (Figure 1(d)). This result suggests that circulating exosomes reproduce features within the tumour, particularly HSP70 status.

Next, we compared circulating HSP70 exosomes with CTCs quantified by Cell-Search (a current FDA-approved approach), as predictors of tumour progression. We performed receiver operating characteristic (ROC) curve analysis to discriminate metastatic from non-metastatic patients. As shown in Figure 2, HSP70 exosomes showed a better discrimination since the area under the curve (AUC) was higher when quantifying HSP70 exosomes in the blood than CTCs (AUC =

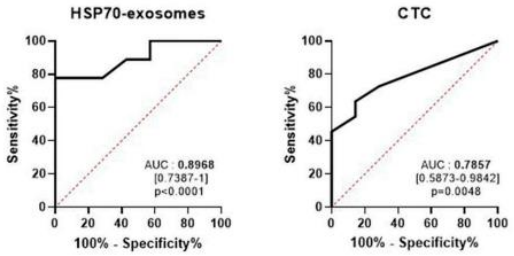

Figure 2. HSP70 exosomes compared to CTCs to discriminate metastatic from non-metastatic patients. ROC curve analysis for the detection level of HSP70 exosomes (left panel) or circulating tumour cells (CTCs, right panel), AUC = 0.8968, CI95% = [0.7387–1], $p < 0.0001$ compared to 0.7857, CI95% = [0.5873–0.9842], $p = 0.0048$.

0.8968, CI95% = [0.7387–1], $p < 0.0001$ for HSP70 exosomes compared with 0.7857, CI95% = [0.5873–0.9842], $p = 0.0048$, for CTCs). Based on curve ROC analysis, we calculated a preliminary HSP70-exosome optimal cut off of 1.92 ng/ml ($p = 0.0288$, 95% Wald confidence limits < 0.001, 0.674). Due to the size of this pilot cohort ($n = 40$), the reliability of this HSP70-exosome cut-off value needs confirmation in larger cohorts. That HSP70 exosomes may be more reliable predictors of cancer dissemination than CTCs is not surprising since CTCs are rare events, less than 10 cells/ml of blood, whereas exosomes are found in large amounts in body fluids. To our knowledge, this is the first study comparing in a clinical cohort tumour-derived exosome with CTCs as biomarkers.

To determine whether HSP70 exosomes could be used as predictors of response to the therapy, we compared HSP70-exosome levels at diagnosis and after treatment, at the same time as the first tumour evaluation by imagery. We found that patients in clinical response had a mean decrease of 77%, whereas patients in clinical progression had a mean increase of 92% (Figure 3(a)). For longer-term follow up, we demonstrated that variation in the level of HSP70 exosomes was associated with the disease status, as determined by scanner imagery. For instance, the level of exosomal HSP70 for patient 1, diagnosed with a metastatic HER2+ breast cancer, T4N1M1, varied according to treatments until the stabilisation after the 12th cure. Between the diagnosis and the second cure, the concentration of HSP70 decreased from 2.62 to 0.835 ng/ml corresponding to the start of the treatment (trastuzumab, pertuzumab and paclitaxel). At the fourth cure, we observed an increase from 0.835 to 3.931 ng/ml corresponding to the replacement of paclitaxel (taxol) by docetaxel (taxotere), due to excessive toxicity. At the sixth cure, we detected a decrease to 1.529 ng/ml that kept stable until the eighth cure. At the 10th cure, the level of HSP70 increased up to 2.874 ng/ml corresponding to a decrease in the dosage of taxotere. Finally, at the 12th cure, the concentration of HSP70 decreased to 0.938 ng/ml and then stabilised until the end of follow up. This stabilisation correlated with clinical stable disease, diagnosed by scanner imagery (Figure 3(b)). Interestingly, when compared HSP70 exosomes with that of the CA-15-3 breast cancer clinical marker, we observed that both reached a sustained low level when the disease stabilised.

Patient 2 was diagnosed with a metastatic NSCLC, T3N2M1. The levels of HSP70 and CEA (a gold standard lung cancer marker) followed the same variations during the different treatments until the disease clinical

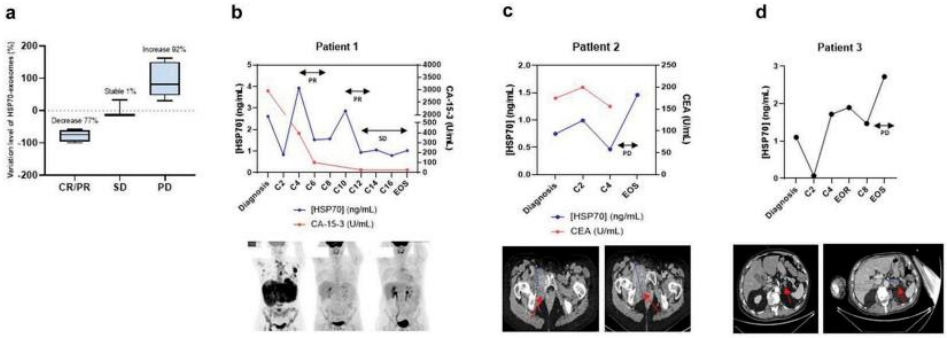

Figure 3. Exosome HSP70 levels correlates with the response to treatment. (a) Exosomal HSP70 variation ratio between diagnosis and the first tumour evaluation according to the clinical status. CR/PR = Complete/Partial response ($n = 6$), SD = Stable disease ($n = 3$), PD = Progression disease ($n = 11$), $p = 0.0043$. (b)–(d): Case study of the correlation between HSP70 levels in exosomes (blue lines) and their response to the different cures (C2, C4, etc.). CA-15-3 and CEA clinical tumour marker measurements, when available, are indicated by a red line. EOR = End of radiotherapy, EOS = End of study, SD = Stable Diseased PD = Progression disease, PR = Partial response. Panels below show the respective scan imagery. Metastasis is visualised by black spots (patient 1) and red arrows (patients 2 and 3).

progression. Indeed, this patient had brain metastases as revealed by scanner imagery. Interestingly, this progression correlated with an important increase in exosomal HSP70 levels (from 0.466 to 1.461 ng/ml) (Figure 3(c)).

Patient 3 was also diagnosed with a metastatic NSCLC, T3N2M1. Exosomal HSP70 level varied depending on the treatment, especially between second and fourth cures where the concentration of HSP70 increased from 0.071 to 1.719 ng/ml, which corresponded to a change in platinum salts due to bad tolerance. Then, after eighth cure, exosomal HSP70 level progressively increased from 1.468 to 2.72 ng/ml, increase that associated with ganglionic cerebral and peritoneal cancer progression, as observed by imagery (Figure 3(d)). Altogether, these results are consistent with data in the literature, since several studies have reported that the expression of HSP70 within the tumour biopsies correlated with tumour volume [16,17]. We hypothesise that the treatments which lead to a reduction in the size of the tumour, consequently lead also to a decrease in the amount of HSP70 exosomes released. Conversely, non-responder patients might have a tumour growth that translates to a higher release of HSP70 exosomes. Although these case reports suggest a correlation between HSP70 exosomes and conventional tumour markers, the curve of HSP70 exosomes have several peaks compared to that of conventional tumoural markers (Figure 3(b,c)). Further studies are needed to determine whether these peaks reflect a higher sensitivity to detect changes in the physiological state of the tumour [18].

High expression of HSPs, particularly HSP70 and HSP90, has been shown to correlate with clinical parameters such as diagnosis, prognosis and/or response to therapy in a wide variety of cancers, both at the intracellular [15,19–25] and extracellular levels [2,11,16,24,26–30]. Our findings here provide for the first time in a prospective cohort an overview of HSP70 expression (circulating, both in exosomes and soluble, and within the tumour) and indicate that exosomal HSP70 hold promises as a potential predictor of tumour growth/spread for the monitoring of cancer patients bearing HSP70-expressing tumours. Further, towards a more personalised patient care, our follow-up studies point out the interest of measuring for each patient the difference between exosomal HSP70 level at baseline and the value after a cure. We intend to perform a multi-centre study that will include larger homogeneous cohorts to refine cut-off values and to confirm whether monitoring variations in circulating exosomal HSP70 levels could be used to detect responders to the therapy.

From a functional point of view, we previously showed that HSP70-exosomes restrain tumour immune surveillance by promoting myeloid-derived suppressor cells' (MDSCs) functions [7,11]. Thus, the abundant HSP70 exosomes found in metastatic patients may lead to a pro-tumoural environment. Since HSP70-targeting therapies are being developed in cancer, quantifying HSP70 exosomes from a simple blood sample might be a strategy to select the patients likely to benefit from these innovative therapies [11,14,31,32].

The main advantage of the HSP70 exosomes is that these tumour-specific nanovesicles are found in large amounts in the peripheral blood, providing a minimally invasive method for serial assessment of predictive and prognostic markers during multi-stage cancer progression. Further progress is needed to bring the exosomes to clinical practice, especially concerning the isolation technique. We are currently pursuing this issue through the development of a nanovesicle isolation technic based on a microfluidic system-on-chip approach.

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Competing interest

The authors declare that they have no competing interests.

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Supplementary material

Materials and methods

Western blotting

Isolated exosomes were tested for the expression of exosomes markers such as TSG101. Nanovesicles were lysed with Laemmli buffer and separated on SDS/PAGE gels. Then, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham GE Healthcare Life Sciences) for western-blotting analysis. After transferring, the membranes were blocked with 5% bovine serum albumin for 1 hour and incubated overnight at 4°C with antibodies specific for HSP72 (ADI-SPA-810-F, EnzoLife), TSG101 (sc-7964, Santacruz), CD9 (ab92726, Abcam), CD63 (NBP2-4225, BioTechne), GRP94 (ab2791, Abcam), β-actin (A3854, Sigma Aldrich). Then, the horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch) was added for 1 h 30 at room temperature (RT). The immunoreactive proteins were revealed using ECL detection reagents (34095, ThermoFisher Scientific). Band intensities on membrane were captured using Chemidoc XRS+ system and image were analyzed using Image Lab software (Bio-Rad Laboratories).

Dosage of HSP70

The dosage of HSP70 has been performed by HSP70 High Sensitivity ELISA kit (Enzo Life science) according to the manufacturer protocol.

CTC detection

Whole blood (7.5mL) was collected in CellSave® preservative tubes. Within 96 h, CTCs were enriched using the CellSearch® CTC Kit (Silicon Biosystems, Menarini), with the automated Celltracks® Autoprep® system according to manufacturer's instructions. Isolated cells were collected in a cartridge and scanned with the CellTracks analyzer II® System. CTCs were blinded counted by experienced investigators. EpCAM- and Cytokeratin-positive and CD45 (common leukocyte antigen)-negative cells are identified as CTCs. We used the established cutoff of ≥ 5 CTCs to define CTC-positive patients.

Tumor response evaluation

Tumor response was determined as best response-based response evaluation criteria in solid tumors (RECIST) version 1.0 measurable disease.

Biolayer Interferometry

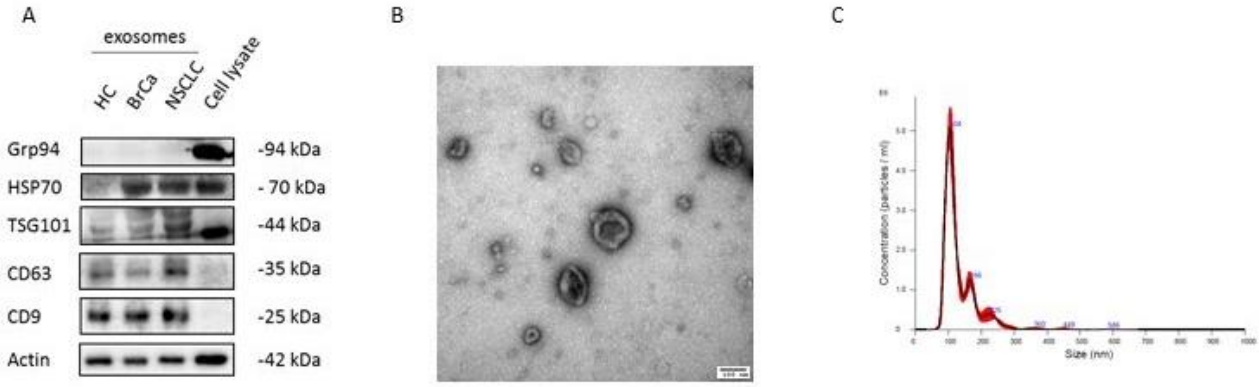
Capture of tumor-derived exosomes have been performed as previously described[9]. HSP70 exosomes capture experiments were conducted with an Octet Red instrument (FortéBio, Menlo Park, CA). The ligand (A8 aptamer) was biotinylated using EZ-Link NHS-PEG4-biotin (2 nM, 30 min, RT, Thermo Fisher Scientific, Germany) and immobilized on streptavidin sensors (black 96-well plate, FortéBio). Functionalized sensors were incubated in PBS (10 min) then incubated with exosomes, recombinant human HSP70 (100nM, ADI-ESP, EnzoLife Sciences) or PBS (for 600 seconds). All sensorgrams were corrected for baseline drift by subtracting a control sensor exposed to running buffer only (FortéBio, Data analysis software version 7.1.0.89).

Supplementary legends

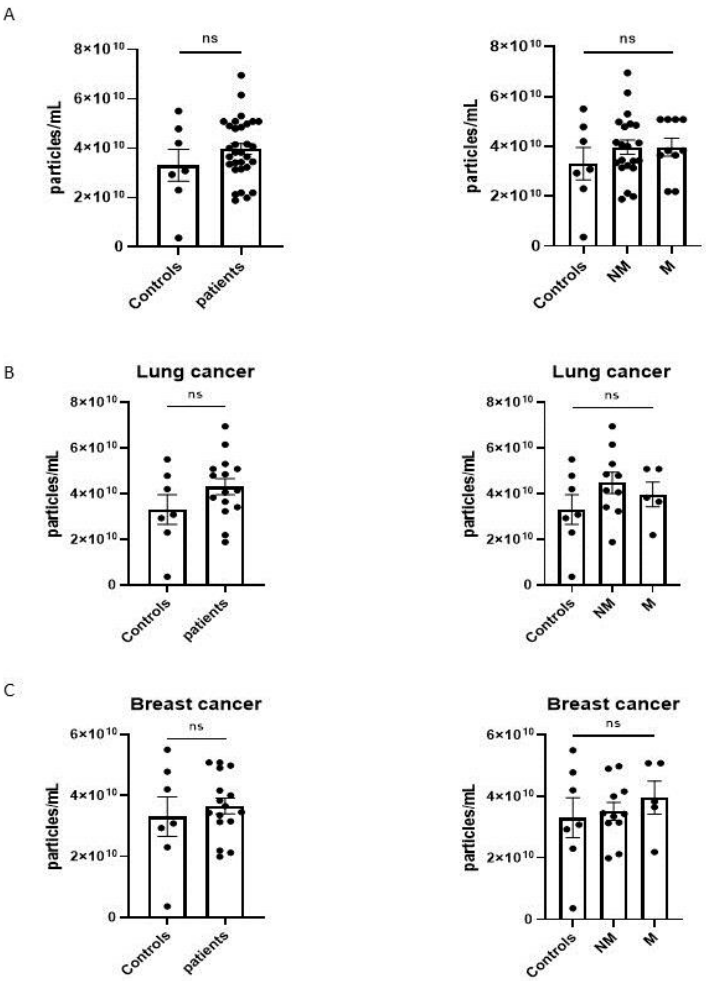
Supplementary Figure 1: Characterization of tumor-derived exosomes. A. Representative western-blot analysis of exosomes isolated from breast (BrCa) and lung cancer (NSCLC) patients, and healthy controls (HC). Cell lysate is used as a control. B. TEM images suggesting the presence of exosomes. C. Representative size/distribution profile determined by NTA.

Supplementary Fig 2: Exosomes concentration does not differ between patients and healthy volunteers. A-C. Exosome concentration in the plasma of breast and lung cancer patients (A, n=31, NM: Non-metastatic n=21, M: Metastatic, n=10) or just in lung cancer (B, n=15, NM: Non-metastatic n=10, M: Metastatic, n=5) or in breast cancer patients (C, n=16, NM: Non-metastatic n=11, M: Metastatic, n=5), versus healthy controls (n=7), Student t test, ns.

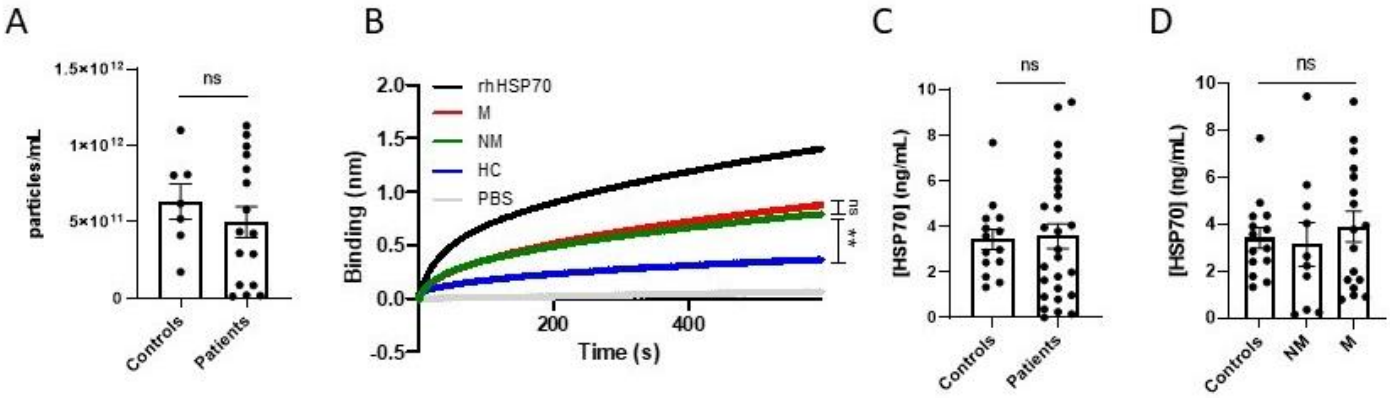
Supplementary figure 3: Analysis of exosomes in urine samples. A. NTA quantification of exosomes in healthy volunteers (controls) and cancer patients. B. Capture of exosomes presenting HSP70 in their membrane by BLI, using the peptide aptamer A8, from urine samples of metastatic (M, n=18), non-metastatic patients (NM, n=19) and healthy volunteers (HC, n=14). Recombinant human HSP70 and PBS were used as controls, 1way ANOVA, $p<0.001$. C-D. Expression of HSP70 within urinary exosomes in all cancer patients (n=27), metastatic (M, n=17), non-metastatic patients (NM, n=10) and healthy volunteers (n=14), Mean +/- SEM. (1way ANOVA, ns).



<u>Supplementary Figure 1:</u> Characterization of tumor-derived exosomes.



<u>Supplementary Fig 2</u>: Exosomes concentration does not differ between patients and healthy volunteers.



<u>Supplementary figure 3:</u> Analysis of exosomes in urine samples.

DISCUSSION ET PERSPECTIVES

Le travail décrit dans ce manuscrit a pour but d'émettre la preuve de concept de l'utilisation des HSP70-exosomes en tant que biomarqueur de tumeurs solides. Pour cela, nous avons ouvert une étude clinique pilote qui inclue 20 patients des deux cancers les plus fréquents en France : le cancer du sein et le cancer du poumon.

Différentes études indépendantes s'intéressant à HSP70 ont démontré sa surexpression dans les tumeurs, à la fois au niveau intracellulaire et membranaire, ainsi que sa sécrétion dans le milieu extracellulaire[325,329,370,371]. Les différents groupes de chercheurs ont également indiqué que l'expression de HSP70, qu'elle soit intracellulaire, membranaire ou soluble dans la circulation, corrélait avec différents paramètres cliniques importants pour le diagnostic, le pronostic ou le suivi des tumeurs[331,348,350,363,371]. Cependant, aucune étude ne s'est appliquée à étudier l'ensemble de ces paramètres dans un même projet. Au cours de ce travail, nous avons pu mettre en évidence que HSP70 extracellulaire était majoritairement dans les exosomes et que la forme soluble était faiblement exprimée dans le plasma des patients. Ceci peut s'expliquer par le fait que la membrane des exosomes est enrichie en cholestérol et en certains lipides qui rendent la double membrane plus rigide et stabilise HSP70, contrairement à la forme soluble qui est exposée à la dégradation[330,370]. Ces résultats indiquent que la sécrétion de HSP70 se fait préférentiellement via les exosomes, ce qui implique certainement un tri spécifique, qui reste néanmoins à démontrer.

De plus, nous avons montré que l'expression de HSP70 dans les exosomes semblait correspondre à celle dans la biopsie tumorale ce qui laisse à penser que les exosomes reproduisent les caractéristiques de la tumeur. Enfin, nos résultats indiquent que les patients atteints d'un cancer à un stade métastatique possèdent plus de HSP70-exosomes que les non métastatiques, et encore plus que les sujets témoins, tandis que le nombre total d'exosomes ne diffère pas. Nous n'avons pas non plus observé de différences dans l'urine des patients ; cette analyse semble être plus pertinente pour les cancers uro-génitaux[264,265,267,372]. Il a été décrit que HSP70 à la membrane des cellules cancéreuses est une caractéristique propre au cancer, et que ce phénomène semble être présent dans une grande majorité des tumeurs solides, faisant de HSP70 membranaire un marqueur tumoral[329,330,353,363,373]. Nos résultats indiquent donc que la quantité de HSP70-exosomes (*ie* dérivés de tumeur) est d'autant plus importante que la maladie est avancée. D'autres recherches sont nécessaires afin de déterminer si la quantité de HSP70-exosomes est corrélée au volume tumoral.

Notre équipe a démontré, en collaboration avec l'équipe du Pr François Ghiringhelli, que les HSP70-exosomes sont capables d'activer les MDSCs et d'induire un environnement immunosuppressif qui favorise la croissance tumorale. Depuis, cette même équipe a observé que les patients atteints d'un cancer du côlon à un stade métastatique présentaient significativement plus de MDSCs circulantes que

les sujets témoins[374]. De notre côté, nous avons démontré que les HSP70-exosomes semblent être universels au cancer[368]. Ainsi, ces données semblent converger vers le fait que plus la maladie progresse, plus le nombre de HSP70-exosomes sécrété augmente et plus le nombre de MDSCs est important, formant ainsi un mécanisme qui permet à la tumeur de croitre. Il est cependant nécessaire de confirmer cette hypothèse via une étude portant sur le même type de tumeur, et qui inclue ces deux paramètres.

Ensuite, nous avons montré via nos courbes ROC que la détection des HSP70-exosomes est plus sensible et plus spécifique que la détection des CTCs, avec une aire sous la courbe bien plus élevée (AUC=0,8968, CI95% = [0,7387-1], p<0,0001 pour les HSP70-exosomes comparé à 0,7857, CI95% = [0,5873-0,9842], p=0,0048, pour les CTCs). C'est à notre connaissance la première étude comparant la détection de ces deux marqueurs circulants. Nos résultats semblent logiques puisqu'une cellule libère des milliers d'exosomes tandis que les CTCs restent un évènement rare et difficile à détecter techniquement[9,126,375]. De plus, les technologies actuelles de détection des CTCs ne permettent pas d'identifier totalement toute l'hétérogénéité de ces cellules, ce qui entraine un certain nombre de faux positifs et faux négatifs[88,376]. Au contraire, dans l'optique d'une analyse combinatoire multiparamétrique, les exosomes, dont les HSP70-exosomes, sont présents en grand nombre dans la circulation sanguine ce qui permet d'envisager moins de contraintes techniques ; je pense notamment à la quantité de matériel à disposition pour réaliser des analyses. Nous avons déterminé un seuil (préliminaire au vu de la taille de la cohorte), au-delà duquel les patients auraient un risque de présenter des métastases : 1,92ng/mL. Ce seuil est bien évidemment à affiner via un jeu de donnés plus important.

Nous avons également démontré que les HSP70-exosomes semblent être utiles dans le suivi des patients comme marqueurs de réponse au traitement. En effet, nos résultats indiquent que la variation du taux de HSP70-exosomes entre le diagnostic et la première évaluation tumorale par imagerie est corrélée à la réponse à la thérapie. Ainsi, nous avons observé que les patients en réponse clinique avaient une diminution moyenne de HSP70-exosomes de 77% tandis que les patients progresseurs avaient une augmentation moyenne de 92%. Les patients stables avaient quant à eux une variation minime, de l'ordre de 1% en moyenne. Ensuite, nous avons également montré que le taux de HSP70-exosomes pouvait être utilisé pour le suivi à long terme puisqu'il est associé au statut de la maladie déterminé par imagerie médicale, chez trois patients que nous avons pu suivre ; au cours du suivi, le patient 1 a été diagnostiqué comme répondeur partiel à deux cures différentes, à chaque fois traduites par une chute du taux de HSP70, puis comme étant stable pendant plusieurs cures, correspondant à une variation quasi nulle du taux de HSP70. Les patients 2 et 3 ont, eux, été diagnostiqués comme progresseurs au cours de leur ligne de traitement, phénomène associé à une augmentation importante

du taux de HSP70. Ces données sont en adéquation avec la littérature qui indique que l'expression de HSP70 dans les biopsies tumorales sont corrélées avec le volume de la tumeur[348,373,377]. L'hypothèse est que le traitement aboutit à une réduction de la taille de la tumeur et donc de la quantité de HSP70-exosomes libérés. Au contraire, les patients non répondeurs ont très certainement une croissance tumorale qui se traduit par une augmentation de cette sécrétion de HSP70-exosomes.

De plus, nous avons pu observer pour les patients 1 et 2 que le taux de HSP70-exosomes suivait l'évolution de la courbe du taux des marqueurs tumoraux conventionnels que sont le CA-15-3 pour le cancer du sein et l'ACE pour le cancer du poumon. Cependant, la courbe des HSP70-exosomes contient plusieurs piques qui sont absent des courbes des marqueurs tumoraux. Nous avons noté une variation à la suite d'une intolérance ou une toxicité excessive de la molécule chimiothérapeutique. Des études complémentaires sont nécessaires afin de déterminer si la détection des HSP70-exosomes est une méthode plus sensible, qui pourrait notamment refléter plus précisément les changements physiologiques.

De fortes concentrations de HSPs ont été corrélé au diagnostic, au pronostic et la réponse au traitement de différents cancers, à la fois au niveau intracellulaire[328,349,356,378–380] et extracellulaire[126,351,351,352,368,381]. Cette étude pilote a permis pour la première fois dans une cohorte prospective de fournir une vue d'ensemble de l'expression de HSP70 dans le cancer du sein et du poumon. Elle a également permis de démontrer l'intérêt des HSP70-exosomes en tant que potentiel biomarqueurs de la croissance tumorale des patients porteurs de tumeurs surexprimant HSP70. Il est important de noter que cette étude est une étude pilote, qui vise uniquement à émettre la preuve de concept de l'intérêt de cette sous-population d'exosomes dérivés de tumeurs en tant que biomarqueurs de tumeurs solides malignes. Afin de démontrer son utilité clinique, il est nécessaire de monter des études plus grandes, avec des cohortes plus homogènes. Nous avons d'ores et déjà commencé l'extension de cette étude sur une cohorte plus importante de patients atteint du cancer colorectal, avec des résultats préliminaires encourageants (Figure 19).

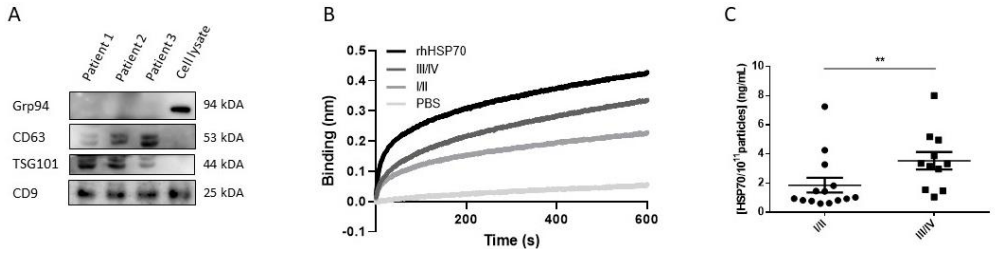


Figure 20 : Résultats préliminaires de l'étude des HSP70-exosomes dans le cancer colorectal. *A. Analyse par western-blot d'exosomes isolés du plasma de patients atteints d'un cancer colorectal. Un lysat cellulaire a été utilisé comme contrôle. B. Capture des HSP70-exosomes des patients métastatiques (M), non-métastatiques (NM) et des sujets sains (HC) par BLI. C. Quantification de HSP70 exosomal par ELISA. Student t test, p<0,005.*

L'identification d'une sous-population d'exosome dérivée de tumeur constitue une première étape dans la perspective de l'utilisation des exosomes en tant que biomarqueur. L'apparition d'un anticorps commercial dirigé contre la partie extracellulaire de HSP70, couplé à l'émergence de technologies innovantes qui permettent l'analyse des VEs, offre la possibilité de déterminer avec précision la proportion de HSP70-exosomes dans le plasma de patients atteints d'un cancer. Il pourrait donc être possible très prochainement de déterminer si le pourcentage d'exosomes dérivés de tumeurs, mesuré de façon quantitative et directe, a une valeur clinique. Cela pourrait également permettre d'isoler spécifiquement cette sous-population, par FACS ou via des billes magnétiques par exemple, de façon à analyser son contenu. En effet, les exosomes possèdent la capacité de refléter toute la complexité tumorale au travers de leur contenu en protéines, en lipides et en matériel génétique. La capacité éventuelle d'analyser le contenu biologique des HSP70-exosomes uniquement, et donc des exosomes dérivés de tumeurs, offre la possibilité d'une analyse combinatoire puissante en vue d'une utilisation diagnostique, pronostique ou de suivi des patients. Le fait de coupler des marqueurs de nature différente au sein des exosomes, comme l'expression d'une protéine couplée à celle d'un micro-ARN particulier, pourrait permettre d'augmenter la sensibilité et la spécificité d'un test diagnostic basé sur les exosomes.

Enfin, un des défis majeurs de la recherche sur les exosomes consiste à mettre au point une technologie permettant une isolation et une analyse plus automatisée, afin de pouvoir envisager son utilisation en clinique. En effet, les techniques actuelles d'isolation des exosomes ne sont que très peu compatibles avec une routine hospitalière. Ainsi, de nombreuses équipes travaillent sur le développement de technologies innovantes, capables d'isoler et d'analyser les exosomes à partir d'un faible volume[252,382–384]. Nous nous penchons également sur le sujet puisque nous travaillons en collaboration avec l'équipe du Dr Wilfried Boireau de l'institut Femto de Besançon qui a mis au point une plateforme NanoBioAnalytique (NBA) combinant trois techniques : l'imagerie par Résonance des Plasmons de Surface (SPRi), la Microscopie à Force Atomique (AFM) et la Spectrométrie de Masse (MS)[385,386]. L'enjeu ici est de coupler leur technologie avec notre aptamère peptidique ou l'anticorps spécifique de la partie extracellulaire de HSP70, afin d'analyser le contenu protéique des HSP70-exosomes.

A ce jour, les exosomes présentent donc un fort potentiel dans le diagnostic du cancer. Cependant, le besoin d'une meilleure connaissance de l'hétérogénéité de ces nanovésicules, le manque de standardisation des protocoles, et le manque d'études réalisées à grande échelle retardent son apparition en clinique. Actuellement, une seule entreprise, appelée ExosomeDx™, possède une technologie sur le marché. Elle est incluse dans les « *National Comprehensive Cancer Center Network Guidelines* » (NCCN) aux Etats-Unis et consiste en la détection de plusieurs mutations dans les

transcrits d'exosomes dérivés d'urine de patients atteints d'un cancer de la prostate avec un taux de PSA compris entre 2 et 10 ng/mL[264,265]. Les chercheurs ont établi un score qui permet d'indiquer au clinicien si les patients au risque élevé de cancer de la prostate doivent subir une biopsie ou non. Cet exemple unique d'application médicale, à la valeur clinique clairement établie, illustre parfaitement le potentiel des exosomes dans le diagnostic du cancer.

CONCLUSION GENERALE

Ce travail a donc permis d'émettre la preuve de concept de l'utilisation des HSP70-exosomes dérivés de tumeurs, dans le diagnostic et le suivi de patients atteints d'un cancer du sein et du poumon. Il analyse pour la première fois l'expression de HSP70 sous toutes ses formes chez les patients atteints de ces cancers, et suggère que l'utilisation des HSP70-exosomes est plus sensible et plus spécifique que celle des CTCs. Enfin, ce travail montre l'intérêt d'analyser les HSP70-exosomes lors du suivi des patients puisque leur taux est corrélé à la réponse à la thérapie. Enfin, cette étude pilote ouvre la voie à une étude plus approfondie et plus généralisée de l'utilité des HSP70-exosomes dans cancer.

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ANNEXES

ANNEXE 1

Tracking the evolution of circulating exosomal-PDL1 to monitor melanoma patients

Marine Cordonnier, Charlée Nardin, Gaëtan Chanteloup, Valentin Derangere, Marie-Paule Algros, Laurent Arnould, Carmen Garrido, François Aubin & Jessica Gobbo

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Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients

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ABSTRACT

In the era of immunotherapies there is an urgent need to implement the use of circulating biomarkers in clinical practice to facilitate personalized therapy and to predict treatment response. We conducted a prospective study to evaluate the usefulness of circulating exosomal-PD-L1 in melanoma patients' follow-up. We studied the dynamics of exosomal-PD-L1 from 100 melanoma patients by using an enzyme-linked immunosorbent assay. We found that PD-L1 was secreted through exosomes by melanoma cells. Exosomes carrying PD-L1 had immunosuppressive properties since they were as efficient as the cancer cell from which they derive at inhibiting T-cell activation. In plasma from melanoma patients, the level of PD-L1 (n= 30, median 64.26 pg/mL) was significantly higher in exosomes compared to soluble PD-L1 (n= 30, 0.1 pg/mL). Furthermore, exosomal-PD-L1 was detected in all patients whereas only 67% of tumour biopsies were PD-L1 positive. Although baseline exosomal-PD-L1 levels were not associated with clinic-pathologic characteristics, their variations after the cures (ΔExoPD-L1) correlated with the tumour response to treatment. A ΔExoPD-L1 cut-off of > 100 was defined, yielding an 83% sensitivity, a 70% specificity, a 91% positive predictive value and 54% negative predictive values for disease progression. The use of the cut-off allowed stratification in two groups of patients statistically different concerning overall survival and progression-free survival. PD-L1 levels in circulating exosomes seem to be a more reliable marker than PD-L1 expression in tumour biopsies. Monitoring of circulating exosomal-PD-L1 may be useful to predict the tumour response to treatment and clinical outcome.

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Introduction

Immune checkpoint inhibitors such as antibodies targeting PD-L1 (programmed death-ligand) are revolutionizing cancer patients' management, particularly in melanoma and non-small cell lung cancer (NSCLC) treatment. Two monoclonal antibodies have already been approved by the FDA (Nivolumab and Pembrolizumab) in both indications [1–3]. In advanced melanoma patients, around 40% of objective response rate is observed whereas in NSCLC patients, Nivolumab response rate is approximately 20%. Therefore, there is an urgent need to validate a biomarker allowing selection of patients who might benefit from an immunotherapeutic approach. With this goal, the first studies aimed to identify PD-L1 expression in patients' biopsies but this test has proven to be unreliable due to the heterogeneity of PD-L1 expression within the tumour [4,5]. Additionally, different studies attempted to measure PD-L1 level in blood samples. However, this protein is very unstable and not easy to detect. Recent studies report the interest of using circulating nanovesicles, such as exosomes, as a diagnostic tool.

Exosomes are 40–150 nm lipid bilayer membrane-bound particles generated and released by most kinds of cells through a defined intracellular trafficking route [6,7]. Exosomes carry nucleic acids, lipids and proteins, potential biomarkers that are protected by an exosome's cholesterol-rich membrane. Among the proteins, exosomes contain membrane proteins (flotillin-1, tetraspanins, annexinV, ICAM-1), immunostimulatory molecules (MHC I/II) and ESCRT proteins (Alix, TSG101) [8–10]. In the setting of cancer, exosomes are released by all tumour cells and they can be isolated from circulating peripheral blood [6,11,12]. Exosome-based diagnostics provide higher sensitivity and

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**Co-direction of the work.

Supplemental data for this article can be accessed here.

specificity over conventional biopsies or liquid biomarkers due to their stability in biofluids. In addition, exosomal markers are readily available from most biofluids and recent isolation technologic advances make exosome-based diagnostics cost and labour effective [13]. Recent studies indicate that PD-L1 expression in extracellular vesicles isolated from cancer patients' blood samples can correlate with tumour features [14–18]. Additionally, studies of tumour-derived exosomes, performed in cultured cells, have demonstrated that these exosomes represent an effective mechanism of immunosuppression [17,19–21].

In this work, we have performed a prospective clinical study to investigate whether PD-L1 levels in exosomes isolated from melanoma patients' plasma could predict a patient's response to immunotherapy.

Material and methods

Cell culture

Non-cancerous human lung cells (MRC-5) human lung fibroblasts, A549 human lung cancer cells, B16F10 mouse melanoma cells and SK-MEL-2 human melanoma cells were purchased from the ATCC. All cell lines were cultured at 37°C, 5% CO_2 in RPMI 1640 medium (Dutscher) supplemented with 10% foetal bovine serum (Dutscher) depleted in exosomes and were tested weekly for mycoplasma contamination.

Exosomes purification from the cell's supernatants

Cells were cultured in medium depleted from serum-derived exosomes. Supernatants were collected from cell lines and sequentially centrifuged at 300*g* for 10 min (4°C), at 2000*g* for 20 min and at 10,000*g* for 40 min. Then, exosomes were ultracentrifugated at 100,000*g* for 70 min and washed in PBS (Beckman Coulter, Optima XPN-100). Supernatants were carefully removed and exosome pellets were suspended either in 50 µl of 1% RIPA lysis buffer or in 50 µl of PBS.

Lymphocyte isolation and co-culture experiment

PBMC were isolated from buffy coats (EFS, Besançon) by centrifugation using a density gradient (Lymphocytes separation medium, Eurobio). Red blood cells were lysed and T cells were enriched (RosetteSep, Stem Cell). Then, purified T cells (10^5) were activated with CD2/CD3/CD28 beads (Miltenyi Biotec) and incubated with either human melanoma cells (10^5) (SK-MEL-2) or human lung cancer cells (A549) or exosomes derived from these cell lines (10^6). After 48 h of culture, T cells were collected and analysed by flow cytometry for the expression of membrane-bound PD-1 (46-1799-42, eBioscience), and intracellular Ki67 (48-5699-42, eBioscience), IFNY (506516, Biolegend) after fixation and permeabilization (BD Perm/Wash Buffer, Biosciences).

EXOMEL clinical study

This prospective study was conducted between January 2016 and December 2018 in the Department of Dermatology, University Hospital of Besançon, France. The study design was approved by the local research ethics committee and a written informed consent was provided before enrolment. Study adhered to the Declaration of Helsinki Principles. Patients with melanoma were included at different clinical stages graded according to the latest American Joint Committee on Cancer staging classification (8^{th} edition, Balch 2018).

Study objectives and endpoints

The primary objective of this study was to quantify exosomal PD-L1 in the blood of melanoma patients. The secondary objectives were to determine whether the amount of ExoPD-L1 could be associated to: (i) stages diseases, (ii) response to treatment, (iii) overall survival (OS) and progression-free survival (PFS), (iv) clinical variable and (v) PD-L1 expression in tumours or soluble in the plasma. The primary endpoint of the study was the blood concentration of PD-L1-exosomes at different time points using ELISA. The secondary endpoints were to compare: (i) the baseline concentration of ExoPD-L1 with clinic/pathologic features (age, gender, primary melanoma histology subtype and location, tumour burden, prior therapy and disease status, biologic data-lactate dehydrogenase, lymphopenia, soluble PD-L1 using ELISA dosage and tumour PD-L1 using immunohistochemistry staining, (ii) the concentration of ExoPD-L1 and their variation of in patients having a complete response (CR), partial response (PR), progressive disease (PD) (based on immune-related irRECIST).

Blood collection and storage

Peripheral blood was drawn into sodium heparin tubes. First sampling at inclusion was labelled S1. Second sampling was labelled S2. Change in ExoPD-L1 from S1 to S2 was labelled ΔExoPD-L1. To ensure exosomes integrity, blood was centrifuged at 2400*g* for 15 min to remove cell debris and dead cells. Plasma samples were stored in 1 mL aliquots at −18°C.

Exosomes isolation from plasma samples

Thawed plasma samples of 5 mL were differentially centrifuged 300*g* for 5 min at 4°C and then 17,000*g* for 10 min at 4°C. Next, supernatants obtained in the previous step were ultra-centrifuged at 200,000*g* for 1 h at 4°C (Beckman Coulter, Optima XPN-100). Supernatants were carefully removed and exosome pellets suspended either in 50 µl of 1% RIPA lysis buffer or in 50 µl of PBS.

Exosomes characterization

Exosomes size and concentration were determined by nanoparticle tracking analysis using a NS300 Instrument (Nanosight, Amesbury, UK).

To determine PD-L1 expression at the exosome's surface, protein–protein interaction experiments were conducted with an Octet Red instrument FortéBio, Menlo Park, CA). The ligand (PD-1) was biotinylated using EZ-Link NHS-PEG_4-biotin (2 nM, 30 min, RT, Thermo Fisher Scientific, Germany) and immobilized on streptavidin sensors (black 96-well plate, FortéBio, USA). Functionalized sensors were incubated in PBS (10 min) then incubated with exosomes (10^6, isolated from either the supernatant of cancer cell lines, normal cells, melanoma patients' plasma, lung cancer patients' plasma or healthy donors' plasma). All sensorgrams were corrected for baseline drift by subtracting a control sensor exposed to running buffer only (FortéBio, Data analysis software version 7.1.0.89).

Isolated exosomes were tested for the expression of exosomal markers. Nanovesicles were lysed and separated on SDS/PAGE gels. Proteins were transferred onto a polyvinylidene fluoride membrane (Amersham GE Healthcare Life Sciences) for western-blotting analysis. After transferring, membranes were blocked with 5% bovine serum albumin for 1 h and incubated overnight at 4°C with antibodies specific for Alix (2171s, Cell Signalling), TSG101 (sc-7964, Santacruz), PD-L1 (sc-50298), CD9 (ab92726, Abcam), CD63 (NBP2-4225, BioTechne), GRP94 (ab2791, Abcam), β-actin (A3854, Sigma Aldrich). Horse radish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) was added and immunoreactive proteins revealed using ECL detection reagents (34095, ThermoFisher Scientific). Band intensities were captured using Chemidoc XRS+ system and images were analysed using Image Lab software (Bio-Rad Laboratories).

Determination of PD-L1 concentration in plasma and circulating exosomes

Soluble PD-L1 and PD-L1 in exosomes levels were measured using an enzyme-linked immunosorbent assay (PD-L1 Human ELISA Kit, Invitrogen), according to the manufacturer's instructions. Protein concentrations were determined according to standard curves.

Immunohistochemistry of PD-L1 in tumour tissue samples

Formalin-fixed paraffin-embedded tumour tissue samples were obtained for correlation analysis of PD-L1 expression between tissue and circulating exosomes. All IHC procedures were performed using Benchmark apparatus (Ventana). PD-L1 expression was evaluated with 22C3 clone (Dako). PD-L1 immunostaining on tumour tissue samples (blind coded) was assessed by a dedicated pathologist.

Tumour response evaluation

Tumour response was based on immune-related irRECIST using unidimensional measurements on contrast-enhanced computed tomography scan or PET-CT (Positron Emission Tomography) [19]. CR, complete response; PR, partial response. Stable disease (SD) was labelled SD. High tumour burden was defined as 10 lesions or more, or only 1 lesion bigger than 3 cm. Imaging re-evaluation was performed in blind-coded samples.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v24.0 and the Graphpad Prism 6 program. Data presented are from at least three independent experiments. Error bars shown in graphical data represent mean ± SEM. When data are presented as box plots, the bar indicates the median, the box shows the interquartile range (25–75%) and the whiskers extend to 1.5 the interquartile range. For normally distributed data, significance of mean differences was determined using two-tailed paired or unpaired Student *t*-test. For data not normally distributed, non-parametric Kruskal–Wallis and Wilcoxon tests were used for unpaired and paired analysis, respectively. To test the relationship between variables, Spearmann correlation coefficients were calculated. Dependence was ruled out with a correlation coefficient (absolute *q*-value) of <0.7. Kaplan–Meier survival curve, Log-rank test and Cox proportional hazards model were used to analyse survival data. Results from Cox proportional hazards models were reported as hazard ratios (HRs) with 95% confidence interval (CIs). A two-tailed value of $p < 0.05$ was considered statistically significant. Quantitative data are expressed as mean ± SEM from at least three independent experiments. Quantitative results were compared using the Mann–Whitney test according to their distribution. The

cut-off value for the change in PD-L1-Exo was calculated by the Receiver Operating Characteristic (ROC) curve analysis to identify melanoma patients with high probability to display disease control.

Results

Cancer cells release exosomes expressing membrane PD-L1

We first studied whether PD-L1 could be secreted through exosomes. We purified exosomes from different cell lines such as human and murine melanoma (SK-MEL-2, B16F10), human lung cancer (A549) and MRC-5. TSG101, Alix, CD9 and CD63 were used as markers of exosomes and Grp94 served as a negative control. As shown in Figure 1(a), PD-L1 was expressed in exosomes from the three cancer cell lines analysed but not in the non-cancerous cell line. We then analysed PD-L1 expression in exosomes (ExoPD-L1) isolated from pooled blood samples of melanoma patients (n = 6) or lung cancer patients (n = 6) or exosomes isolated from healthy donors (n = 5). As previously observed for cell lines, we only detected PD-L1 in the exosomes isolated from cancer patients (Figure 1(b)). While there was a difference between cancer patients and healthy donors in the detection of PD-L1 expressing exosomes, no difference was found in the total number and size of the exosomes (Supplementary Figure 1A-B).

To determine whether PD-L1 was expressed at the membrane of the vesicles, we used an interference biolayer approach to capture exosomes with a PD-1 recombinant protein. As shown in Figure 1(c–d), exosomes could be captured both from cancer cell lines and cancer patients' blood samples (melanoma, lung cancer), indicating the presence of PD-L1 in their membrane. In contrast, no ExoPD-L1 (or negligible amounts) could be captured from the non-cancerous cells' supernatant or from the blood of healthy donors. These results indicate that PD-L1 is present at the membrane of tumour-derived exosomes.

Next, we determined whether isolated PD-L1-exosomes were functional. Primary T cells were activated (CD3, CD28) and then co-cultured for 24 h with either human melanoma SK-MEL-2 cells (positive control) or with exosomes purified from those cells' supernatant (ExoPD-L1). As shown in Figure 1(e), the exosomes were as efficient as cancer cells in inhibiting T-cell activation, as determined by the expression of PD-1, Ki67 and IFNϒ. Indeed, we observed an almost two-fold decrease in the percentage of PD-1, Ki67 and IFNϒ in the T cells cultured in the presence of SK-MEL-2 cells and SK-MEL-2-derived exosomes (respective comparisons: PD-1: 52.43% ± 7.56 and 54.84% ± 7.60 vs 100%; 57.54% ± 5.95, Ki67: 67.51% ± 6.20 vs 100%; 43.71% ± 3.45 and IFNϒ: 68.01% ± 3.44 vs 100%) (Figure 1(e)). Similar results were observed with lung cancer cells (Supplementary Figure 1C).

Altogether, these results show that PD-L1 is secreted in exosomes derived from cancer cells and that this molecule is functional since it is able to mediate T-cell immunosuppression.

Patient characteristics: the EXOMEL cohort

To study ExoPD-L1 as potential biomarkers, we opened a prospective clinical study. Over the study period, 100 melanoma patients were included. Among these patients, 43% received anti-PD-1 antibodies, 10% ipilimumab and 18% BRAF and MEK inhibitors. Treatment information and clinical outcomes are detailed in Supplemental Table 1. Overall, 12 had a CR, 28 had a PR, 10 had SD, and 46 had PD). In the cohort, 46 patients were evaluated after a median follow-up of 16 months. Among them 5 had a CR, 23 had a PR, 8 had SD, and 10 had PD, giving an overall risk ratio of 60.8%. Mean age was 64 years old (SD, 13.7) and 39% of patients were female. Most primary melanomas were cutaneous (84%) and carried a BRAF mutation (51%). At the time of inclusion, most patients were AJCC stage IV (74%) with M1c and M1d disease (80%) (Table 1).

Expression of PD-L1 in plasma or in circulating exosomes versus tumour biopsies

First, we compared the expression of PD-L1 within exosomes in blood samples versus PD-L1 tumour expression in biopsies by IHC. Then, in plasma samples, we compared by ELISA the levels of soluble PD-L1 with PD-L1 in exosomes (Figure 2(a)). As expected, the level of ExoPD-L1 (n = 30, median 64.26 pg/mL) was significantly higher compared with soluble PD-L1 in the plasma, which was barely detectable (n = *30*, 0.1 pg/mL). Interestingly, ExoPD-L1 was detected in all patients (100%) (Figure 2(b)) whereas, only 67% were PD-L1 positive in tumour biopsies (Figure 2(c–d)). We conclude that PD-L1 was much easier to detect and quantify in circulating exosomes rather than in biopsies or when soluble in plasma.

Association between baseline ExoPD-L1 expression and clinical variables

Baseline ExoPD-L1 levels (S1) were not associated with clinical/pathologic characteristics such as age, gender, primary melanoma histology subtype and location,

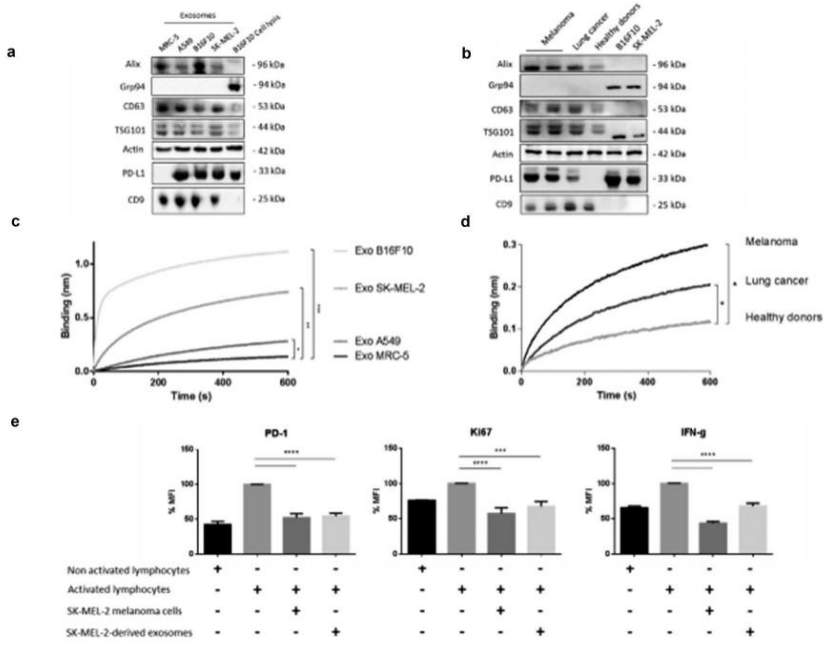


Figure 1. PD-L1 expression in tumour-derived exosomes. (A/B) Representative immunoblots showing expression of Alix, CD63, TSG101, PD-L1 and CD9 in exosomes derived from cell lines (a) MRC-5 (normal human lung cells), A549 (human lung cancer), B16F10 (mouse melanoma) or SK-MEL-2 (human melanoma) or (b) plasma-derived exosomes from melanoma patients, lung cancer patients and healthy donors. Grp94 is used here as an exosomal negative control and actin as a loading control. Cell lysates are also included. (C/D) Binding of exosomes (nm) derived from cell lines (MRC-5, A549, SK-MEL-2, B16F10) or from plasma samples of melanoma patients ($n = 6$) or lung cancer patients ($n = 6$) and healthy donors ($n = 5$) to immobilized biotinylated PD-1 determined by biolayer interferometry. Binding curves represent mean signal of triplicate measurements for each sample. Mann–Whitney. (c), $^{***}p < 0.001$, $^{**}p = 0.0081$, $^{*}p = 0.0041$. (d) $^{*}p = 0.0039$ melanoma, $^{*}p = 0.0031$ lung cancer). (e) Percentage of PD-1, Ki67 and IFNY mean fluorescence intensity in lymphocytes cultured 24 h in the presence or absence of SK-MEL-2 or SK-MEL-2-derived exosomes, determined by flow cytometry ($^{***}p = 0.0006$, $^{****}p < 0.0001$).

tumour burden, prior therapy, or disease status, with the exception of lymphopenia that associated with a significantly higher ExoPD-L1 level ($p = 0.048$) (Supplementary Table 2).

Association between changes in exosomal-PD-L1 expression and tumour response

We evaluated whether a variation in ExoPD-L1 expression could be predictive of tumour response to therapy. Two blood samples, before and after treatment (S1+ S2), were obtained from 46 patients. Median interval between S1 and S2 was 4.5 months. Reasons for S2 sample unavailability were either patient death ($n = 23$) or the patient was lost to follow-up ($n = 31$). Among these 46 patients, 36 patients received anti-PD-1 therapy. The other patients received Ipilimumab ($n = 2$, including 1 in association with anti-PD-1 therapy), anti-BRAF and anti-MEK targeted therapies ($n = 8$, including 4 in association with anti-PD-1 therapy). The mean change in exosomal-PD-L1 (ΔExoPD-L1) between S1–S2 was 43.4 pg/mL. Association between ΔExoPD-L1 and disease status is shown in Figure 3(a–b). In patients experiencing complete and PR ($n = 27$), ΔExoPD-L1 decreased without statistical significance (mean S1: 121.06 ± 26.65 vs mean S2: 104.78 ± 17.11, $p = 0.8607$). In patients experiencing progression ($n = 9$), ΔExoPD-L1 increased significantly (mean S1 85.90 ± 24.46 vs mean S2 344.20 ± 70.30 $p = 0.0002$), (Figure 3(a)). Furthermore, ΔExoPD-L1 was significantly associated with disease status, i.e. responders (CR+PR+SD) vs non-responders ($p = 0.001$) (Figure 3(b–e)).

Using ROC curve analysis, ΔExoPD-L1 showed good discrimination between patients experiencing disease response ($n = 36$) and those experiencing disease

Table 1. Clinical characteristics of melanoma patients with available ΔExoPD-L1 (n = 46).

		Patients N = 46
Age, years		
	<70	28 (60.9%)
	=70	18 (39.1%)
Gender		
	Male	28 (60.9%)
	Female	18 (39.1%)
ECOG performance status		
	0	36 (78.3%)
	=1	10 (21.7%)
Anatomic location of primary melanoma		
	Cutaneous	36 (78.3%)
	Head and neck	7
	Lower limb	8
	Upper limb	6
	Trunk	14
	Hand and foot	1
	Mucosal	4 (8.7%)
	Uveal	3 (6.5%)
	Unknown	3 (6.5%)
Histological subtype*		
	SSM	14 (30.4%)
	NM	8 (17.4%)
	LMN	-
	ALM	1 (2.2%)
	Desmoplastic	1 (2.2%)
	Unknown	12 (26.1%)
	NA	10 (21.7%)
Breslow, mm*		
	0–1 mm	7 (15.2%)
	1.01–2 mm	7 (15.2%)
	2.01–4 mm	12 (26.1%)
	=4 mm	9 (19.6%)
	Unknown	1 (2.2%)
	NA	10 (21.7%)

progression (n = *10*) (AUC = 0.867, SE: 0.057, CI): 95% = 0.755–0.978; $p < 0.001$) (Figure 3(d)). Based on ROC curve analysis, we calculated an optimal cut-off. The ΔExoPD-L1 cut-off >100 pg/mL demonstrated an 83% sensitivity, a 70% specificity, a 91% positive predictive value and a 54% negative predictive value for disease progression.

Association between changes in ExoPD-L1 expression and clinical outcome

The median follow-up was at 9.9 months. No significant difference was found between the two groups of patients for either the total number of circulating exosomes or exosome size (Supplementary Figure 2A-B). In univariate analysis, ExoPD-L1 blood levels at S1 were not associated with OS and PFS. The small number of events regarding OS and PFS (5 events for OS and 15 events for PFS) precluded multivariable analysis. However, ΔExoPD-L1 was associated with PFS (HR: 1.003, CI 95% = 1.001 – 1.006; p = 0.006) and OS (HR: 1.004, CI: 95% = 1.001–1.008, p = 0.034) (Supplemental Table 3). The use of a ΔExoPD-L1 cut-off allowed stratification in two groups of patients statistically different in terms of OS (median not reached, $p = 0.048$) and PFS (ΔExoPD-L1 < 100, median PFS not reached vs. ΔExoPD-L1 > 100, median PFS = 9 months, CI: 95% = 4–14.2, p = 0.011) (Figure 4(a–b)). Altogether, these results suggest that monitoring changes in circulating ExoPD-L1 might be used to predict tumour response and clinical outcome.

ExoPD-L1 as a biomarker to detect early responders

Finally, we explored the possibility of following the evolution of the disease by measuring changes in ExoPD-L1 levels. For eight patients, we studied in detail whether PD-L1 levels in circulating exosomes could be used as a potential marker of response to therapy. At key points during different therapies, tumour progression/response was evaluated by medical imagery scanning and, in parallel, we quantified ExoPD-L1 in blood samples. As shown in Figure 5(a–d), tumour response detected in the scan was associated with a decrease in the level of circulating ExoPD-L1 and, inversely, tumour progression in the scan associated with an increase in circulating ExoPD-L1. Interestingly, this inverse association between ExoPD-L1 level and response to the treatment was observed independently of the therapy used (anti-PD -1 or targeted therapies). These encouraging results open the possibility of using exosomes expressing PD-L1, which can be easily isolated from blood samples during cancer patients' follow-up assessments, as predictors of response to the treatment.

Discussion

In this study aiming to explore ExoPD-L1 as a potential tumour biomarker in melanoma cells and patients, we first confirmed previous reports showing that PD-L1 was found in exosomes including on their surface [14]. However, in contrast to what was reported by Liao X et al [23], we found that PD-L1 within the exosomes was detectable and measurable in all patients examined in our EXOMEL cohort but soluble PD-L1 could not be detected (or at very low levels). Soluble PD-L1 is probably not a reliable marker as compared with ExoPD-L1 and the most likely explanation is that in exosomes, PD-L1 is stabilized by the cholesterol-rich membrane [10]. We demonstrate that while PD-L1 within the circulating exosomes was detected in all 100 patients of the cohort (although at different levels, providing a rationale for a cut-off value), only 67% of tumour biopsies were positive for PD-L1. This lack of

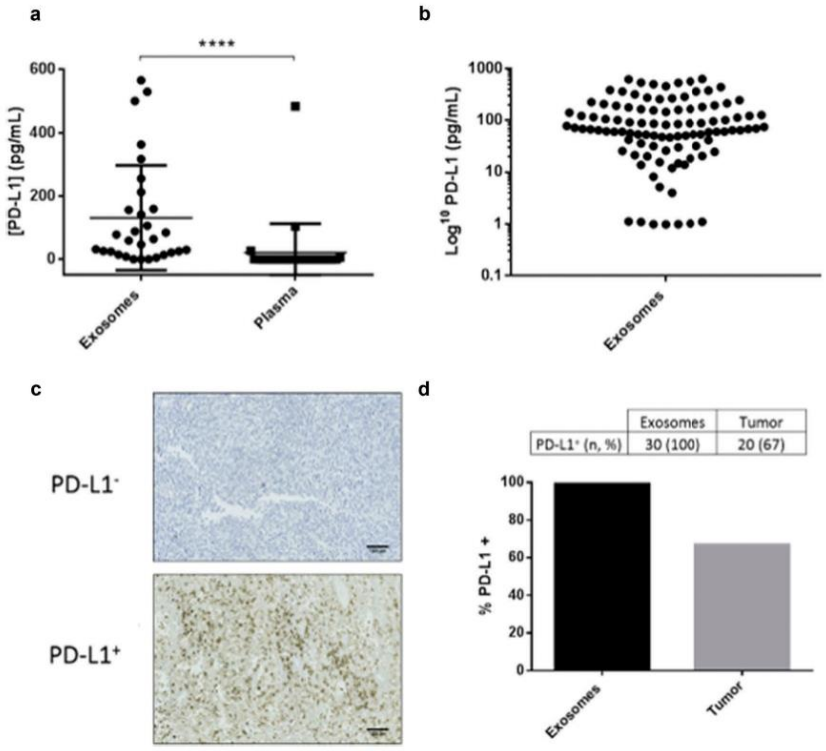
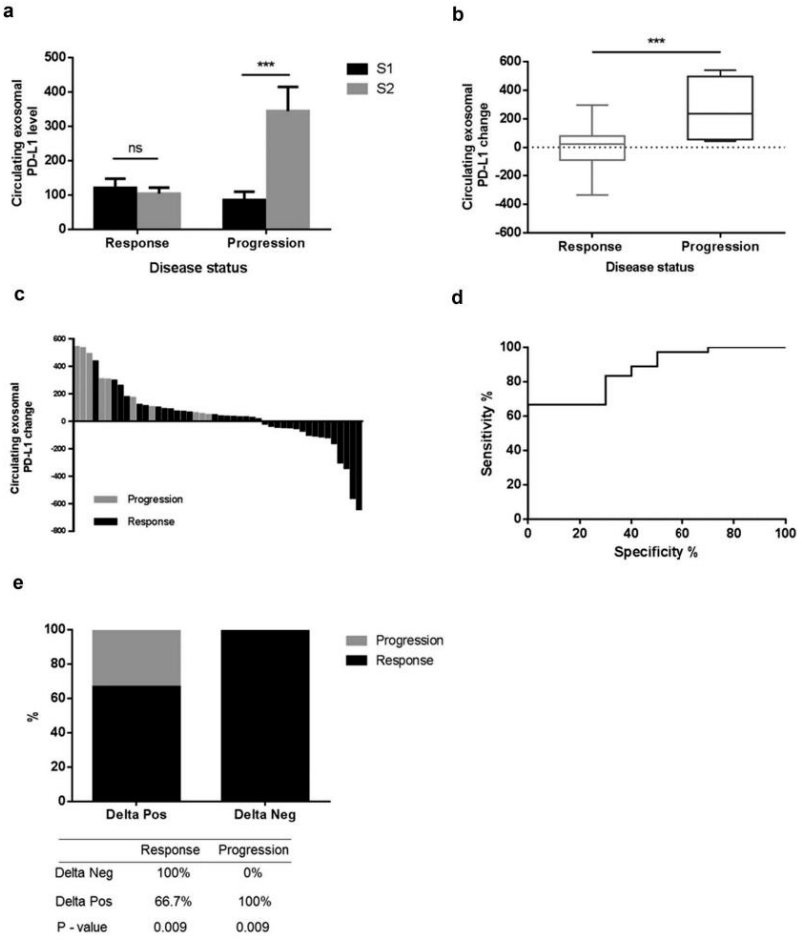


Figure 2. PD-L1 is easily detected in exosomes, when compared with soluble PD-L1 in plasma or in tumour biopsies. (a) Levels of PD-L1 in exosomes isolated from the plasma of melanoma patients compared with PD-L1 levels free in the plasma ($n = 30$) (****$p < 0.0001$), determined by ELISA. (b) Levels of ExoPD-L1 isolated from the 100 patient plasma samples of the EXOMEL cohort. (c) Representative IHC image of PD-L1 negative (PD-L1$^-$) or positive (PD-L1$^+$) tumours (22C3 antibody). Scale bars indicated 100 µm. (d) Percentage of patients positive for PD-L1 when measured in circulating exosomes versus tumour biopsies.

reliability on tumour biopsies can be explained by heterogeneity and dynamic PD-L1 expression changes within the tumour. Moreover, not only do circulating exosomes provide a reliable way to quantify PD-L1 levels, but also they allow for a non-invasive procedure with a blood sample. This is a clear advantage considering that biopsies have inherent risks. The abundance of circulating ExoPD-L1 can be explained by the fact that a single cancer cell can release thousands of exosomes, thereby resulting in a signal amplification [24]. In addition, other cells such as myeloid-derived suppressive cells, macrophages, or dendritic cells can also release exosomes with PD-L1 [22,25–29,30], thereby contributing to the overall number of circulating exosomes expressing PD-L1 observed in patients' blood. The identification of each subpopulation of circulating exosomes expressing PD-L1 with cell-specific markers may help to improve the use of PD-L1 as a circulating predictor of tumour growth.

We also demonstrate here that PD-L1 is present at the exosome membrane in the supernatant of cancer cells (but at very low levels in normal cells) and in the blood of cancer patients (but not in healthy donors). The exosomes expressing PD-L1 are as efficient as the cancer cells from which they derive to render T-cells anergic, thereby blocking immune surveillance. These observations are consistent with the work of Chen et al [17]. We can hypothesize that, through exosomes, PD-L1 not only acts on tumours and close tumour microenvironments but also at distal sites. ExoPD-L1 can, therefore, play a key role in immunosuppression involving PD-1/PD-L1 by targeting T lymphocytes in secondary lymphoid organs. Although there are no data concerning the circulating ExoPD-L1 threshold beyond which immunosuppression is induced,



	Response	Progression
Delta Neg	100%	0%
Delta Pos	66.7%	100%
P - value	0.009	0.009

Figure 3. Changes in the level of ExoPD-L1 stratify melanoma patients according to disease status. (a) Mean value of circulating ExoPD-L1 evaluated at S1 and S2 in patients grouped according to disease status (response, $n = 36$) or progression, $n = 10$). Mann–Whitney (***$p = 0.0002$). (b) Comparison of changes in level of ExoPD-L1 in melanoma patients between S1/S2, according to disease status. Mann–Whitney (***$p = 0.0002$). (c) Waterfall plots showing changes in level of ExoPD-L1 between S1/S2, according to disease status. Black bars represent responder patients ($n = 36$) and grey bars represent progressive patients ($n = 10$). (d) ROC curve analysis of changes in level of ExoPD-L1 (S1/S2) in responder patients compared with non-responders (AUC = 0.867, SE 0.057, CI95% 0.755–0.978; $p < 0.001$). (e) An increase in ExoPD-L1 is associated with disease progression $p < 0.009$. A decrease in ExoPD-L1 is associated with response $p < 0.009$. All patients with a decrease in level of ExoPD-L1 experienced a tumour response and all patients with an increase in level of ExoPD-L1 experienced progression.

this observation provides a rationale to combine PD-1/PD-L1 inhibitors with a targeted therapy.

In agreement with these results, analysis of the EXOMEL cohort indicates that the level of ExoPD-L1 inversely correlates with the response to the therapy: A high increase in ExoPD-L1 is associated with tumour progression while a decrease is associated with tumour regression, as determined by imaging scan analysis. The rate of circulating PD-L1 might reflect anti-tumour immunity involving CD8 + T-cells elicited by different therapies including, but not exclusively, checkpoint inhibitors. Since it has been reported that the tumour mutation burden

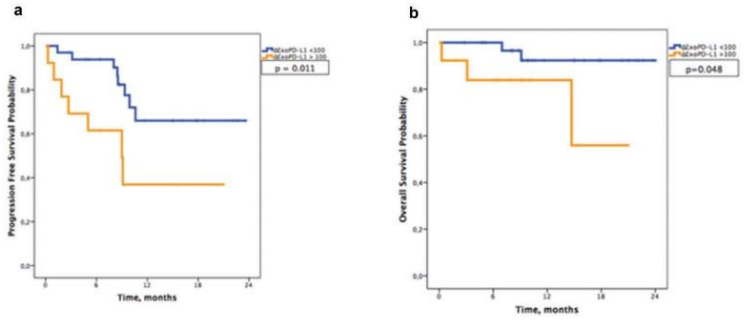


Figure 4. Melanoma-derived ExoPD-L1 can be used as a marker of survival. Kaplan–Meier estimates of (a) progression-free survival (p = 0.011) and (b) overall survival (p = 0.048) in patients according to ΔExoPD-L1 (n = 46).

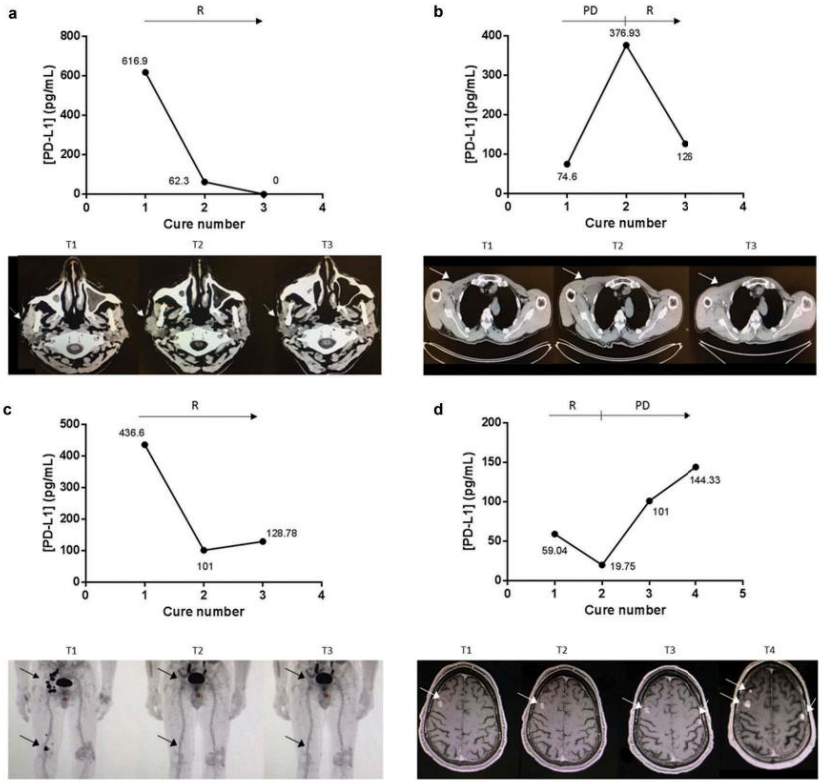


Figure 5. ExoPD-L1 can be used as follow-up markers in melanoma patients. Case study of the correlation between ExoPD-L1 levels in plasma samples from melanoma patients and response to PD-L1-based therapy. Concomitant imaging and ExoPD-L1 sampling in four patients experiencing (a) response as observed in the parotid gland and cervical lymph nodes on CT scan; (b) complete response in the subcutaneous tissue, popliteal and ilioinguinal lymph nodes on PET-CT; (c) disease response in the muscle and infraclavicular lymph nodes on CT scan; (d) initial response (at cure 1) then disease progression as detected in the brain on MRI. R: response, PD: progression of the disease. Tumour metastases in the scans are indicated by an arrow.

(TMB) is positively associated with the response to therapy [31,32], it will be interesting to correlate ExoPD-L1 levels with patient TMB.

Conclusions

The present study offers the rationale for using ExoPD-L1 as a predictor of treatment response in melanoma patients, with the advantages of its non-invasive collection and real-time monitoring. It has already been suggested as a marker of PD-1/PD-L1 response [14,17]. In this work, we go further and demonstrate in a larger and prospective cohort that ExoPD-L1 may be a marker of different therapies (immuno- or targeted-therapy). We are currently conducting a multicenter clinical study with melanoma patients to establish precise cut-off values. The interest in ExoPD-L1 as a marker of patients' response to therapy undoubtedly goes beyond melanoma and might be useful in other cancer types in which tumour evasion from T-cell surveillance plays a crucial role in patients' survival and response to therapy, such as with lung cancer. The ability to capture Exo-PD-L1 directly from blood using a microchip requiring only a few microlitres of blood demands urgent investigation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Trial registration

AC-2015-2496/DC-2014-2086

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ANNEXE 2

Membrane-anchored heat-shock protein 70 (Hsp70) in cancer

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review

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Mini-review

Membrane-anchored heat-shock protein 70 (Hsp70) in cancer

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ABSTRACT

Hsp70 is a highly conserved and inducible heat shock protein that belongs to the *HSP70* family of molecular chaperones and plays a central role in protein homeostasis. The main function of Hsp70 is to protect cells from physiological, pathological and environmental insults, as it assists an ATP-dependent manner the process of protein folding. Since Hsp70 provides critical cell survival functions, cancer cells are assumed to rely on this chaperone. Strong evidence suggests that Hsp70 is upregulated in different type of cancers and is involved in tumor growth, invasion, migration and resistance to anti-cancer therapy. Interestingly, this Hsp70 upregulation induces Hsp70 re-location into plasma membrane. In this review, the role of Hsp70 in cancer will be discussed focusing particularly on the extracellular membrane-bound Hsp70. The mechanism by which Hsp70 is translocated to plasma membrane of tumor cells and the recent discoveries of drugs targeting this Hsp70 in cancer therapy will be also highlighted.

1. Introduction

Heat shock protein-70 (Hsp70 also called Hsp72 or HSPA1) is a stress-inducible 70-kDa molecular chaperone of the highly conserved *HSP70* family [1]. The main physiological functions of Hsp70 within the cell are: (1) folding of both nascent and denatured proteins to native state, (2) refolding of intracellular aggregated proteins, and (3) degradation of aggregated proteins by ubiquitin proteasome system or via the process of molecular chaperones-mediated lysosomal autophagy [2]. It can also contribute to several cellular homeostatic events including the transportation of macromolecules like proteins and RNA, secretion, stimulation of signal transduction, regulation of transcription factors, cell division, migration, and differentiation (Fig. 1A) [3]. Structurally, Hsp70 has two distinct domains: a nucleotide-binding domain (NBD) and a substrate binding domain (SBD), where the unfolded peptide binds. Although the molecular mechanism by which Hsp70 assists the folding of denatured proteins into their native state still remains enigmatic, the common suggested mechanism is mainly assigned to the binding of the chaperone to hydrophobic residues of client proteins thereby preventing intermolecular interactions in an ATP-dependent manner [4,5]. In normal unstressed cells, Hsp70 is expressed at low or undetectable levels. However, its expression is upregulated in response to different types of cellular stress particularly protein damaging conditions like heat shock, oxidative stress, hypoxia, altered pH, and heavy metals [6,7]. A large number of studies including from us describe a cytoprotective role of Hsp70 toward different types of cell death like caspase-dependent/-independent apoptosis, necrosis or autophagic programmed cell death [8–15]. In addition, Hsp70 can be localized on the endolysosomal membrane of transformed cells and plays a major role in the resistance of the cancer cells to lysosomal cathepsine-induced cell death (Fig. 2B) [16]. In cells overexpressing Hsp70 such as cancer cells, Hsp70 can translocate to plasma membrane or can be extracellularly secreted. Although the function of membrane Hsp70 is still largely unknown, it may provide an additional stress inducible role for Hsp70 such as stimulation of antitumor immune responses [10]. Extracellular Hsp70 has been shown to have a protective role against injury in different tissues such as liver and spleen since it stimulates the immune system to remove the unwanted cells from circulation [17]. As Hsp70 may confer survival advantage to tumor cells, several studies revealed Hsp70 major contribution to cancer cell resistant to chemotherapy. This includes *in vitro* resistance to cisplatin in prostate cancer [18], imatinib in chronic myeloid leukemia [19],

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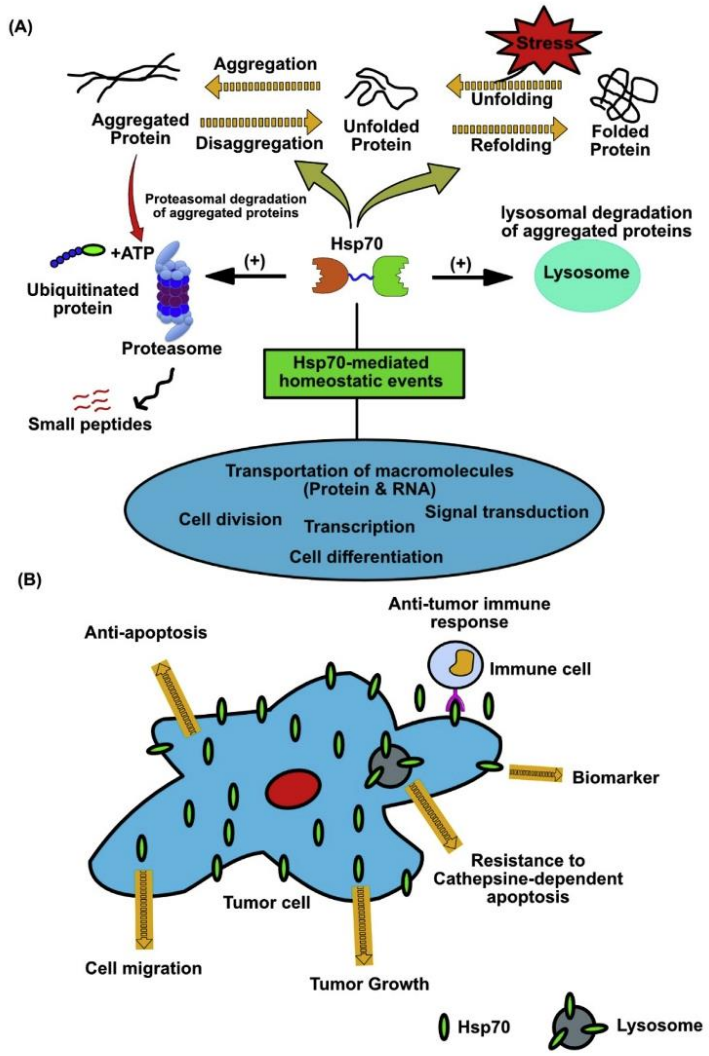

Fig. 1. (A) Illustrating the physiological functions of Hsp70: assists protein folding into its native form, proteasomal degradation of aggregated proteins, transport of client proteins across the cellular membrane, and some other homeostatic events. (B) The suggested role of Hsp70 in different type of cancers. Overexpression of Hsp70 in cancer cells can mediate several oncogenic events like anti-apoptotic response, antitumor immune response, tumor growth, and cell migration.

topotecan and gemcitabine in fibrosarcoma [20], and oxaliplatin and 5-fluorouracil in colon cancer [21,22]. Therefore, Hsp70 inhibition can provide a novel therapeutic strategy to treat different types of cancers by targeting several oncoproteins and signaling pathways required for cancer initiation and progression. In this review, the role of membrane-anchored Hsp70, particularly in cancer, the mechanism by which Hsp70 translocated to plasma membrane and its pharmaceutical targeting in cancer therapy will be discussed.

2. Overexpression of Hsp70 in tumor cells

Tumor cells because they have to re-wire their metabolism have a strong need of chaperones for their survival particularly Hsp70. Besides, tumor micro environment (TME) is considered to be a setting where cells are subjected to various types of environmental, physiological and pathophysiological stress including elevated level of oxidative stress, lack of nutrients, hypoxia and increased expression of mutant proteins. These factors are believed to play a central role in cancer initiation and progression [23] and contribute to the overexpression of Hsp70 in different types of cancer cells including hepatocellular

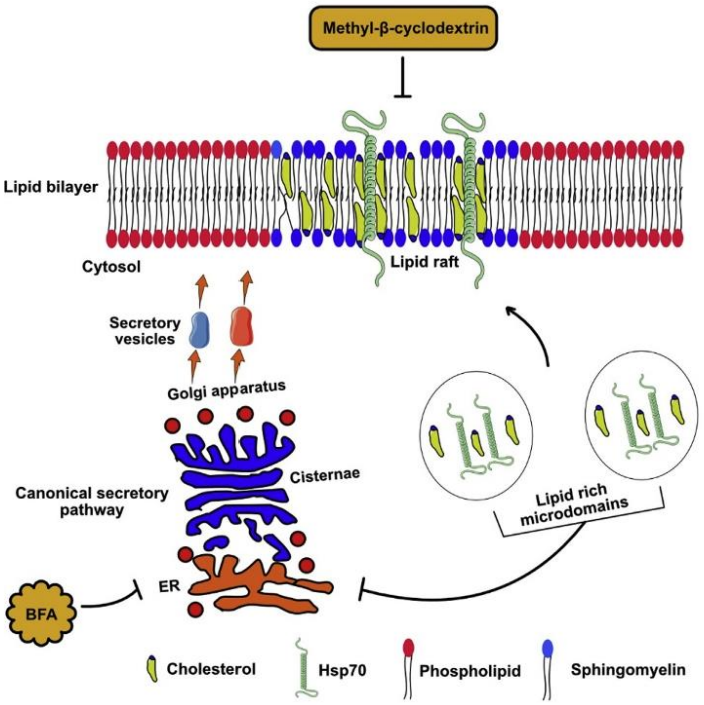

Fig. 2. Illustrating the suggested mechanism by which Hsp70 is translocated to plasma membrane. As Hsp70 lacks a consensual sequence necessary for its transportation across plasma membrane through the canonical secretory pathway, it is believed that Hsp70 can be transported within detergent-resistant lipid rich microdomains (DRM). Methyl-β-cyclodextrin can inhibit Hsp70 release, but not classical secretory pathway inhibitor like brefeldin A (BFA).

carcinoma [24], cervical cancers [25], and acute myeloid leukemia [26].

Several *in vivo* and *in vitro* studies revealed that increased expression level of Hsp70 significantly stimulates both tumor growth [27–33] and cell migration [34–39] of different tumor cells (Fig. 2B). Correlation linking Hsp70 increased expression level to cancer development and progression prompted several research scientists to consider Hsp70 as a putative diagnostic biomarker for poor prognosis in cancer. For instance, Hsp70 was reported to be a potential diagnostic marker in hepatocellular carcinoma [40], prostate cancer [41], esophageal adenocarcinoma [42], lymph node metastasis in colorectal carcinoma [34], lung [43], ovarian [44] and breast cancers [45]. Hsp70 expression level has been associated to the clinical stage in melanoma [46], oral cancer [47], and to overall survival in bladder cancer [48]. Moreover, poor survival and prognosis in several tumors like acute myeloid leukemia, breast, and cervical cancers is greatly linked to high staining of Hsp70 in biopsies [49–51].

3. Membrane Hsp70

3.1. Mechanism of Hsp70 translocation to the cytoplasmic membrane

The expression of inducible Hsp70 in the membrane of various tumor cells, but not normal cells, was reported by several studies in response to the aforementioned stressful conditions [52–54]. The molecular mechanism by which Hsp70 is translocated to plasma membrane still remains under investigation. However, it is well reported that when anchored, the chaperone only leaves extracellular a 14-amino acid loop (aa 450–463) located in its C-terminal domain (TKDDNNLLGRFELSG, termed TKD) [55]. Hsp70 protein lacks a consensual sequence/signal peptide for the canonical (ER/Golgi) secretory pathway. Moreover, the translocation of Hsp70 across the plasma membrane of tumor cell is unaffected by the common inhibitor of the canonical secretory pathway such as brefeldin A (BFA) [56,57]. Here we summarize the suggested mechanisms for Hsp70 translocation to cancer cells plasma membrane (Fig. 2).

The influence of lipid rafts as an alternative molecular transport system on the delivery of Hsp70 to the cell membrane was studied. The isolated soluble and detergent-resistant microdomain (DRM) fractions from colon cancer Caco-2 cell line revealed the localization of Hsp70 in the DRM fraction. Exposure of cells to heat shock treatment obviously increased the Hsp70 expression and its efficient translocation to DRM. It has been also revealed that Caco-2 cells are able to release Hsp70 into culture medium under heat stress conditions. Treating with classical secretory pathway inhibitors did not affect this result. Interestingly, Hsp70 release was disrupted by treating cells with the cholesterol sequestering agent methyl-β-cyclodextrin (MβCD). This result supports the significant impact of DRM on Hsp70 membrane association and release [57]. Similarly, Hunter-Lavin and colleagues [58], also confirmed the Hsp70 release in PBMCs under normal culture conditions. Viable cell count and lactate dehydrogenase enzyme assay indicated that Hsp70 release occurs in the absence of cell damage. Indeed, Hsp70 release was inhibited by treating the cells with MβCD, but not the classical secretory pathway inhibitor BFA. The interaction of Hsp70 with an artificial membrane bilayer mainly consisting of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) was investigated. The results displayed a rapid interaction of liposome-incorporated recombinant Hsp70 with the artificial membrane, accompanied by an opening of ion channel conductance. In addition, Hsp70 was detected on the surface of the plasma

membrane of heat-stressed HepG2 using immunostaining with FITC conjugated cmHsp70 monoclonal antibody that targets the TKD peptide of Hsp70. These results indicate that Hsp70 might translocate to the plasma membrane within DRM [59,60]. It has been reported that both Hsp70 proteins, i.e. the stress inducible (Hsp70) and the constitutively expressed (Hsc70), have the ability to interact and to aggregate in an artificial liposome, mainly consisting of phosphatidylserine (PS) in a time and a protein concentration-dependent manners. These results suggested a role for the heat shock proteins in the proper folding of membrane proteins and in client proteins translocation across the membrane [61]. Liposome insertion assay detected the interaction of Hsp70 with liposomes enriched in PS. The authors also reported high molecular weight Hsp70 oligomers following insertion into the lipid bilayer. Moreover, Hsp70 accumulation on the liposomal surface was increased in the presence of PS-enriched saturated fatty acids. The results of this study suggested that Hsp70 interaction with the plasma membrane may favor a rigid membrane structure [62]. Hsp70 can specifically interact with PS moieties on the membrane of pheochromocytoma PC12 cells in the early apoptotic phase. This was accompanied by rapid Hsp70 integration into the cell membrane. Addition of Hsp70 to the medium resulted in a significant reduction of PC12 cell viability. Hsp70 binding to PS was disrupted following cell treatment with annexinV. This suggested a role for Hsp70 in the phospholipid flip flop during early apoptotic cell death [55]. It has been reported that the interaction of Hsp70 with liposomes depends on their lipid compositions. A weak binding of Hsp70 to the liposomal surface that mainly consisting of phosphatidylcholine (PC), was reported. Fluorescence microscopic analysis revealed the insertion of both tryptophan residues W90 and W580 of Hsp70 in the liposome bilayer hydrocarbon region. In contrast, Hsp70 was found to peripherally bind to a liposomal surface mainly consisting of phosphatidylserine, cardiolipin, or bis-monoacylglycero phosphate [63]. In heat-stressed primary and differentiated adipocytes, Hsp70 was found to be localized within a lipid droplet enriched in a triacylglycerol, cholesterol ester, and surrounded by a monolayer of phospholipids. Alkaline treatment of adipocyte revealed that Hsp70 may bind to droplet surface monolayer through noncovalent interactions. Results suggested the essential role of Hsp70 stabilizing the droplet monolayer, protein transport to lipid droplet, and proper folding of denatured protein on the surface of lipid monolayer [64].

3.2. Membrane-anchored Hsp70 in tumor cells

Membrane Hsp70 in cancer cells was reported to have additional stress-inducible roles such as induction of cell survival and contribution to antitumor immune response. These properties are often associated to the membrane Hsp70 extracellular 14 amino acids TKD sequence [55,65,66]. In this context, the role of membrane-associated Hsp70 in the pathogenesis of cancer was investigated. Flow cytometry analysis revealed a significant increase of Hsp70 surface expression level on both dysplasia and carcinoma cells via *in vitro* enhancement of the cytolytic activity of NK cells in oral [67], colon cancer [68] and fibrosarcoma MethA tumors [69]. Increased expression level of another Hsp, Hsp90, was also observed in the membrane of poorly differentiated tumor cells as well as in advanced clinical stage of tumors (T3/T4). *In vitro* activation of NK cells by both Hsp70 peptide (TKD) and interleukin-2 (IL-2) followed by reinfusion of activated cells, greatly enhanced NK cells cytolytic activity against Hsp70 membrane-positive colon carcinoma cells [67]. Activation and maturation of dendritic cells were also found to be mediated by Hsp70 via the TRL4 pathway [70,71]. Elevated Hsp70 level enhances the proliferative responsiveness of T-lymphocytes toward Hsp70 positive in osteosarcoma cell lines [72]. It has also been reported that Hsp70 is localized on the endolysosomal membrane of CX2 human colon carcinoma cells and plays a major role in the resistance of cancer cells to lysosomal cathepsine-induced cell death. Depletion of Hsp70 significantly enhanced lysosomal membrane permeability and subsequent release of hydrolyzing enzymes to the cytosol, suggesting a pro-survival function of Hsp70 in colon cancer [16].

3.3. Membrane Hsp70 in tumor-derived exosomes

Accumulating evidence suggests that Hsp70 can be secreted through extracellular vesicles (EVs), particularly exosomes. These are nanovesicles (50–200 nm) generated within the endosomal compartment by most eukaryotic cells [73,74]. It is well known that tumor cells produce larger number of exosomes when compared to normal cells and the content of the tumor-derived exosomes (TDEs) is quite different from normal cells-derived exosomes. One of the specific surface biomarkers associated with TDEs is the stress protein Hsp70 [75].

It has been reported that Hsp70 interacts with death silencing domain (Bag4) on the surface of colon (CX+) cancer cells-derived exosomes. Localization of Hsp70/Bag4 on the exosomal surface significantly stimulated NK cells migration and cytolytic activity against a Hsp70-positive tumor [76]. Considering possible vaccination strategies against hepatocellular carcinoma, it has been reported that exosomes derived from the chemotherapeutic resistant cell line HepG2 possessed high amounts of Hsp70 both within exosomes and on exosomal membrane. Moreover, exosomes bearing Hsp70 showed immunoregulatory properties by stimulating NK antitumor response and production of granzyme B. Since expression of the inhibitory receptor CD94 was upregulated, the expression level of activating receptors CD69, NKG2D, and NKp44 was decreased [77]. The ability of TDEs to stimulate antitumor immune response was analyzed in an engineered myeloma (J558HSP) cell line. Cells were manipulated to express the endogenous P1A tumor antigen and membrane-bound Hsp70. It was revealed that exosomal Hsp70 derived from such tumor cell line stimulated the maturation of dendritic cells accompanied by an upregulation of CD40 and CD80 (Fig. 3). Furthermore, immunization of BALB/c inbred mice with TDEs enriched with Hsp70 leads to stimulation of T-helper cells and $CD8^+$ cytotoxic T-lymphocytes suggesting exosomal Hsp70 antitumor activity [78].

Hsp70-enriched exosomes derived from heat-treated CT26 mouse colon carcinoma cells significantly enhanced antitumor effect of TDEs via the expression of MHC II in an *in vitro* antigen presenting cell model. Investigating the antitumor effect in an allogenic mouse model, authors found a stimulation of Th1-polarization followed by an increased production of IgG2a and Interferon gamma (IFN-γ) [79]. A study was conducted to test the immunomodulatory effect of tumor-derived

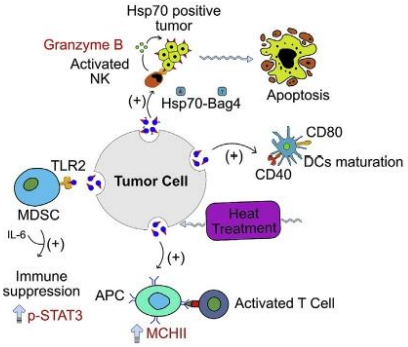

Fig. 3. Illustrating the suggested role of tumor-derived exosomal Hsp70 in cancer. The presence of Hsp70 on the membrane of TEDs may exert an antitumor immune response via NK cells activation, maturation of DCs-mediated T-cell cytotoxicity, and overexpression of MCHII on the surface of APCs. It can also enhance tumor growth via stimulation of the immune suppressive activity of MDSC and overexpression of phosphorylated STAT3.

exosomal Hsp70 on the suppressive activity of myeloid-derived suppressor cells (MDSCs). It was shown that TDEs enriched in membrane-bound Hsp70 triggered MDSCs activation. This occurred via association of Hsp70 to the toll like receptor 2 (TLR2) on MDSCs, resulting in up-regulation of phosphorylated signal transducer and activator of transcription 3 (STAT3) and autocrine secretion of IL-6 (Fig. 3). These effects were no longer observed if exosomes previously treated with a Hsp70 specific antibody or peptide aptamer binding to the extracellular domain (TKD) of membrane-bound Hsp70. Moreover, treatment of tumor cells with amiloride decreased exosomes production led to the enhancement of the antitumor effect of the chemotherapeutic drug cyclophosphamide [75]. TDEs from the supernatants of Renca cell cultures were found to exhibit more Hsp70 on the membrane. In accord with the previous reports, addition of these Hsp70-exosomes to MDSCs stimulated proinflammatory cytokines activation, tumor growth factors production, and tumor progression. However, this observation was totally reversed when exosomes were preincubated with an anti-Hsp70 antibody. The effect of exosomal Hsp70 on the stimulation of MDSCs suppressive activity via STAT3 phosphorylation was confirmed in renal carcinoma. This mechanism might provide a future therapeutic approach [80]. Our previous results indicated that several cancer cell lines including breast, lung, and ovarian cancer were able to produce high amount of exosomes when compared to their normal counterparts. Moreover, interferometric analysis of exosomes using the Hsp70 aptamer A8 indicated a significant Hsp70 expression on the surface of TDEs from a wide panoply of cancer cell lines, compared to their normal counterparts where membrane Hsp70 remained undetected. Combined treatment of tumor-bearing mice with cisplatin/A8 or 5-fluorouracil/A8, but not single treatments, induced a complete regression of tumor that was linked to the inhibition of Hsp70 immuno-suppressive activity, suggesting that Hsp70 role in MDSCs activation may contribute to its overall effect on tumor growth [22].

4. Membrane Hsp70 as a promising target in cancer therapy

The higher metabolic activity of tumor cells associated to the overexpression of oncogenes, mutated proteins and down regulation of tumor suppressor genes explains the essential role of chaperones like Hsp70 in tumor cells survival [81]. Targeting membrane Hsp70 can provide a novel strategy in cancer immunotherapy. Since it is well reported that the amount of Hsp70 in the membrane is dependent on the total cellular amount of Hsp70 (approximately 10% of cytosolic Hsp70 is anchored to plasma membrane [10,22]), here, we will discuss both direct inhibitors, which target the extracellular Hsp70 TKD domain, and indirect inhibitors that target intracellular Hsp70.

The first molecule described targeting specifically inducible Hsp70 was a neutralizing peptide, which contained the apoptosis inducing factor (AIF) domain that interacts with Hsp70. This peptide, known as ADD70 for AIF-derived decoy for Hsp70, was found to sensitize rat colon cancer cells (PROb) and mouse melanoma cells (B16F10) toward apoptosis-induced by cisplatin (see Fig. 4). ADD70 had the ability to bind the substrate binding domain (SBD) of Hsp70 blocking Hsp70-AIF association and subsequent cytoprotective activity [84,85]. 2-Phenylethynesulfonamide (PES), also known as Pifithrin-μ, is a chemical molecule that blocks Hsp70 by binding to its SBD thereby preventing Hsp70 association with several client proteins like APAF-1 and p53 and its co-chaperone Hsp40 [16]. The molecular mechanism by which PES triggers tumor cell death was mainly attributed to induction of apoptosis, degradation of the misfolded proteins and destabilization of lysosomal membrane [86]. The combined treatment between Pifithrin-μ and oxaliplatin greatly enhanced the cytotoxic effect of oxaliplatin against both colon (HCT116 and LoVo) and prostate (LNCaP, PC-3, and DU145) cancer cell lines [87]. In additions, PES displayed an obvious cytotoxic effect in several leukemia cell lines in combination with the histone deacetylase inhibitor vorinostat (SAHA) or with the Hsp90 inhibitor, geldanamycin analog (17-AAG) [88]. A similar interesting

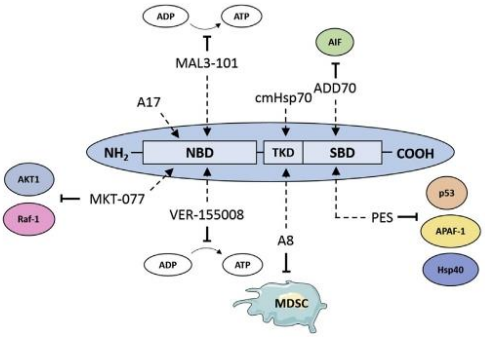

Fig. 4. Compounds targeting Hsp70 in cancer therapy. Most of the inhibitors bind to the nucleotide binding domain (NBD) blocking ATPase activity like MAL3-101, A17 aptamer, MKT-088, and VER-155008. The extracellular motif of membrane Hsp70 (TKD) is recognized by the monoclonal antibody cmhsp70 and the aptamer A8. Finally, Pifithrin (PES) and ADD70 target the substrate binding domain (SBD) of Hsp70.

combinational effect was obtained when PES was associated to the proteasome inhibitor bortezomib [89] or MG-132 to treat primary multiple myeloma cells [90]. MAL3-101 is an allosteric Hsp70 inhibitor targeting the nucleotide binding domain (NBD) by inhibiting its ATPase activity. It showed an anti-proliferative effect against breast (SK-BR-3) cancer [91], multiple myeloma [89], and Merkel cell carcinoma [92] cell lines. The adenosine-derived compound VER-155008, a specific Hsp70 inhibitor that blocks NBD, was found to induce apoptotic cell death in colon carcinoma cells [93], and to exert a significant reduction of cell viability in both melanoma [94] and colon cancer [93] cell lines, when combined with the Hsp90 inhibitor tanespimycin. The rhodacyanine dye analog of MKT-077 is also an allosteric Hsp70 inhibitor that binds the NBD. This compound revealed a marked cytotoxic effect in breast cancer MDA-MB-231 and MCF-7 cell lines via destabilization of the chaperone client proteins Akt1 and Raf1 and subsequent apoptosis induction [95].

In a more targeted approach, the monoclonal antibody cmHsp70 was designed to specifically bind the extracellular TKD domain of membrane Hsp70. This antibody showed an antitumor effect in colon tumor bearing BALB/c mice resulting in a significant reduction of tumor growth and an enhancement of overall survival. Moreover, these effects were associated to an increase of NK cells, macrophages, and granulocytes tumor infiltration. The cmHsp70 has also shown efficacy in combination with radiotherapy in NSCLC patients [10]. Concerning Hsp70-peptides-based vaccines, several studies reported the immunogenic potential of oncogenic peptides chaperoned by Hsp70. These peptides showed specific immune response against cancer indicating their broad application in clinical trials. For instance, a Hsp70-peptide complex purified from cell lysates of autologous tumors were evaluated in clinical trials (phase I and II) against different cancer types including malignant melanoma, chronic lymphatic leukemia, glioblastoma, colon carcinoma, NSCLC and HCC. The results revealed a significant immune response in a large number of patients treated either with the Hsp70-peptide complex alone or in combination with certain agents like GM-CSF and IFN-γ [97]. Peptide aptamers were also developed to inhibit Hsp70 as novel lead compounds in cancer therapy. For example, A8 is a peptide aptamer that binds to the extracellular TKD domain of membrane Hsp70. As a result, A8 blocks MDSCs activation by TDEs expressing Hsp70 on the surface. Treatment of tumor-bearing mice with A8 has been shown to induce a marked decrease of tumor growth by decreasing number and the activation of splenic MDSCs. Furthermore, this treatment has also been reported to improve

the development of an antitumor immune response via enhancement of the number of anti-tumor macrophages (M1-like) and $CD8^+$ T cells. When combining with chemotherapeutic treatments such as cisplatin or 5-fluorouracil, the antitumor effect of A8 was further improved [22]. A17 is another peptide aptamer that binds Hsp70 ATP-binding domain, has been shown to exert cytotoxic effects when combined to chemotherapeutic drugs cisplatin in different cancer cell lines and to induce cell death and tumor regression in B16F10 tumor bearing mice [96]. Studies combining A8, to develop an efficient anticancer immune response, and A17 to induce cancer dell death, are currently under investigation. Another phase I clinical trial investigated the stimulatory effect of Hsp70 peptide TKD along with interleukin-2 (IL-2) on NK cells in patients with advanced colorectal and non-small cell lung cancer (NSCLC). Results evidenced that NK cells killed Hsp70-positive tumor cells after activation with TKD and low doses of IL-2 [67]. Recently, targeting membrane-anchored Hsp70 in glioma malignant cells by magnetic nanoparticles (MNPs), such as the biocompatible superparamagnetic iron oxide nanoparticles (SPIONs) loaded with cmHsp70, was found to increase the retention of the nanoparticles within the tumor cells [98,99]. Another *in vitro* study indicated that conjugation of cmHsp70 to gold nanoparticles (AuNPs-Hsp70) enhanced both targeting and uptake of AuNPs-Hsp70 within the membrane Hsp70-positive mouse colon carcinoma (CT26) cells [59,100]

5. Concluding remarks

The stress inducible molecular chaperone Hsp70 shows a high level of expression in a great variety of cancers, explaining why TDEs express Hsp70 in their membrane. The process by which a part of cytosolic Hsp70 re-locates to the plasma membrane is still a matter of debated issue, as the protein is deprived of a signal peptide enabling transport via the canonical secretory pathway. Several studies suggested Hsp70 transportation within lipid rich microdomains. Hsp70 can therefore be used as a diagnostic marker for cancers. Clinical results so far indicate that Hsp70 is present in tumor liquid biopsies, notably in circulating TDEs, and is associated with a poor prognosis. From a functional point of view Hsp70 has been involved in cancer initiation and progression. The signaling pathway induced by Hsp70 seems to depend on the type of cancer cells and stress conditions. This include uncontrolled cell proliferation, cytoprotective effect, enhanced cell survival and cell migration. The expression of Hsp70 on the surface of cancer cells was also reported to have additional roles in cancer development. Pharmaceutical targeting of Hsp70 has become an interesting trend towards the development of a novel therapeutic strategies against different types of cancer. Several compounds are now under preclinical evaluation. Molecules specifically targeting membrane Hsp70 (antibodies and peptide aptamers) might have particular interest. Those molecules block Hsp70 activation of MDSCs thereby inducing an efficient anti-cancer immune response. They could be used in combination with any anti-cancer therapy inducing Hsp70 (most chemotherapeutic drugs or Hsp90 inhibitors). Those peptides or antibodies could be used both for therapeutic and diagnosis purposes (to detect circulating Hsp70, particularly nanovesicules expressing Hsp70 on the membrane). These molecules may open new avenues for the use of Hsp70 as a target in cancer theranostics.

Author contributions

ME, JG, and CG conceived the idea for the review. ME and MC searched the literature and wrote the manuscript. ME and MC generated the figure panels. GC and VV edited the final version of the manuscript.

Declaration of competing interest

The authors declare no financial conflict.

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ANNEXE 3

Exosomal miRNA: Small Molecules, Big Impact in Colorectal Cancer

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review

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Review Article

Exosomal miRNA: Small Molecules, Big Impact in Colorectal Cancer

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Colorectal cancer (CRC) is one of the major causes of cancer-related deaths worldwide. Tumor microenvironment (TME) contains many cell types including stromal cells, immune cells, and endothelial cells. The TME modulation explains the heterogeneity of response to therapy observed in patients. In this context, exosomes are emerging as major contributors in cancer biology. Indeed, exosomes are implicated in tumor proliferation, angiogenesis, invasion, and premetastatic niche formation. They contain bioactive molecules such as proteins, lipids, and RNAs. More recently, many studies on exosomes have focused on miRNAs, small noncoding RNA molecules able to influence protein expression. In this review, we describe miRNAs transported by exosomes in the context of CRC and discuss their influence on TME and their potential as circulating biomarkers. This overview underlines emerging roles for exosomal miRNAs in cancer research for the near future.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in men and the third in women in Europe [1]. 772,000 newly diagnosed cases were registered in 2018, and the estimated number of CRC-related deaths is 242,000. Recently, despite the development of therapies revolutionizing cancer treatment like immune checkpoint inhibitors (e.g., anti-PD-1, anti-PD-L1, or anti-CTLA-4 antibodies), clinical prognosis in CRC remains unsatisfactory, with a 5-year survival rate neighboring 13% at the metastatic stage IV of the disease [2]. An area of study carrying hope for future therapies is the understanding of the relationship between patient prognosis and tumor landscape in primary colorectal tumors. Genetic and epigenetic background of the tumor, as well as tumor microenvironment (TME) composition, are the main factors explaining heterogeneity of response to therapy observed in patients. The TME contains many cell types including stromal cells, immune cells, and endothelial cells. The resulting intra- or intertumoral heterogeneity is of prime importance for all aspects of tumor metabolism and explains the differences in tumor abilities to proliferate, invade, and escape therapy [3–6].

In this context, exosomes are emerging as major contributors in cancer biology. Exosomes are lipid-bilayer, cup-shaped nanovesicles (diameter: ~50–150 nm) secreted by cells and originating from the endosomal pathway. Exosome release is a common mechanism, and a broad range of cells secrete exosomes, including tumor cells. As a result, exosomes have been detected in a wide variety of biological fluids (e.g., blood, urine, saliva, malignant ascites, and breast milk) [7, 8]. Cumulative evidence suggests that exosomes

can establish a fertile environment to support tumor proliferation, angiogenesis, invasion, and premetastatic niche formation. Moreover, they may also facilitate tumor growth and metastasis by inhibiting immune surveillance and by increasing chemoresistance via removal of chemotherapeutic drugs. It has been often reported that tumor cells generate more exosomes than normal cells and that circulating exosome levels are increased in the blood of cancer patients when compared to healthy individuals [9–11]. These features make exosomes interesting reservoirs of potential cancer biomarkers such as proteins, lipids, and RNAs. Although there are some CRC tumor markers used worldwide, there is a particular need for new biomarkers due to technical constraints concerning their detection [12]. In this context, exosomes have become in the last few years an important area of research.

Given their role in TME, exosomes have an essential function in cell-to-cell communication, but they also have specific biological functions. The bioactive cargos received by a recipient cell can modify its physiology by tempering with a vast range of processes [13–17]. Exosomes are implicated in tumor cell proliferation [18], increased migration and invasive properties [19–21], resistance to chemotherapy [22], angiogenesis [23], and escape from the immune system [24]. Although miRNA proportion in exosomes may drastically change depending on the physiological context, tissue, or cell type, they often represent one of the predominant RNAs contained in exosomes [25–27]. Exosomes protect miRNAs from degradation, enabling them to be stably expressed in the extracellular space and to be efficiently integrated by specific recipient cells [28]. Consequently, exosomal miRNAs are also deeply implicated in cancer progression. Therefore, modification or inhibition of exosomal miRNAs might be a potential therapeutic strategy in cancer. In this review, we focus on the impact of miRNA on TME in CRC. First, a description of miRNAs and their biogenesis will be presented, followed by a description of exosome biogenesis and composition. We will conclude by a description of the action of exosomal miRNAs in CRC.

2. miRNAs

miRNAs are short single-stranded noncoding RNAs, with a size varying generally between 18 nt and 25 nt (usually 22 nt), that possess the ability to bind complementary target messenger RNAs (mRNAs). miRNAs can induce either translational repression or sometimes degradation of their mRNA targets, thereby constituting a crucial part of post-transcriptional regulation of mRNA expression. Several studies reported the importance of miRNAs in cancer progression, including tumor proliferation, invasion, migration, cell survival, regulation of the immune response, angiogenesis, epithelial-mesenchymal transition (EMT), and cellular stemness [29–35].

In the canonical pathway, miRNAs are at first expressed by the RNA polymerase II as immature stem-loop structure-containing precursors, known as pri-miRNA, of a few hundred to several thousand nucleotides long [36]. However, some pri-miRNAs can be transcribed by RNA polymerase III and some, like miRtrons, are not issued from dedicated transcriptional units but are matured from mRNA introns. A whole cellular machinery is devoted to their processing and nucleocytoplasmic export into functional cytoplasmic miRNAs. First, pri-miRNA precursors are processed into smaller stem-loop pre-miRNAs (approx. 70 nt) by the Microprocessor complex [37]. This complex consists of the Drosha protein, carrying the RNAse activity and DGCR8, that helps determining the proper endonucleolytic cleavage site [38, 39]. Pre-miRNAs are then recognized and exported to the cytoplasm by Exportin-5, where they undergo further endonucleolytic cleavage at the extremities of the stem structure by the RNAse Dicer [40]. The resulting product corresponds to a duplex of 2 complementary miRNAs, the leading strand miRNA or 5p miRNA (formerly at the 5′ extremity of the pre-miRNA) and the passenger strand or 3p or star (∗) miRNA (formerly at the 3′ extremity of the pre-miRNA). This duplex is loaded into a protein complex containing notably Argonaute protein (Ago2), which retains only one of the 2 miRNA strands to form the functionally active RISC complex [41]. The miRNA within RISC complex can recognize and bind to a crucial guide sequence in the target mRNA, located in the vast majority of cases in the 3′-untranslated region (3′-UTR). This sequence, called "seed," corresponds typically to the position 2 to 8 at the 5′ extremity of the miRNA [42]. Mostly, miRNA pairing with its target is rather imperfect and leads to translational repression or destabilization of the mRNA target [43, 44]. Occasionally, complementarity with the mRNA target is almost total, leading to mRNA cleavage and degradation [45]. As of today, there are around 2,000 entries for human miRNAs in the miRBase database (v22.1) (http://www.mirbase.org/index.shtml). Even if the function of most of them is still unknown, miRNAs are predicted to target most existing mRNAs. Over the years, evidence for their involvement in almost all biological processes accumulated, especially concerning their ability to target oncogenic or tumor suppressor genes in multiple cancer-related cellular pathways [46, 47]. miRNAs are present in significant proportions in blood (and several biological fluids such as saliva, urine, and semen), either incorporated in nucleoprotein complexes with Ago2 protein, nucleophosmin1 protein, within high-density lipoproteins (HDL) particles, or finally encapsulated within exosomes or other extracellular vesicles (EVs) [48–50].

3. Exosome Biogenesis and Composition

Exosome biogenesis is initiated by inward membrane invagination of early endosomes to form intracellular multivesicular bodies (MVBs) and then released into the extracellular environment by MVB fusion with the plasma membrane (Figure 1). They differ from other EVs, like ectosomes, which are created by outward budding of the plasma membrane, and apoptotic bodies created during the apoptosis process [51]. Using complex signaling and molecular machineries, like the Endosomal Sorting Complex Required for Transport (ESCRT), newly forming exosomes can incorporate various biologically active molecules. These

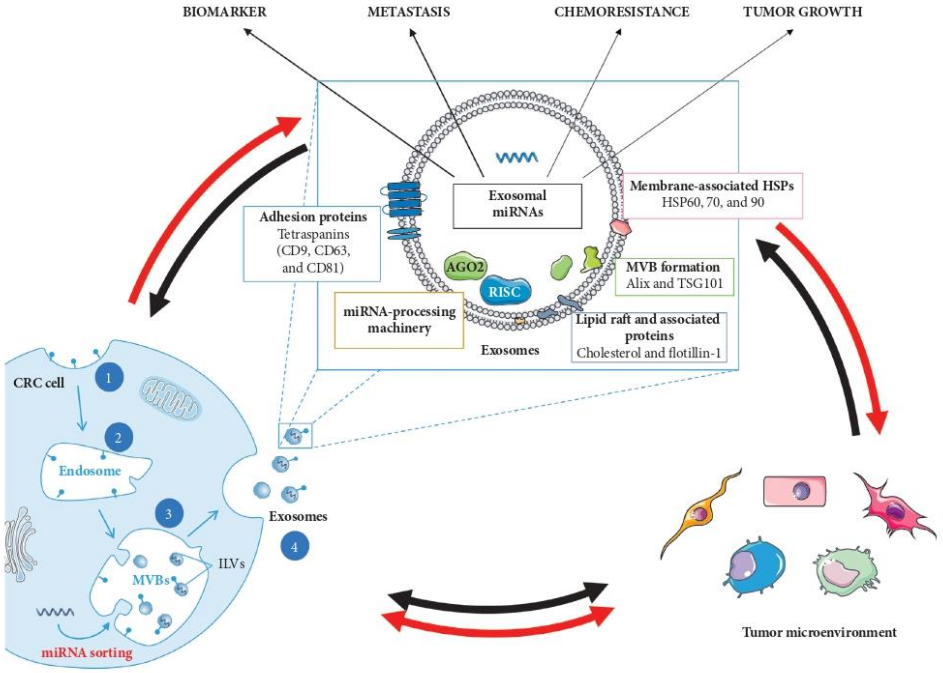

FIGURE 1: Scheme of exosome biogenesis, composition, and major role in TME modification, in the context of CRC. The biogenesis of exosomes involves 4 different steps: (1) the membrane invagination; (2) endosome formation; (3) generation of the exosome precursors, called intraluminal vesicles (ILVs), by inward budding of endosomes (these accumulations of ILVs are termed as multivesicular bodies (MVBs)); and (4) the fusion of MVBs with the plasma membrane release the ILVs in the extracellular space by exocytosis and become exosomes. Composition: exosomes are composed of different types of enzymes and proteins involved in adhesion, intracellular signaling, immunostimulatory molecules, multivesicular body (MVB) formation, and heat shock proteins (HSPs). Exosomes contain nucleic acids, including miRNA, mRNA, DNA, and small noncoding RNA (snRNA and tRNA). In addition to direct interactions between CRC cells and TME, exosomes, especially exosomal miRNAs, play a key role in the cross talk between cells in TME. CRC cells can release exosomes that will modify TME cells and promote tumor growth, metastasis formation, and chemoresistance. Inversely, stromal cells can also release exosomes that influence tumor cell metabolism. Differential expression of miRNAs within exosomes could also be useful in CRC as biomarker for diagnosis and monitoring.

include different types of nucleic acids and soluble and transmembrane proteins [52, 53]. Among the proteins present in secreted exosomes, some are involved in its biogenesis, like tetraspanins (CD9, CD63, and CD81), Tsg101, and Alix (Figure 1). These proteins are often used as markers, validating exosome enrichment during exosome isolation. Coupled to exosome physical-chemical characteristics (size, density, and buoyancy), they can help discriminating exosomes from other EVs and extracellular particles [54, 55]. Besides, exosome membranes are enriched in lipids (e.g., ceramide, cholesterol, phosphatidylserine, and sphingolipids) and lipid rafts, also playing an important role in their biogenesis and conferring exosomes reinforced rigidity compared to plasma membrane [53]. In particular, ceramide accumulation resulting from conversion of sphingomyelin by sphingomyelinases participates in the formation MVBs [56]. Exosomes also contain proteins that play a functional role in cellular communication, like in antigen presentation. Proteins of the molecular histocompatibility complex (MHC) and various heat shock proteins (Hsp60, Hsp70, and Hsp90) are present in exosomes [57–62]. The incorporation of secreted exosomes into the recipient cell takes place by several mechanisms including macropinocytosis, phagocytosis, endocytosis, or interaction through surface receptors [63, 64].

During their formation, exosomes naturally incorporate cytoplasmic medium. Initially, it was hypothesized to be a nonselective process, resulting in a similar miRNA concentration both in exosomes and parenting cells. Some studies using miRNA for cancer diagnosis or prognosis purposes were implicitly based on the fact that circulating exosomal miRNA levels, especially in body fluids, should reflect accurately the miRNA content of their cells of origin. However, it was rapidly shown in several contexts that the

most expressed endogenous miRNAs in tumor or normal cells were not necessarily the ones predominantly secreted into the extracellular environment [65–67]. It is to note, however, that while some miRNA proportions are very different between the cell and the released EVs, this is not always the case. For example, some miRNAs among the most commonly present in both parent cells and exosomes, and that may be potential CRC diagnostic biomarkers present in tissue, plasma, and serum, are miR-192-5p, miR-10a-5p, and miR-191-5p [68, 69].

4. Exosomal miRNAs in CRC

The way miRNAs are selectively transported into exosomes for secretion (exosomal sorting) is still not completely clear, although several mechanisms have been proposed [70]. In this section, we will address those hypotheses and the role of different types of biomolecules in miRNAs selective transport into exosomes in the context of CRC.

4.1. Role of miRNA Putative Sequence Signals. Several studies suggest the requirement of intrinsic sorting signal sequences in miRNAs, needed for their incorporation into exosomes [71, 72]. One of those sorting mechanisms was described in exosomes from peripheral blood mononuclear cells. It involves recognition of 4-bp RNA motifs, GGAG, by the RNA-binding hnRNPA2B1 protein, provided that it is sufficiently sumoylated [72]. hnRNPC and hnRNPA1, members of the same family of protein, can also bind exosomal miRNAs. Nevertheless, no associated motif has been identified. Another RNA motif, GUUG, was found to be enriched in miRNAs present in exosomes derived from a CRC cell line (SW620) and resembles the GGAG motif recognized by hnRNPA2B1 [73]. This motif was also suggested to be involved in miRNA loading, but it is not known whether it constitutes a specificity of cancer cells or if some RNA-binding proteins, like hnRNPA2B1, intervene in the recognition of this motif.

4.2. Role of Exosome Membrane Lipid Composition. It has been reported that the lipid composition of exosome membranes directly influences exosome biogenesis and composition [53, 56, 74]. This also affects miRNA sorting into exosomes. For instance, the level of neutral sphingomyelinase2 (nSMase2), regulating ceramide synthesis, can influence the quantity of miRNA exported through exosomes [70, 75]. In CRC and hepatocellular carcinoma cell lines, it has been shown that sphingomyelin phosphodiesterase 3 (SMPD3), which also generates ceramide from sphingomyelin, is also involved in miRNA encapsulation [76]. SMPD3 inhibition leads to a decrease in exosomal miRNA levels, while the intracellular miRNA level in CRC cells increases. This influence of SMPD3 was, for example, reported for mir-638, a miRNA also downregulated in exosomes of CRC patients which has been proposed as a biomarker [77, 78].

4.3. Role of Proteins Involved in miRNA Biogenesis and Functions. The miRNA maturation process is connected with miRNA export in exosomes and endosomal trafficking. Knockout of Ago2 leads to the selective decrease of certain miRNA populations in exosomes from several cell lines [79]. In addition, components of the RISC complex can colocalize with MVBs, when MVBs turnover into lysosomes is blocked [80]. In exosomes derived from different cancer cell types, all the essential elements required for pre-miRNAs processing into mature miRNAs, including Dicer and Ago2, are available [10, 81]. When transfected with *C. elegans* pre-miRNA, those exosomes were able to process this pre-miRNA into mature miRNA. This was confirmed to be a Dicer-dependent process. In contrast, miRNA maturation machinery was not detected in exosomes from nontumorigenic cancer cells. CD43, a suspected mediator of active protein transported into exosomes, is enriched in those exosomes. This protein is responsible for the increased level of Dicer, further linking exosome processing with miRNA biogenesis [82, 83]. Probably also related to miRNA biogenesis, it was observed that passenger-strand (3p) miRNAs seem predominant in CRC cell-derived EVs compared to their 5p counterparts [84].

One mechanism highlighted in CRC cells underlines a possible role of the small GTPase KRAS in miRNA sorting. KRAS mutations occur in more than a third of sporadic colorectal cancers, and it has been associated with several other cancers, in particular, regarding tumor aggressiveness [85–87]. Exosomes secreted by KRAS mutant CRC cells can induce growth and migration of wild type (WT) cells [88, 89]. KRAS mutations can influence the recruitment of Ago2, involved in miRNA maturation and secretion, into the nascent exosome [90, 91]. In particular, KRAS mutations affect exosomal encapsulation of several miRNAs implicated in CRC, such as the oncogenic miR-10b, which is selectively retained in WT KRAS-cell exosomes [90]. A higher rate of tumor-suppressor miRNA sequestration and decreased level of oncomiRs were observed in exosomes compared to their parent CRC cells [92]. This process seems to depend on the major vault protein (MVP), a proposed miRNA-binding protein responsible for sorting miRNA to exosomes that is overexpressed in multidrug-resistant cancer cells [93, 94]. Since tumor cells can selectively retain oncomiRs, it was suggested as a phenomenon favoring tumor growth and progression [90, 92]. Moreover, exosome secretion could be used as a way to discard tumor-suppressor miRNAs or other molecules that promote apoptosis, cell cycle arrest, or differentiation, thus also enhancing tumor cell growth and metastasis. This selective secretion was, for example, observed for several tumor-suppressor miRNAs, like miR-23b, miR-224, and miR-921 [95]. In that study, it was shown to be dependent on an important exosome transporter, Rab27, and to significantly affect metastasis and angiogenesis potential of bladder carcinoma cell lines. Because most studies rather focused on how the miRNAs secreted from tumor cells influence their environment, these interesting data need further investigation.

As we will see in the following sections, there are hints that these mechanisms can be disturbed during the tumorigenic process in CRC, explaining the differences systematically observed in miRNA content between exosomes from healthy individuals and CRC patients.

5. Exosomal miRNAs Influence CRC Tumor Microenvironment

Exosomal miRNAs in the tumor microenvironment (TME) have a significant influence on tumor development and progression but are also able to transfer the ability to resist to the anticancer therapy [96–98]. The following section will present the main exosomal miRNAs (exomiRs) proven to be functionally implicated in CRC tumor metabolism. These include miR-21, the miR-200 family, the miR 17~92 cluster, and miR-1246 alongside other relevant miRNAs. Information on expression, role as a biomarker, and function of each miRNA in CRC will be further detailed in the following sections. Available data are summarized in Table 1.

5.1. Exosomal miR-21 and miR-155

5.1.1. Expression and Role as Biomarker. miR-21 was the first shown to be expressed at high levels in the exosomes of 3 different CRC cell lines (HCT-15, SW480, and WiDr) [99]. Interestingly, these tumor-derived exosomes were found to be transferred to normal hepatic and lung cell types, preferred metastasis targets for colon tumors. Later, it was confirmed that miR-21 was overexpressed not only in colon tumor tissue and in liver metastases tissue, but also in plasma exosomes of CRC patients [11, 100]. Exosomal miR-21 expression in plasma has been significantly correlated to its expression on tumor tissue, but also to disease stage, occurrence of liver metastasis, and prognosis. Other studies have reported that this exomiR can be used as a biomarker in CRC [101] but also as a general biomarker of gastrointestinal cancers including esophagus, rectum, and pancreas [102].

miR-21 was systematically found in miRNA populations characterizing circulating exosomes from plasma, feces, and serum in the context of colorectal cancer, as well as in exosomes from different CRC cell lines [84]. It is thereby possible that the circulating biomarker value of miR-21 comes mostly from its presence in exosomes. Nevertheless, it was recently shown that nonvesicular Ago2-associated miR-21 was actively released from HT29 CRC cell lines and that its levels could surpass those of EV-encapsulated miRNA in the absence of chemical lysis [120].

5.1.2. Function in CRC. Exosomal signal of stromal origin, such as exosomes produced by normal fibroblasts (NOFs), can be transferred to CRC cell lines (DLD1 or SW40) and lead to an increased expression of miR-21-5p. This transfer also leads to increased phosphorylation of cell-signaling factors Erk, Akt, and Bad, resulting in an increased resistance to the anticancer drug oxaliplatin (Figure 2(a)).

Overexpression of mir-21 observed in exosomes from CRC tissues leads to a drastic reduction of endothelial progenitors cell (EPC) migration, proliferation, and invasion properties [121]. EPCs are circulating progenitor cells of different types, able to differentiate into functional endothelial cells and to participate in new vessel formation and blood vessel regeneration. This effect on EPCs occurs most likely through direct targeting of interleukin 6 receptor (IL6R) (Figure 2(b)). Since EPCs promote thrombus repair and resolution, it was hypothesized that it led to a higher incidence of deep-vein thrombosis, a prognostic factor in cancer patients.

In the context of CRC, stromal cells themselves can also release miR-21 into the TME, in agreement with previous observations based on stromal microdissections [122]. The altered cancer-associated fibroblasts (CAFs) produce miR-21 rich exosomes, both in regards to intracellular levels but also to the exosome content of NOFs. This increased expression in exosomes is associated with an increase in liver metastasis. These data were confirmed *in vivo* in mouse orthotopic xenografts (Figure 2(c)) [103].

miR-21 is enriched in exosomes produced by M2 macrophages, as is the oncomiR miR-155 [104]. M2 macrophages serves as *in cellulo* model for tumor-associated macrophages (TAMs) present in the TME. These macrophages promote proliferation, invasion, and metastasis of cancer cells, angiogenesis, and immune escape [123]. In CRC cells, both miR-21 and miR-155 are able to target the transcriptional regulator BRG1, resulting in increased migration and invasive behavior (Figure 2(d)). Exosomal miR-21 and miR-155 were thus suggested to be partly responsible for TAM's effects on CRC cells.

5.2. Exosomal miR 17~92 and 25~106b Clusters

5.2.1. Expression and Role as Biomarker. Members of the 17~92 miRNA cluster (miR-17, -18a, -19a, -19b-1, -20a, and -92a-1) were detected in high proportions in exosomes from the LIM1863 CRC cell line, alongside the members of its paralog cluster miR 106b~25 (mir-25b, -93, and -106b) [84]. Interestingly, miR-17, -19a, -20, and -92a are specifically enriched in exosomes as compared to several of their parent CRC cell lines, indicating their potential importance in exosomal communication [106]. miR-19a, -19b, and -92a are also upregulated in serum exosomes of CRC patients compared to those of healthy individuals, which has been linked to liver metastasis recurrence [9]. miR-19a-5p, in particular, was also suggested as a convincing biomarker for CRC severity and lymph node metastasis appearance and prognosis. The upregulation and biomarker value for disease stage of miR-19a-5p, as well as miR-19a-3p and miR-17-5p, were confirmed in serum exosomes of CRC patients [101, 106]. Moreover, miR-17-5p, -18a-5p, -19a/b-3p, -20a-5p, and -92a-1-5p expression is significantly upregulated in exosomes derived from metastatic CRC cell line SW620 compared to exosomes derived from the nonmetastatic SW480 cell line [107].

5.2.2. Function in CRC (miR-25-3p). Concerning the precise role of those two miRNA clusters in exosomal regulation, the main set of evidence comes from miR-25-3p and its action on the formation of premetastatic niche. Serum-derived exosomal miR-25-3p expression has been associated with higher rate of metastases in CRC patients [23]. *In vitro* data suggest that CRC cell-derived exosomes containing miR-25-3p can enter endothelial cells and induce migration, angiogenesis, and vascular permeability. This was confirmed *in*

Table 1: Summary of exosomal miRNA with known functions in CRC. The main source of exosomes in the publications is indicated. Exosomes isolation techniques employed in the literature are indicated with their respective exosome enrichment validation procedures. Without any other mentions, plasma and serum are to be considered from human origin.

miRNA	Exosome source	Isolation technique	Exosome validation technique	Associated function	References
miR-21	(i) Cell supernatant (a) HCT-15, SW480, WiDR (b) CAFs, NOFs (c) Macrophages (ii) Serum (iii) Plasma (whole/mesenteric vs peripheral)	(i) UC (ii) UC/ExoQuick (iii) UC	(i) (a) WB : CsD81, (b) NTA/TEM/WB, (c) TEM/WB (ii) WB : CD81/none (iii) TEM/TEM + WB : Tsg101	Diagnosis biomarker, migration, invasion, liver metastasis, chemoresistance	[11, 99–105]
miR-155	(i) Plasma (whole/mesenteric vs peripheral) (ii) Cell supernatant (macrophages)	UC	(i) TEM/TEM + WB : Tsg101 (ii) NTA + TEM + WB	Migration, invasion	[104, 105]
miR-17~92 and 25~106b clusters	(i) Cell supernatant (SW480, SW620) (ii) Serum	(i) UC + OptiPrep (ii) qEV SEC/ExoQuick/UC	(i) TEM + WB (ii) EM + WB/-/TEM/TEM + WB	Diagnosis biomarker	[9, 23, 101, 106, 107]
miR-25-3p	(i) Cell supernatant (SW480, HCT116) (ii) Serum	UC	TEM + WB	Migration, angiogenesis, vascular permeability, pre-metastatic niches	[23]
miR-200 family	(i) Cell supernatant (a) SW640, SW480 (b) CCL27 (spheroid cultures) (ii) Plasma (mesenteric vs peripheral)	(i) (a) UC, (b) ExoQuick-TC (ii) UC	(i) (a) TEM, (b) None (ii) TEM, WB : Tsg101	EMT reversion marker, preventing permeation, associated with lower survival in exosomes	[98, 108–110]
miR-200b	Cell supernatant (SW480, HCT116)	UC	TEM + NTA	Proliferative activity	[98]
miR-1246	(i) Cell supernatant: (HCT116, HT29, SW480, Colo201, WiDR) (ii) Serum (iii) Plasma	UC	(i) WB : CD81/NTA/TEM (ii) WB : CD81 (iii) NTA + TEM + WB	Diagnosis biomarker, proliferation, migration, angiogenesis, pre-metastatic niches induction, TAM reprogramming	[11, 23, 102, 111]
mir-96 and mir-149	(i) Tissue (ii) Plasma	ExoCapTM + SG + FACS	TEM + WB : CD63	Tumor suppressor	[112, 113]
mir-486-5p	Plasma	Total exosome isolation kit	None	Tumor suppressor	[78, 114]
mir-6869-5p	Serum	Total exosome isolation kit	None	Prognostic biomarker, tumor suppressor	[78, 115]
mir-8073	Cell supernatant-HCT116	UC	None	Tumor suppressor	[116]
mir-193a	(i) Modified CT26 cells xenograft (ii) Serum	(i) UC + SG, pulldown (ii) exoEasy	NTA	Tumor suppressor	[92]
mir-10b	(i) Tissue (ii) Cell supernatant (HCT116)	UC + ExoQuick + exosomes precipitation solution	TEM-IG + WB	Oncogenic, CAF transformation	[117]
mir-142-3p	Cell supernatant (HCT-116, HT-29, SW480, MSCs)	UC	NTA + TEM-IG + WB	Induces stemness	[118]
mir-196b-5p	Serum	—	—	Induces stemness prognostic biomarker	[119]
mir-210	Cell supernatant (HCT-8)	Exosome precipitation solution	TEM	Induces EMT transition	[19]

EMT: epithelial-mesenchymal transition; FACS: fluorescence-activated cell sorting; MSCs: mesenchymal stem cells; OptiPrep: commercial density gradient medium; SG: sucrose gradient; TAM: tumor-associated macrophages; TEM/TEM-IG: electron microscopy (transmission/transmission coupled with immunogold labelling); UC: ultracentrifugation (may include differential centrifugation steps and eventual additional filtering); WB: western blot. Total exosome isolation kit (Invitrogen), ExoQuick (System Biosciences), exoEasy or exoRNeasy (Qiagen), qEV SEC (Izon), ExoCapTM (JSR), and exosome precipitation solution (Macherey-Nagel): commercial exosome purification solution or kits.

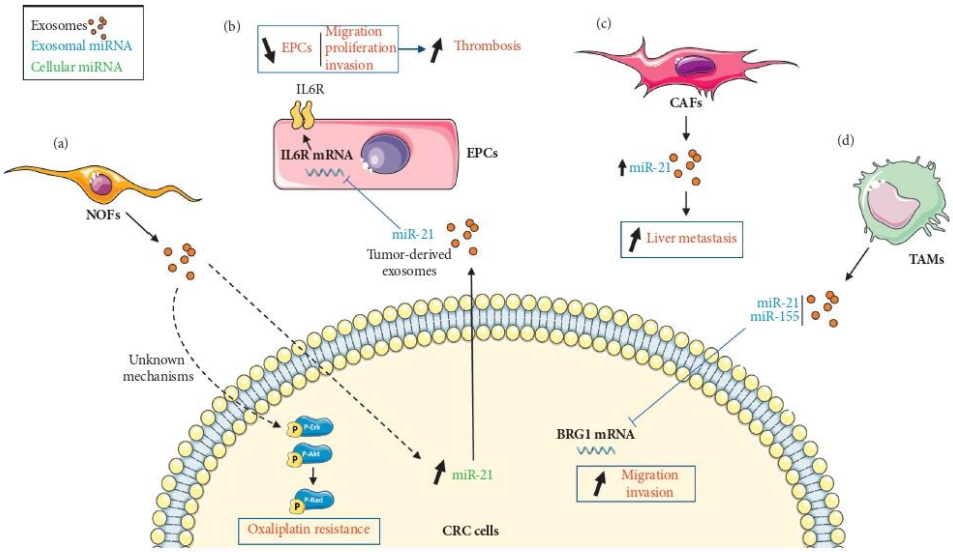

FIGURE 2: Proposed models for the role of exosomal miR-21 in CRC development. (a) Fibroblast-derived exosomes have an effect on CRC cells. The internalization of normal fibroblast- (NOF-) derived exosomes into CRC cells leads to an increase of cellular miR-21 and to the activation of phospho-Erk/Akt pathway, leading to oxaliplatin resistance. (b) CRC cells release miR-21-containing exosomes that are able to inhibit endothelial progenitor cell (EPC) IL6R mRNA transcription, leading to a reduced migration, proliferation, and invasion and favoring thrombosis in CRC. (c) Cancer-associated fibroblasts (CAFs) secrete miR-21-overexpressing exosomes which increase liver metastases. Tumor-associated macrophages (TAMs) also release miR-21-containing exosomes that can negatively regulate BRG1 mRNA in CRC cells and lead to an increased migration and proliferation.

vivo by tail vein injection of exosomes in mice, leading to a higher rate of metastases formation in liver and lungs, in a miR-25-3p-dependent manner. It was suggested to result from miR-25-3p targeting of the transcription factor KLF2. KLF2 negatively regulates expression of angiogenesis factor VEGFR2 and of KLF4, a transcription factor regulating the integrity of endothelial barrier and tight junctions.

5.3. Exosomal miR-200 Family

5.3.1. Expression and Role as Biomarker. Another important family of exosomal miRNAs in CRC is the miR-200 family, which encompasses two miRNA clusters. The first regroups miR-200a, -200b, and miR-429, and the second regroups miR-141 and 200c. Lower expression levels of miR-200c and miR-141 were significantly associated with better survival, in both the tumor draining vein (mesenteric) plasma and the corresponding exosomal fraction [124].

5.3.2. Function in CRC. On one hand, miR-141, -200c, and -429 have a protective effect against tumor progression, but only seemingly active in absence of the epithelial-mesenchymal transition (EMT), a crucial feature of cancer cells acquiring metastatic properties. Indeed, CRC metastatic cells (SW640) treated with the anticancer drug decitabine (DAC) reacquire epithelial characteristics by undergoing EMT reversal (MET). This includes inhibition of their migration and invasion properties. During this phenomenon, exosomal miR-141 and -200c expression increases, while remaining unaffected when DAC has no effect on EMT, like in the corresponding primary tumor cell line (SW480) [108]. This suggests that miR-141 and -200c expression in exosomes is negatively impacted by EMT and positively impacted by the mesenchymal-epithelial transition (MET) (Figure 3(a)).

miR-200c and also miR-141 and miR-429 are expressed in exosomes of naïve CCL27 CRC cell spheroids in 3D culture models [109, 110]. In cells surrounding the tumor, they directly target several members of the ZEB family, which are transcription factors involved in EMT (Figure 3(b)). As a result, miR-200c inhibits EMT in the lymphatic endothelial cells (LECs) co-cultured with CRC spheroids [110], and miR-200c, -141, and -429 inhibit EMT in co-cultured blood endothelial cells (BECs) [109]. Since exosomal expression of those miRNAs is lost in 5-FU (5-fluorouracil) chemoresistant spheroid cultures, surrounding cells engage in EMT transition, visibly weakening the lymphatic (LEC) and blood (BEC) endothelial barriers. By facilitating the crossing of those barriers by CRC cells, this phenomenon could explain increased metastasis occurrence in chemoresistant CRC. Thus, the data suggest that transfer of those miRNAs through exosomes contribute to preventing cell permeation into epithelia and maintaining tissue and organ integrity in normal physiological cell conditions (Figure 3(c)).

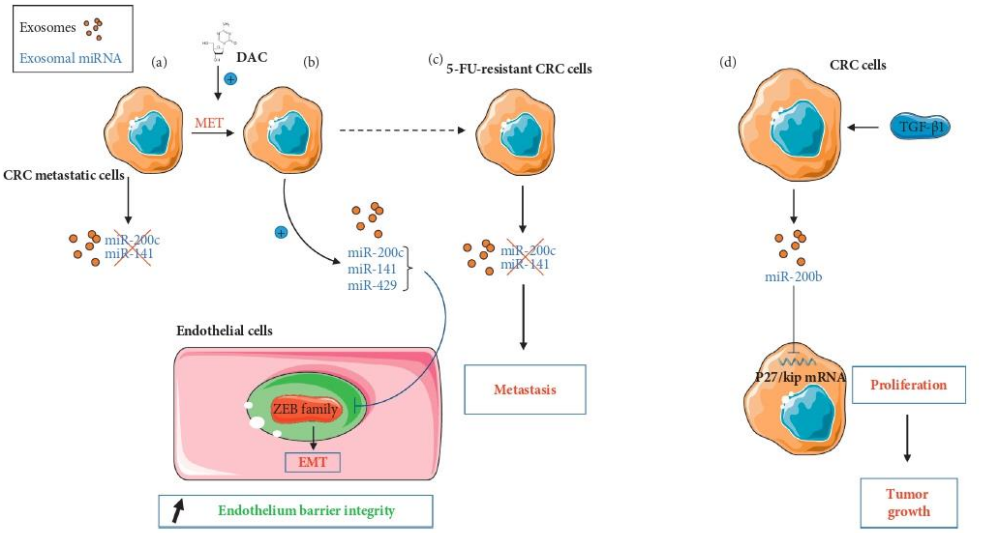

FIGURE 3: Proposed models for the dual roles of exosomal miR-200 family members on TME in CRC. (a) Upon decitabine (DAC) treatment, CRC cells enter a MET process that stimulates the release of miR-141/miR-200c enriched exosomes. (b) In endothelial cells, exosomal miR-200c, -141, and -429 can also inhibit the expression of transcription factors belonging to the ZEB family, activators of EMT. (c) On the contrary, 5-FU-resistant CRC cells release exosomes without miR-200 family members, favoring EMT. (d) CRC cells exposed to TGF-β1 release miR-200b-enriched exosomes that inhibit p27/kip mRNA, leading to an increased proliferation.

On the other hand, an oncogenic effect of exosomal miR-200b derived from CRC cells (HCT-116 and SW480) was also reported [98]. Exosomal miR-200b level is increased in cells treated with TGF-β1 in a dose-dependent manner and is responsible for the proliferative properties of the resulting exosomes, observed on another CRC cell line. These results were assigned to direct targeting of the antiproliferative cyclin-dependent kinase inhibitor 1B (p27/kip) by miR-200b (Figure 3(d)). The decrease of p27/kip expression was confirmed *in vivo* following miR-200b injection in xenograft of tumor cells. This also led to an increase in tumor size, as expected.

5.4. Exosomal miR-1246

5.4.1. Expression and Role as Biomarker. It was reported that miR-1246 is specifically upregulated in exosomes derived from several CRC cell lines and carcinoma cell lines from the cervix (HeLa), bladder (T24), prostate (PC-3), and liver (HepG2) [11, 23]. In a meta-analysis encompassing literature data from blood, urine, and other bodily fluids, it was the best performing miRNA biomarker for gastrointestinal cancers in terms of specificity and sensitivity [102]. This was in agreement with a previous high-throughput experimental study in serum exosomes, concluding that miR-1246 was the best potential miRNA biomarker for CRC diagnosis in serum together with miR-23a [11].

5.4.2. Function in CRC. Through its action on inflammation, exosomal miR-1246 holds an important role in TME. It was shown that this action was linked to the presence of p53 (*TP53*) mutations in CRC cells. These alterations are one of the most frequent genetic traits of human cancers [111, 125]. The experimental proofs obtained both *in vitro* and *in vivo* allowed to establish a model, in which the presence of *TP53* mutations in CRC cells, specifically resulting in a gain of function (mutp53), led to an increase of miR-1246 levels in exosomes [111]. Exosomal miR-1246 can induce reprogramming of macrophages towards a TAM phenotype, a hallmark of solid tumors associated with poor prognosis (Figure 4). Indeed, those mutp53-reprogrammed TAMs possess an anti-inflammatory cytokine secretion signature (e.g., IL-10, TGF-β, or VEGF). Moreover, their proinflammatory cytokine secretion (e.g., IL-8, IFN-γ, and ICAM-1) is decreased. Mutp53-reprogrammed macrophages also revealed a marked stimulation of extracellular matrix (ECM) degradation activity and enhanced migration and invasion properties. As a consequence, the anti-inflammatory, immunosuppressive, promigration, and proinvasion properties obtained by such macrophages promote tumor growth and metastatic burden to liver and lungs, as confirmed in mice hetero- and orthotopic xenograft models.

Interestingly, a pull-down experiment revealed an association of miR-1246 with hnRNPA2B1, which is suggested to be responsible for miRNA sorting in exosomes in its sumoylated form [72, 111]. The motif recognized by hnRNPA2B1 (GGAG) is carried by miR-1246, and hnRNPA2B1 sumoylation is 3 times higher in mutp53 CRC cells than in the WT CRC cells, suggesting that changes in this mechanism are involved in exosomal miR-1246 oncogenic properties.

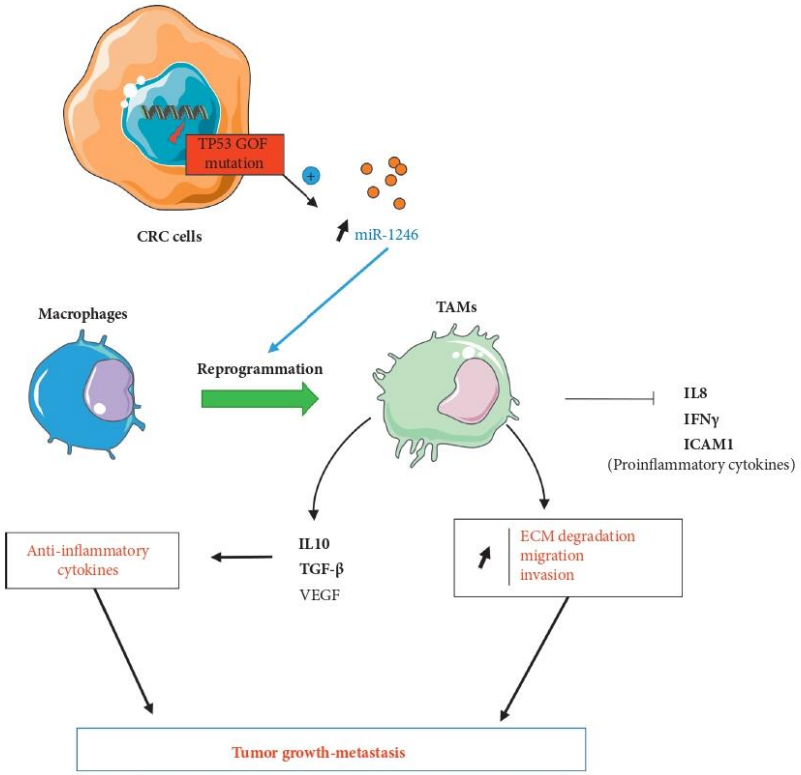

FIGURE 4: Proposed models for the effects of exosomal miR-1246 on the TME. p53 mutations resulting in gain of function (GOF) in CRC cells induce the release miR-1246-overexpressing exosomes. Exosomal miR-1246 can induce a switch of macrophage phenotype towards a tumor-associated phenotype (TAM), modifying tumor inflammatory state. It leads to a decreased secretion of proinflammatory and increased secretion of anti-inflammatory cytokines. TAMs also present enhanced ECM degradation, migration, and invasion properties. Exosomes are represented as small orange circles.

5.5. miR-149 and miR-96-5p, -486-5p, -6869-5p, -8073, and -193a: Tumor Suppressors. GPC1 (glypican-1) is a member of the heparan sulphate proteo-glycan family and is an important biomarker, found in several types of cancer (breast, pancreatic, and glioma) and involved in angiogenesis and tumor growth [126–129]. It was shown that GPC1 overexpression in CRC cells induces EMT and promotes cell invasion and migration [112]. The miR-149 gene is located within an intron in the *GPC1* gene. miR-149 and miR-96-5p are both able to directly target GPC1 mRNA, resulting in proapoptotic and antiproliferative effects in CRC cells *in vitro* and *in vivo*. Both miRNAs are downregulated in exosomes from CRC tissues or in plasma when compared to those of healthy individuals, while the exosomal GPC1 level is increased [113].

It was shown that exosomal miR-486 was upregulated within plasma exosomes and whole plasma of CRC patients. Therefore, it was suggested as a CRC diagnosis biomarker [114]. However, miR-486-5p possesses a tumor suppressor activity via inhibition of cell proliferation. Indeed, it targets directly PLAGL2, a transcription factor for β-catenin and IGF2 that promotes both proliferation and metastasis and inhibits apoptosis. Nevertheless, in CRC tissues, it has been shown that miR-486-5p expression is inhibited due to a high rate of DNA methylation of its promoter region. The consequent upregulation of PLAGL2/β-catenin/IGF2 pathway leading to proliferation and migration was confirmed in CRC cells.

miR-6869-5p downregulation was also observed in tumor tissues and serum exosomal fractions from CRC patients, and it was proposed as a potential biomarker of CRC prognosis [115]. The tumor-suppressor activity of miR-6869-5p was supported by direct targeting of TLR4, subsequently inhibiting TNF-α and IL-6 production in CRC cells via the TLR4/NF-κB signaling pathway, leading to a decrease in cellular proliferation.

While there is no difference between intra- or extracellular miR-8073 in normal colorectal cells, it is at least 60 times more

expressed in exosomes from CRC cells than in the intracellular extracts. Mizoguchi et al. demonstrated that it can directly target several factors involved in survival, proliferation, and antiapoptosis (FOXM1, MBD3, CCND1, KLK10, and CASP2), resulting in its antiproliferative properties *in vitro* and its effect on tumor growth *in vivo* [116].

Finally, miR-193a was shown to have a tumor-suppressor activity by targeting Caprin1, an upstream activator of the G1/S-specific cyclin-D2 (Ccnd2) and the proto-oncogen transcription factor c-Myc [92]. This causes G1 cell cycle arrest, leading to inhibition of cell proliferation. miR-193a is present at high levels in the exosomal fraction of CRC patients' serum, particularly in advanced stages, with high risks of metastasis. It was also demonstrated that miR-193a sorting into exosomes, which is increased in CRC, was caused by the MVP transporter [92].

5.6. miR-10b: Indirect Oncogenic Activity via CAF Transformation. miR-10b was detected in exosomes derived from multiple types of cancer cells and was particularly enriched in exosomes from CRC cells [90], but also breast cancer [130] and non-small cell lung cancer [131]. It can target directly PIK3CA, thus inhibiting PI3K/Akt/mTor pathway activity, closely associated with the inhibition of cell migration and invasion [117, 132]. Moreover, exosomes derived from CRC cells that contain miR-10b can be transferred to fibroblasts. In the target cells, this results in increased expression of TGF-β and SM α-actin. Expression of those genes are characteristics of myofibroblast-like CAF phenotype [103], and should stimulate tumor cells proliferation. miR-10b was shown to be particularly sensitive to mutation in the exosomal sorting protein KRAS, as KRAS mutations lead to a decreased incorporation of miR-10b in secreted exosomes [90].

5.7. miR-142-3p and 196b-5p: Stemness-Inducing miRNAs. Bone marrow-derived progenitors are an additional important type of stromal cells present in tumors, which are able to release cytokines and exosomes and influence TME. Bone marrow mesenchymal stem cells (BM-MSCs) are indeed able to release exosomes that increase markers of stemness (Oct4, lin28, KLF, Bmi-1, CD44, and SOX2) in CRC cells and their subsequent invasion, adhesion, and drug resistance properties [118]. It has been shown that this effect relies on the influence of miR-142-3p, present in exosomes, which can directly target Numb, an inhibitor of the Notch stem cells pathway [133]. Consequently, exposure to miR-142-3p-containing BM-MSC exosomes results in a boost of tumorigenesis and tumor metastasis, but not tumor weight and size, as shown in orthotopic grafts in mice [118].

miR-196b-5p influences stemness by targeting directly SOCS1 and SOCS3, modulators of stemness pathways, resulting notably in increased STAT3 transcription factor activity in CRC cells and tissues. It increases the production of antiapoptotic factors, like Bcl-2, Bcl-xL, and BIRC, and stem cell factor markers, like NANOG, Bmi-1, OCT4, and SOX2, and increases resistance to the drug 5-fluorouracil [119]. miR-196b-5p was confirmed to be dramatically upregulated in serum exosomes of CRC patients, in a much more distinct manner than in the whole serum, and associated with poor prognosis.

5.8. Lead on miR-210 Importance in EMT. It has been observed that a subpopulation of HCT-8 CRC cells became nonadherent after a few days of culture. Additionally, their proportion increased with culture time, and they developed chemoresistance properties by undergoing EMT [19, 134]. This metastatic-like phenotype can be spontaneously reverted in new cell-free cultures, but not in the presence of other HCT-8 cultured cells. Indeed, the reverse MET phenomenon was inhibited by exosomes produced by cultured cells. As miR-210 is significantly upregulated in HCT-8 exosomes, the authors suggested that it may play a role in MET inhibition [19].

5.9. Other miRNAs. Finally, additional miRNAs found in exosomes were also identified as potential biomarkers in CRC patients. Even if they are not described to play a role in exosomes mode of action, we tried to make a list as exhaustive as possible of the main reported ones in the current state of the art. Data are outlined in Table 2 [105, 135–139].

6. Concluding Remarks

Cell-to-cell transfer of miRNAs by means of exosomes, released by both stromal and tumor cells, plays an important role in tumor progression and metastasis. Several technical obstacles should be overcome to allow improved exosome characterization and further research in particular subjects that remain less covered. Study of miRNA targets and role in CRC provides great hopes for better understanding and characterization of tumor properties, diagnosis and personalized medicine, and innovative therapeutic approaches. These aspects will be briefly discussed in the following sections.

6.1. Limitations regarding Exosome Isolation Methods and Exosome Purity. Exosomes constitute great reservoir of biomarkers since they preserve miRNAs from extracellular environment and have dedicated roles and a specific biology. For example, in one of the first high-throughput characterization of exosomal miRNAs in CRC cells by Ji et al., almost a third of miRNAs from a subpopulation of exosomes were not reported as implicated in colon cancer before [84]. However, exosomal miRNA studies are limited due to a few technical issues. It is currently almost impossible to achieve a very high degree of purity without sacrificing yield when isolating exosomes. There are many approaches to isolate exosomes from the same medium, which are fundamentally different in their principles, resulting in different yields and degree of purity and often used according to the objectives of downstream applications [140].

Unfortunately, it has been shown that the purification method has a great impact on the exosome population

Table 2: Summary of exosomal miRNAs with a potential biomarker role in CRC or whose expression is associated with CRC diagnosis and progression. The main source of exosomes referred in the literature is indicated. Exosome isolation techniques employed in the diverse references are indicated with their respective exosome enrichment validation procedures. Without any other mentions, plasma and serum are to be considered from human origin.

miRNA	Exosome source	Isolation technique	Validation	Associated effect	References
mir-221	Cell supernatant (HT-15, SW480, WiDR)	UC	WB : CD81	Biomarker for tumor size, TNM stage, Dukes stage, lymph node metastasis, recurrence	[99, 135]
mir-215	(i) Cell supernatant (a) HT-15, SW480, WiDR (b) CAFs, NOFs	(i) UC (ii) UC	(i) WB : CD81/ NTA + TEM + WB (ii) NTA + TEM + WB	Upregulation in CAF exosomes	[99, 103]
mir-23a	(i) Serum (ii) Cell supernatant (SW48, SW480, SW620, HCT116, HT29, RKO)	(i) UC (ii) UC	(i) TEM/WB (ii) TEM/WB	Diagnosis biomarker associated with liver metastasis recurrence	[9, 11]
mir-320a, -4476	(i) Tissue (ii) Serum	UC	TEM	Associated with liver metastasis recurrence	[9]
mir-125a	Plasma	ExoQuick	SEM/none	Early tumor stage biomarker	[27, 136]
mir-320c	Plasma	ExoQuick	SEM	Higher levels in CRC patients	[136]
mir-328	Plasma (mesenteric vs. peripheral)	UC	TEM + WB : Tsg101	Liver metastasis biomarker (better performing in mesenteric vein)	[105]
mir-4472-3p	Serum	ExoQuick	TEM + WB	Diagnosis and tumor recurrence biomarker	[137]
mir-6803-5p	Serum	Total exosome isolation kit	None	Diagnosis and prognostic biomarker, associated with stage and lymph node metastasis	[138]
mir-4644	Meta-analysis	—	—	GI cancer diagnosis biomarker	[102]
mir-7641	Cell supernatant (HT-15, SW480, WiDR)	UC + OptiPrep	TEM + WB : Alix, Tsg101	Diagnosis biomarker	[107]
mir-638	Serum	Total exosome isolation kit	None	Biomarker for TNM stages III-IV and liver metastasis	[77, 78]
mir-548c	(i) Serum (ii) Plasma (mesenteric vs peripheral)	(i) Total exosome isolation kit (ii) UC	(i) None (ii) TEM + WB : Tsg101	Diagnosis and prognostic biomarker, associated with faster relapse	[78, 105, 139]
mir-let-7a, -1229, -150, -223	(i) Cell supernatant (HCT116, HT29, RKO, SW48, SW480) (ii) Serum	UC	WB	Potential diagnosis biomarkers	[11]

GI cancers: gastrointestinal cancers; OptiPrep: commercial density gradient medium; TEM/SEM: electron microscopy (transmission/scanning); UC: ultracentrifugation (may include differential centrifugation steps and eventual additional filtering); WB: western blot. Total exosome isolation kit (Invitrogen), ExoQuick (System Biosciences), and qEV SEC (Izon): commercial exosome purification solution or kits.

obtained, including on miRNA content [141, 142]. To help ensure that the obtained isolates are enriched in exosomes, several validation tests also have been proposed. These tests typically include nanoparticle tracking analysis (NTA), exosomes markers detection by western blot, or examination of exosomes by electron microscopy. Studies on the research of biomarkers, mentioned along this review, used different methods of purification, as summarized briefly in Tables 1 and 2. Both the purification method and validation of exosomal enrichment experiments have to be taken into account during result interpretation.

6.2. Nonexosomal vs. Exosomal Extracellular RNAs. Concerning the vesicle-free part of circulating miRNAs secreted by cells by other means, the involved mechanisms are still unclear. Their release could also largely result from cellular lysis. Compared to miRNAs contained in EVs, it is not clear if their role in tumor transformation and progression is negligible or not. To elucidate these roles entirely will remain difficult due to current technical impediments limiting the purity of isolated exosomes and EVs in general [53, 140]. This state of the art was recently backed up by a study in rat serum and plasma, showing that vesicle-free miRNAs are also present in EV fractions after isolation. Moreover, even Ago2-associated part of vesicle-free circulating miRNAs could result from either cellular or exosomal lysis [10, 143].

If the proportion of circulating miRNAs present in exosomes compared to free circulating miRNAs remains elusive, it seems that only a small fraction (down to 10%) of plasma miRNAs are vesicular, whereas in serum or saliva, the majority of miRNA are concentrated in exosomes [48, 49, 144, 145]. In plasma, the fraction of miRNAs present in the vesicle fraction is strongly dependent on the identity of the miRNA considered. Some, like let-7a, were found predominantly enriched in vesicle fractions compared to vesicle-free fractions, while others like miR-16 and miR-92a are preferably associated with circulating Ago2 and seemingly absent from vesicles under physiological conditions [48]. However, it is worth noting that at least in one report (in high-risk colorectal adenomas), 2 serum exosomal miRNAs were considered less efficient biomarkers than their whole serum miRNA counterparts despite their correlated expressions [146]. Although it seems that isolated vesicle-incorporated miRNAs are more stably expressed and constitute more reliable cancer biomarkers than their vesicle-free counterparts [28, 147].

6.3. Exosome Subpopulations in CRC and Their Advantages. Different CRC cell types produce different populations of exosomes. For example, it was shown that CRC cell line exosomes do not contain the same combination of tetraspanin proteins, exosomal markers involved in exosome biogenesis [99]. On the same note, Chen et al. showed that miRNA composition of SW480- and SW620-derived exosomes is significantly different, with more than a third of the miRNAs detected being differentially expressed between the 2 types of exosomes [107]. Moreover, while miRNAs are sorted into exosomes in a differentiation state and cell-type dependent fashion, several types of exosome populations with diverse morphologies have been reported to be secreted by the same cells, in particular in colorectal cancer [148, 149]. The LIM1863 CRC cell line can produce two mutually distinct populations of exosomes, one presenting A33 and the other EpCAM surface proteins, an important cancer-initiating marker in CRC and pancreatic cancer [150, 151]. Their protein and miRNA populations vary significantly between each exosome type and previously determined proteomes of other exosomes, suggesting different effects and/or target recipient cells [84, 150]. Indeed, it was shown that exosomes are tailored to target specific types of recipient cells [152, 153]. This could provide an explanation for the site-specific formation of metastases in colorectal cancer, e.g., the liver, lungs, and lymph nodes. Moreover, their compositions reflect not only this tailoring, but also regulatory events arising in the secreting cell [80]. Exosomes could thus give great advantages for both study of TME and discovery of biomarkers. Indeed, a given exosome population could thereby directly inform us about particular cell types and events they were exposed to, with great specificity. These subpopulations may contain multiple determinants of tumor metastatic potential. The complex interplay between exosome subpopulations, their specific contents, and their potential target cells needs further investigation.

6.4. A Word on Therapeutic Perspectives. Studies on exosomal miRNAs may soon be applied to the clinical setting, as new therapeutic approaches using delivery of miRNA mimics or miRNA antagonist on tumor sites are in development. Several clinical trials concerning the use of miRNAs in the treatment of CRC are currently ongoing and gain more and more interest from biopharmaceutical companies [154]. Furthermore, exosomes themselves constitute a great strategy for the delivery of those new therapeutic actors. Freely circulating miRNAs are rather instable in blood [48] and are also negatively charged, rendering delivery through cell membranes difficult even *in vitro*. Exosomes, on top of their low immunogenicity and cytotoxicity, may enhance therapy deliverability and protect molecules from RNAse activity, making them suitable therapeutic vectors for CRC treatment [155, 156]. Treatment of cells with FF/CAP18 (analog of cathelicidin LL-37), a peptide limiting cancer cell proliferation, induces production of exosomes with antiproliferative properties [157]. This effect is suspected to come from the expression of exosomal miRNAs miR-584-5p, -1202, and -3162-5p. Kyuno et al., have also shown that it is possible to tailor exosomes for therapeutic purposes by transfection with tumor-suppressor miRNAs [158]. The miRNAs in question were miR-342 and -498, which target Claudin7 (cld7) and EpCAM, respectively. Coupled with exosomal expression of ectopic Tspan8, shown to enhance internalization by cancer cells [152], it was sufficient to inhibit tumor growth, motility, and invasion, especially by affecting stemness traits.

To conclude, exosome-carrying miRNAs originating both from the stromal and the tumor cells have a major role in CRC TME. Exosome encapsulation enables miRNA expression in extracellular space and involvement in cell-to-cell communication. Therefore, miRNAs can influence cell inflammatory environment, differentiation status, proliferation, survival, migration, invasion, and angiogenesis properties. Being stably released in the circulatory system, it has been shown at least through venal injection that they can influence distant cell barrier permeability, underlining the role they play in premetastatic niche formation. The delivery of exosome cargo into specific types of target cells may also be one of the mechanisms explaining organ preference of cancer metastasis. For all those reasons, exomiRs constitute a key target for the discovery of biomarkers and new therapeutic approaches in CRC, and an important axis of research in the near future.

Abbreviations

CRC:	Colorectal cancer
TME:	Tumor microenvironment
mRNA:	Messenger RNA
EV:	Extracellular vesicle
MVB:	Multivesicular body
MVP:	Major vault protein
exomiR:	Exosomal miRNA
NOF:	Normal fibroblast
EPC:	Endothelial progenitor cell
CAF:	Cancer-associated fibroblast
TAM:	Tumor-associated macrophage
EMT:	Epithelial-mesenchymal transition
DAC:	Decitabine
MET:	Mesenchymal-epithelial transition
LEC:	Lympho-endothelial cell
BEC:	Blood endothelial cell
5-FU:	5-Fluorouracil
BM-MSC:	Bone marrow mesenchymal stem cell
MHC:	Molecular histocompatibility complex
HSP:	Heat shock protein.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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ANNEXE 4

The vesicular transfer of CLIC1 from glioblastoma to microvascular endothelial cells requires TRPM7

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The vesicular transfer of CLIC1 from glioblastoma to microvascular endothelial cells requires TRPM7

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ABSTRACT

Chloride intracellular channel 1 (CLIC1) is highly expressed and secreted by human glioblastoma cells and cell lines such as U87, initiating cell migration and tumor growth. Here, we examined whether CLIC1 could be transferred to human primary microvascular endothelial cells (HMEC). We previously reported that the oncogenic microRNA, miR-5096, increased the release of extracellular vesicles (EVs) by which it increased its own transfer from U87 to surrounding cells. Thus, we also examined its effect on the CLIC1 transfer. In homotypic cultures, miR-5096 did not increase the expression of CLIC1 in U87 nor in HMEC. However, the endothelial CLIC1 level increased after exposure to EVs released by U87, and even more by miR-5096-loaded U87. The EVs-transferred CLIC1 was active in HMEC, promoting endothelial sprouting in matrigel. Cell exposure to EVs induced cytosolic Ca^{2+} spikes which were dependent on the transient receptor potential melastatin member 7 (TRPM7). TRPM7 silencing prevented Ca^{2+} spikes and the subsequent CLIC1 delivery into HMEC. Our data suggest that the vesicular transfer of CLIC1 between cells requires TRMP7 expression in recipient endothelial cells. How the vesicular transfer of CLIC1 is modulated in cancer therapy is a future challenge.

INTRODUCTION

Extracellular vesicles (EVs) are membrane-enclosed particles released from either endosomes or the cell surface [1–3]. EVs are composed of an array of proteins, nucleic acids, lipids, and other metabolites that reflect the cell of origin. They offer an intercellular route to transfer oncogenic material that change the functions of non-malignant cells, i.e. proliferation, invasion, and angiogenesis [2]. Their secretion is correlated to the cell's ability to produce invadopodia (actin-rich cellular protrusions with proteolytic activity); i.e., inhibition of invadopodia formation decreased exosome release [3–5]. Importantly, glioblastoma (GBM)-derived EVs can cross the brain–blood-barrier and are detectable in the systemic blood circulation [6]. Profiling the composition of GBM-derived EVs may, therefore, offer a non-invasive means of assessing tumors *in situ* [4].

Studies have described extensive RNA expression analyses of GBM-derived EVs, however, proteomic profiles are currently limited [4, 7]. Among the vesicular proteins, one study identify the chloride intracellular channels (CLIC) carried by exosomes between GBM cells [8]. The CLIC family form a class of proteins that do not fit the paradigm set by classical ion channels (for review see; [9–11]). They can exist as both soluble globular proteins and integral membrane proteins with ion channel function. The first member of CLIC, namely

CLIC1 (also known as NCC27), holds pathological implications in a variety of tumors, being involved in cell proliferation, motility, and angiogenesis [12–15]. CLIC1 is overexpressed in glioblastoma (GBM), with highest expression in patients with poor prognosis [13]. CLIC1 is also secreted in extracellular vesicles (EVs) by cancer cells [8] and is detected in biological fluids [8, 16, 17], fostering the hypothesis that secreted CLIC1 protein may increase GBM growth. Interestingly, Setti et al [8] have shown that the secretion of CLIC1 via EVs is common to all human GBM cell lines (U87MG, A172, LN405, U118MG, T98G, DBTRG-05MG and U373 MG). If the number of secreted EVs differs from one type of lineage to another, the membrane markers and biophysical properties of EVs are similar.

Using U87 GBM cell line, we have recently described that miR-5096 increases the outgrowth of filopodia in glioma cells, and promotes the extracellular release of EVs by U87 thereby promoting its own transfer to surrounding cells [18]. Here, we show that EVs also contain active CLIC1 whose amount is not significantly increased by miR-5096. The transfer of CLIC1 to human microvascular endothelial cells (HMEC) requires Ca^{2+} spikes and TRPM7 for their uptake, and contributes to endothelial sprouting [19, 20].

RESULTS

Extracellular vesicles from GBM cells transfer active CLIC1 to HMEC

Both U87 and HMEC expressed CLIC1 proteins, as already reported [12, 21]. Immunoblot analysis of whole cell lysates (WCL) from homotypic cultures revealed that the cell loading with miR-5096 mimic or inhibitor did not significantly change CLIC1 expression after 48h in both U87 and HMEC (Figure 1A). This is in agreement with the absence of miR-5096 effect on CLIC1 mRNA expression (not shown) and predictions from bioinformatics tools which failed to identify any target site for miR-5096 in CLIC1 gene and mRNA. However, the endothelial CLIC1 level was increased after 24h-exposure of HMEC to U87-conditioned media (Figure 1B). We next separated EVs from the effluent (soluble fraction) of culture media as described previously [18]. In all cases, EVs and effluents were adjusted to the same number of U87 (i.e. 4 x 10^6 cells), then applied to homotypic HMEC cultures for 24h. Cell exposure to EVs released from miR-5096-loaded U87 significantly increased CLIC1 levels in HMEC, while the effluent (EVs-free) did not (Figure 1B). The immunoblot analysis of EVs showed an enrichment in the exosome specific protein tsg101 (tumor susceptibility gene 101) [8, 18] (Figure 1C). Clearly, EVs contained CLIC1 proteins and their level seemed to be higher in EVs from miR-loaded U87 than from empty-loaded U87. A possible explanation might be that miR5096 induced an increase in EVs release [18], rather than a significant increase in CLIC1 vesicular content. To confirm the transfer of CLIC1 to HMEC, endogenous CLIC1 was silenced by using *si*RNA in a series of experiments (i.e. relative OD of 0.406±0.061 and 0.015±0.007, respectively before and after CLIC1 silencing; $P<0.05$, $n=3$). As shown in Figure 1C, both cellular (WCL) and vesicular (EVs lysates) CLIC1 contents were suppressed in HMEC by CLIC1 *si*RNA. The CLIC1 immuno-labelling showed that CLIC1 was mostly found in perinuclear areas of control HMEC (Figure 1D). No labelling was observed after silencing CLIC1 in HMEC. After 24h of cell incubations with EVs from miR-5096 loaded U87, we detected CLIC1 in both the cytosol and the plasma membrane of control and silenced HMEC. We also overexpressed a fluorescent-tagged version of human CLIC1 in U87 and collected conditioned media after 48h (see Supplementary Figure 2). Exposure of HMEC to isolated EVs resulted to the fluorescent labelling of HMEC after 24h. Thus, the increase of CLIC1 in HMEC resulted more from a vesicular transfer of CLIC1 rather than an up-regulation of its endogenous expression in recipient HMEC. Are the transferred proteins active in the recipient cells? Using a 24-hour three-dimensional *in vitro* angiogenesis assay, we explored the ability of vesicular CLIC1 to induce endothelial spheroid sprouting [22]. As shown in Figure 1E, EVs stimulated HMEC sprouting even more when collected from miR-loaded U87 (M) (see also Supplementary Figure 1A). This effect was partially prevented by silencing CLIC1 in U87 (Msi). To confirm the contribution of vesicular CLIC1 to angiogenesis, EVs were also tested on branching morphogenesis *in vitro* [22]. HMEC control and CLIC1 silenced were plated in ECM gel and exposed or not (Co) to EVs collected from the same number of U87. Quantification of average number of processes per cell was performed after 12h (Figure 1F; see also Supplementary Figure 1B). Silencing CLIC1 in HMEC decreased the branching as expected for its contribution to *in vitro* angiogenesis [12]. Exposure to EVs stimulated the branching even more when collected from miR-5096-loaded U87 (M). This effect was partially prevented by silencing CLIC1 in secretory U87 (Msi). Thus, the vesicular CLIC1 was active in recipient HMEC and contributed to early steps of *in vitro* angiogenesis [12].

EVs-mediated CLIC1 transfer to HMEC requires TRPM7-dependent Ca^{2+} signaling

In order for EVs to elicit a signaling response from recipient cells, they can fuse with plasma membrane or are taken up via endocytosis or attach to the cell surface [23] (see Supplementary Figure 2). Since endocytosis, receptor internalization and trafficking are regulated by cytosolic Ca^{2+} level [24, 25], we determined whether the endothelial uptake of vesicular CLIC1 was associated with cytosolic Ca^{2+} fluctuations. Spatio-temporal Ca^{2+}

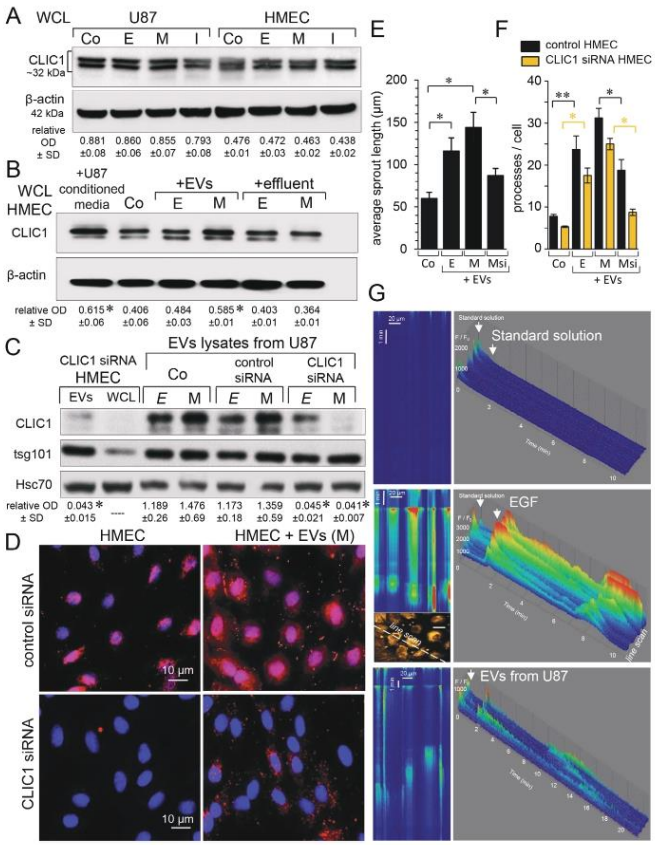

Figure 1: Active CLIC1 protein is transferred via vesicles from GBM to endothelial cells. Immunoblot analysis of CLIC1 in whole cell lysates (WCL) from homotypic cultures of U87 and HMEC, 48 h after loading. Untreated cells were used as control (Co). Cells were loaded empty (E) or with 30nM miR5096 mimic (M) or inhibitor (I). β-actin as loading control (60µg proteins/lane). Numbers indicate mean values of optical densities (OD) of CLIC1 relative to β–actin (± SD; $P>0.05$ *vs* Co; n = 3). **(B)** CLIC1 increased in HMEC after 24 h of incubation with EVs. Cell-conditioned media were collected from homotypic U87 miR-loaded (M) or not (E), 48h after loading. HMEC were exposed to EVs or effluent (soluble fraction) separated from U87-conditioned media. Numbers indicate mean OD values of CLIC1 relative to β-actin (± SD; $^{*}P<0.05$ *vs* Co; n = 3). **(C)** EVs contained CLIC1. Lysates of EVs were immunoblotted for the marker tsg101. Homotypic U87 were loaded (M) or not (E) upon transfection of control siRNA or siRNA targeting CLIC1. Silencing CLIC1 was also tested in HMEC (WCL) and HMEC-released EVs (Hsc70 as loading control). Numbers indicate mean OD values of CLIC1 related to tsg101 for EVs lysates (± SD; $^{*}P<0.05$ *vs* Co (E); n = 4). **(D)** Endothelial cell localization of CLIC1. HMEC were silenced by siRNA CLIC1 then exposed to EVs collected from homotypic U87 (M) (n = 3). CLIC1 stained with alexa Fluor 594 (red) and nuclear DNA with Dapi (blue). **(E)** CLIC1 effect on the length of endothelial sprouts formed from spheroïds in Matrigel for 24h, in the absence (Co) or the presence of EVs collected from homotypic U87 empty (E) or miR5096-loaded (M). When indicated by Msi, CLIC1 was silenced by siRNA in U87 (M). Data are means ± SD ($^{*}P$-values<0.05 *vs* control; n=10) in two independent experiments. **(F)** Contribution of CLIC1 to the branching morphogenesis in HMEC cultured in collagen ECM gel for 12 h, in the absence (Co) or presence of EVs. Histogram shows the average number of processes per cell. Control and CLIC1 siRNA HMEC are filled black and yellow, respectively. Data are means ± SD ($^{*}P<0.05$, $^{**}P<0.01$ *vs* Co; n=10) in two experiments. **(G)** EVs induced Ca^{2+} spikes in HMEC. Representative line scan images of cytosolic [Ca^{2+}] in Fluo-4-loaded HMEC exposed to the standard solution, to EGF (10 ng/ml) and to EVs (from U87) as indicated by arrows. Space and time ordinates are displayed in the horizontal and vertical directions, respectively (scan rate 22.3 µsec/line). Amplitudes of Ca^{2+} signal are expressed as the fluorescent rapport F/F_0 (pseudo-colors) in a tridimensional histogram (F/F_0 *vs* space/time). In all cases, the line scan crossed both cytosol and nuclei of 4 adjacent cells, as shown in the 1024x1024 pixel panel. Note that Ca^{2+} spikes were observed at the beginning of all recordings (due to the initial cell perfusion) and were not reproduced by reapplying the standard solution.

variations were recorded in HMEC loaded with Fluo-4 by using line scanning of confocal microscopy [26]. No Ca^{2+} spike was observed in standard external conditions (Figure 1G; upper panel). As a positive control, HMEC were exposed to epidermal growth factor (EGF), inducing a typical pattern of Ca^{2+} signal [27], i.e. a rapid increase in cytosolic Ca^{2+} which was maintained several minutes before decaying to the resting level, followed by a second transient increase observed about 6 min later. The endothelial cell exposure to EVs from U87 elicited a similar Ca^{2+} signal although with a lower amplitude than did EGF (i.e. max F/F_0 of 1000 ± 500 with EVs and 3000±600 with EGF; $P<0.05$, n=6). Of note, the lag time between successive Ca^{2+} waves was similar (6.61 ± 1.25 min; $P>0.05$; n=6). The well-known inhibitor of Ca^{2+} pathways, 2-APB [28–31], suppressed EVs-evoked Ca^{2+} signal (Figure 2A, lower panel) as well as the subsequently CLIC1 delivery to HMEC (Figure 2B). Indeed, incubations of 2-APB-treated HMEC with EVs for 24 h did not increase CLIC1 labelling, especially in CLIC1-silenced HMEC (Figure 2B). Among 2-APB-sensitive channels in plasma membranes, the melastatin-subfamily of TRP (TRPM7) is a non-specific divalent cation channel upregulated in GBM [29]. To estimate its contribution in both Ca^{2+} waves and CLIC1 delivery to HMEC, endogenous TRPM7 was silenced by using *si*RNA (Figure 2C, 2D). Cell exposure to EVs already produced cytosolic Ca^{2+} waves which were decreased by silencing TRPM7 (Figure 2E). Unfortunately, we could not knockdown both TRPM7 and CLIC1 since HMEC did not survive. After 24h of cell incubations with EVs, CLIC1 labeling was also decreased in cytosol or plasma membrane of TRPM7-silenced HMEC, attesting the partial contribution of TRPM7 in this process (Figure 2F). Of note, cell exposure to soluble fractions (EVs-free effluent) from U87-conditioned media did not increase TRPM7 levels in HMEC, while the miR5096 loading or Kir4.1 silencing did (Figure 2C, 2G). Thus, the vesicular transfer of CLIC1 to HMEC required Ca^{2+} signaling mediated, at least in part, by TRPM7.

DISCUSSION

We report here that CLIC1 protein is transferred via EVs from the GBM cell line U87 to microvascular endothelial cells where it remains active, i.e. induces endothelial sprouting. When applied onto HMEC, EVs elicit Ca^{2+} "spikes" which can be prevented by 2-APB. Although 2-APB inhibits numerous channels including IP_3 receptors [28], store-operated Ca^{2+} channels [30] and TRP channels [29, 31], silencing TRPM7 prevents Ca^{2+} spikes and the subsequent CLIC1 uptake by HMEC. Altogether, our data show that CLIC1 secreted by cancer cells via extracellular vesicles, modulates the activity of neighboring endothelial cells in a TRPM7 dependent manner, promoting tumor angiogenesis.

We previously reported that miR-5096 favors its own transfer from U87 to HMEC via an increased release of EVs, two days after its loading in U87 [18]. Here, we show that EVs also contain CLIC1 and mostly ensure its transfer to HMEC (Figure 1; see also Supplementary Figure 2). EVs are composed of an array of proteins, nucleic acids, lipids, and other metabolites that reflect the cell of origin [4]. We report that EVs, secreted by the same number of U87, produce a greater increase in active CLIC1 in the recipient HMEC when the donor U87 are previously loaded with miR-5096. The most likely explanation is that miR-5096 induces an increase in EVs release [18], rather than a significant increase in CLIC1 vesicular content. How miR-5096 exerts such an effect is currently unknown and not explored in our study. Nevertheless, evidence increasingly points to a connection between lipid metabolism and cancer, characterized by an alteration in the mechanisms that regulate cholesterol homeostasis [32]. It is known that the survival of GBM cells is dependent on uptake of cholesterol [33] in which some microRNAs are the fine tuners [34, 35]. Interestingly, cholesterol promotes the conversion of CLIC1 from cytosolic to transmembrane proteins [36], thus facilitates its docking to the membranes [37, 38]. Drawing on the data above, we propose a pure speculative model where miR-5096 increases cholesterol and CLIC1 is involved in recruiting EVs, leading to an increased secretion of EVs by U87 cells (see Supplementary Figure 3).

By overexpressing fluorescent-tagged CLIC1 proteins (CLIC-OFP) in U87, we observe CLIC1-OFP inside the invadopodia (see Supplementary Figure 2B). Upon exposure to EVs, HMEC become fluorescent and change their morphology; i.e. showing invadopodia formation. Invadopodia act as multivesicular endosome docking sites and are a site of EVs release, meaning the cell's ability to form invadopodia determines their ability to release of EVs [4, 5]. In this process, CLIC1 would contribute to the formation of invadopodia in endothelial and tumor cells, by inducing integrin-mediated actomyosin dynamic [15]. Changes in CLIC1 location from cytosolic to transmembrane proteins are associated with malignant transformation [15]. Our study does not allow to distinguish the two forms of CLIC1 (i.e. soluble form and membrane-inserted chloride conducting pore). However, immuno-labeling of CLIC1 confirms its previously described nuclear location in steady HMEC [9]. Following exposure to EVs, CLIC1 is also detected in cytosol and weakly at the plasma membrane of HMEC within 24h. On the other hand, the endothelial sprouting in matrigel is increased by EVs within 24h. This effect is partially prevented by silencing CLIC1 in donor U87 and is not attributed to miR-5096 itself [39]. Our results are in agreement with the literature showing that a low CLIC1 expression in endothelium decreases capillary-like sprouting in matrigel [12, 14, 15]. We observe a functional difference between silencing all CLIC1 and preventing

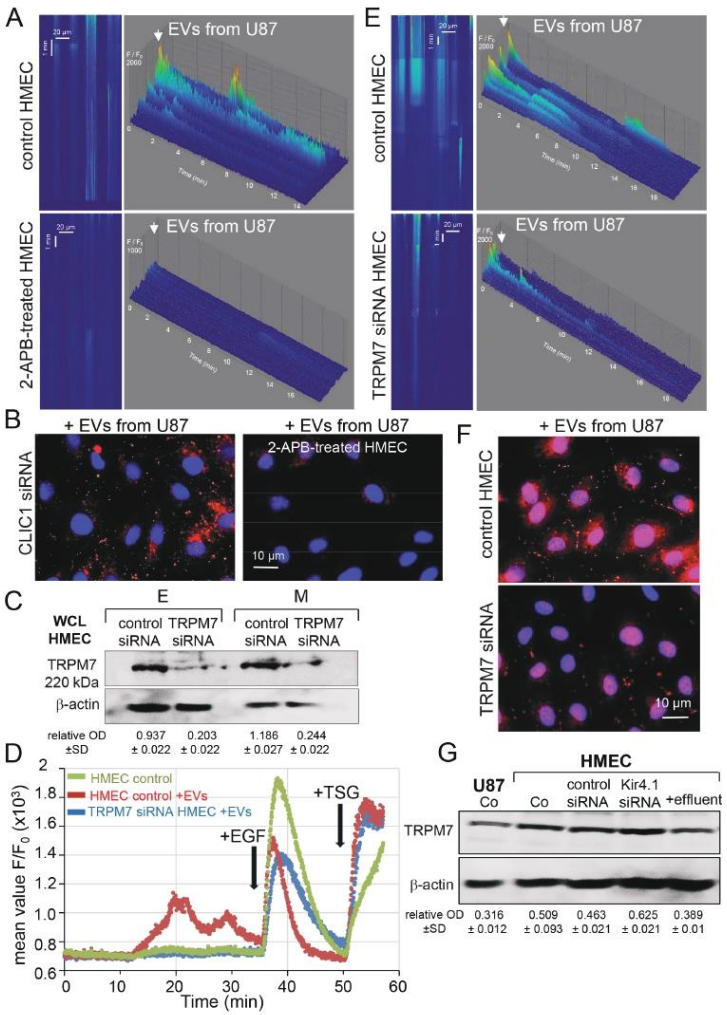

Figure 2: The vesicular transfer of CLIC1 requires cytosolic Ca^{2+} increases in HMEC. (A) Line scan images of cytosolic [Ca^{2+}] increases in Fluo-4 loaded HMEC exposed to the same amount of EVs collected from the same homotypic U87 culture. HMEC were pre-treated with 2-APB (50 µM; lower panel). Tridimensional histogram (F/F_0 *vs* space/time) are representative of 3 experiments. **(B)** Transfer of vesicular CLIC1 was blocked by 2-APB (50 µM). HMEC silenced by siRNA CLIC1 were exposed to EVs from U87 (n = 3). After 24h of incubation with EVs, HMEC were stained for CLIC1 (red) and nuclei (blue). **(C)** Expression of TRPM7 in HMEC. Homotypic HMEC were loaded (M) or not (E) with miR-5096 upon transfection of control siRNA or siRNA targeting TRPM7. Numbers indicate mean OD values of TRPM7 related to β-actin (± SD; n = 2; 80µg proteins/lane). **(D)** Spatially average Ca^{2+} profile showing the dynamic change of Ca^{2+} signals with time and induced by EVs (applied at the beginning of the records) then EGF (10 ng/ml) applied at the time indicated by arrow. Cytosolic Ca^{2+} store depletion was performed by the addition of thapsigargin (TSG, 5µM) at the end of recordings. Values are means of fluorescent ratio F/F_0 ± SD; n = 3. **(E)** Silencing TRPM7 in HMEC reduced the Ca^{2+} signal induced by EVs collected from homotypic U87 for 48h. **(F)** Control and silenced TRPM7 HMEC were exposed to EVs and stained for CLIC1 (red) after 24 h of culture (representative of 3 experiments). **(G)** Expression of TRPM7 in homotypic HMEC upon transfection of control siRNA or siRNA targeting Kir4.1 [18]. HMEC were exposed to the effluent (soluble fraction) from homotypic U87. Numbers indicate mean OD values of TRPM7 related to β-actin (± SD; n = 2).

only the U87-derived CLIC1 delivery. This cannot be easily explained since EVs probably contain proangiogenic factors, miRNAs and extracellular proteases which are required by endothelial cells to proliferate, migrate, and organize into new tubular structures [4, 40]. For instance, EVs from xenografts of glioblastomas contain an oncogenic variant of the epidermal growth factor receptor (EGFRvIII), which can be transferred to endothelial cells, producing proliferation and tubulogenesis [41, 42]. A broad array of cell surface and signaling proteins is involved in tubulogenesis [43], and could explain why the perivascular invasion is more important in vascular endothelial growth factor (VEGF)-deficient glioblastoma cells and brain tumor xenografts treated with anti-VEGF blocking antibodies such as bevacizumab [44, 45].

We hypothesize that EVs bind to recipient endothelial cells (see Supplementary Figure 4). These EVs may remain at the plasma membrane [46] or may be internalized by endocytosis either mediated by clathrin [47, 48] or via caveolae and lipid rafts [49]. These mechanisms require Ca^{2+} increase at the submembrane level [50, 51]. While the precise mechanism of CLIC1 uptake and processing in HMEC remains unclear, we show for the first time that CLIC1 transfer requires an initial Ca^{2+} signaling in recipient cells within 1h. Moreover, the EVs-induced Ca^{2+} signal is suppressed in nominally Ca^{2+}-free standard solution (i.e. no $CaCl_2$ added; data not shown), attesting the involvement of an external Ca^{2+} entry. Among the putative Ca^{2+} entry pathways, the presence of functional TRPM7 channels is known in human endothelial cells [52–55]. By using specific *si*RNA, we identify TRPM7 as a mediator for this Ca^{2+} entry needed for CLIC1 uptake by HMEC. Of note, TRPM7 contributes to the EGF-induced Ca^{2+} signal, without affecting the Ca^{2+} content of internal Ca^{2+} stores sensitive to thapsigargin (see Supplementary Figure 1C).

High CLIC1 expression is involved in the progression of GBM and other tumors [56–58] and correlates with a poor patient outcome [13]. Our data foster the hypothesis that CLIC1 transfer to endothelial cells via EVs contributes to GBM growth by promoting capillary formation [12, 14, 15, 56]. Moreover, the pharmacological inhibition or silencing of TRPM7 inhibits adhesion or invasion in cancer cell lines [19, 59–61] as well as migration of HMEC [53]. Of note, TRPM7 expression is increased in miR-5096 loaded U87 (see Supplementary Figure 1D). Because the silencing of potassium Kir4.1 channels [18] also produces this increase, this up-regulation of TRPM7 should result more from a membrane potential variation than from a direct effect of miR-5096 on TRPM7 gene or mRNA [62]. Nevertheless, it is still not clear whether other proteins and miRNAs could be transferred via EVs to modulate channels in recipient cells [63, 64]. Further investigations are required to fully resolve the functional capabilities of EVs [65–67].

MATERIALS AND METHODS

Cells

Human primary microvascular HMEC (HMVEC-D; Lonza) and U87-MG cells (ATCC HTB-14) were grown in DMEM plus 10% FCS (5% CO_2; 37°C). Cells were incubated 48 h in FCS-free media before use.

Reagents

Monoclonal anti-Tsg101, anti-Hsc70 and anti-β-actin were purchased from Santa Cruz Biotech (Clinisciences, Fr). Monoclonal anti-CLIC1 (ab77214) and anti-TRPM7 (ab109438) were from Abcam. Fluo-4 acetomethyl (AM) ester was from Invitrogen (ThermoFisher). 2-Aminoethyl diphenylborinate (2-APB), thapsigargin (TSG) and other chemicals were from Sigma-Aldrich.

Transfection

Cells were transfected by lipofectamine RNAiMAX according to the manufacturer's protocol (Invitrogen). Human hsa-miR-5096 mimic (mirVana TM miRNA, 4464066-MC22429) and inhibitor (4464084-MH22429) were purchased from Ambion (Invitrogen; ThermoFisher) [18]. Human TRPM7 siRNA (ID 1490) and CLIC1 siRNA (ID 145733) were purchased from (Ambion, AM51331). The sequences were: siRNA CLIC1 (5'-GAGCUUGUGUUGUGCUGAAtt-3' and 5'-UUCAGCACAACACAAGCUCtt-3'); siRNA TRPM7 (5'-GGACCCUCACAGAUGCCUUtt-3' and 5'-AAGG CAUCUGUGAGGGUCCtt-3'). To downregulate Kir4.1, cells were transfected with human *KCNJ10* siRNA SMARTpool (30 nM) purchased from Dharmacon (ThermoFisher), as we described previously [18]. To overexpress fluorescent CLIC1 proteins, we transfected U87 cells with the human CLIC1/NCC27 gene ORF cDNA clone expression plasmid, C-OFPSpark (HG15242-ACR, Sino Biological Inc.; purchased from Interchim, Montluçon, Fr). Cells were used after 48h.

Immunoblotting

Cells were lysed in RIPA buffer, and Western blots were performed as previously described [18]. EVs pellets were lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN) then sonicated for 10 s. Insoluble material was pelleted by centrifugation for 15 min at 14,000 g at 4°C.

Immunofluorescence

Cells were fixed in 4% PFA and permeabilized with 0.1% Triton X-100. Images were performed using a Leica SP2 RS confocal microscope (Z-series of 0.6 µm-optical sections; 512x512 pixels).

EVs isolation

After 48h of culture in FCS-free conditions, cell-conditioned media were collected and sequentially centrifuged at 300 g for 10 min (4°C) then at 2,000 g for 10 min to remove cell debris [18, 68]. EVs were collected by ultracentrifugation at 100,000 g for 90 min. Concentrations were adjusted to the same number of cells (i.e. corresponding to the secretion from 4 x 10^6 cells).

Cell sprouting assay in collagen gels

Sprouting of HMEC spheroids was performed as previously described [69]. For each gel, 8 spheroids (each containing 400 – 500 cells) were seeded into 0.7 ml collagen solution in 24-well plates (PromoCell GmbH) and incubated with the tested solutions for 24h (5% CO_2; 37°C). The cumulative sprout length of 8 randomly selected spheroids was measured for each tested group.

Endothelial tube formation assay in collagen gels

Control and silenced CLIC1 HMEC were plated in DMEM ECM gel, with or without EVs collected from U87, according to the manufacturer's instructions (from Cell Biolabs, Inc). After 12 hours of incubation at 37°C, 80 single cells were scored for the number of processes per cell. Each well is duplicated for each experiment, and each experiment was repeated three times.

Calcium imaging

Spatiotemporal Ca^{2+} variations were recorded in HMEC cultured on uncoated glass then loaded with 4µM Fluo4/AM in FCS-free conditions for 40 min at 37°C [26]. Cells were bathed in the standard solution containing (in mM): 136 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 0.3 NaH_2PO_4, 10 Glucose, and 10 HEPES (pH 7.4). Measurements were performed by a line scan crossing 3-4 cells in a 1024x1024 pixel panel using a confocal microscope (Nikon C1Si) with 100x objective (Nikon, Melville, NY). Excitation was at 488 nm and emission-selected at 500-570 nm. Line scan images in pseudo-colors were acquired at a sampling rate of 22.3 µsec per line (32 lines/sec). To compare cytosolic [Ca^{2+}] fluctuations, fluorescent measurements (F) were expressed as the F/F_0 ratio, where F_0 refers to the basal [Ca^{2+}] fluorescence at rest [70]. The signal amplitudes were shown in a tridimensional histogram (F/F_0 as a function of time/length).

Statistical analysis

Results are expressed as mean ± SD. A Mann-Whitney *U* test was used to compare data groups. Statistics were also made with Tanagra software using a Kruskal-Wallis 1-way ANOVA. In all cases, $^*P$ values < 0.05 were significant.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ANNEXE 5

Exosomes in cancer theranostic: Diamonds in the Rough

Marine Cordonnier, Gaëtan Chanteloup, Nicolas Isambert, Renaud Seigneuric, Pierre Fumoleau, Carmen Garrido & Jessica Gobbo

review

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REVIEW

Exosomes in cancer theranostic: Diamonds in the rough

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ABSTRACT

During the last 10 years, exosomes, which are small vesicles of 50–200 nm diameter of endosomal origin, have aroused a great interest in the scientific and clinical community for their roles in intercellular communication in almost all physiological and pathological processes. Most cells can potentially release these nanovesicles that share with the parent cell a similar lipid bilayer with transmembrane proteins and a panel of enclosed soluble proteins such as heat shock proteins and genetic material, thus acting as potential nanoshuttles of biomarkers. Exosomes surface proteins allow their targeting and capture by recipient cells, while the exosomes' content can modify the physiological state of recipient cells. Tumor derived exosomes by interacting with other cells of the tumor microenvironment modulate tumor progression, angiogenic switch, metastasis, and immune escape. Targeting tumor-derived exosomes might be an interesting approach in cancer therapy. Furthermore, because a key issue to improve cancer patients' outcome relies on earlier cancer diagnosis (metastases, as opposed to the primary tumor, are responsible for most cancer deaths) exosomes have been put forward as promising biomarker candidates for cancer diagnosis and prognosis. This review summarizes the roles of exosomes in cancer and clinical interest, focusing on the importance of exosomal heat shock proteins (HSP). The challenges of clinical translation of HSP-exosomes as therapeutic targets and biomarkers for early cancer detection are also discussed.

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Introduction

For many years, researchers thought that intercellular communications were ensured only by hormones, cytokines or neurotransmitters. However, it is now well established that cells can communicate by means of extracellular vesicles (EVs). EVs are a generic name for all vesicles that are small spherical structures surrounded by a lipid bilayer (of similar structure to that of cell membranes) and that contain hydrophilic soluble components. Through the extracellular vesicles, cells can transfer information from the plasma membrane or internal compartments.[1] There are different EVs: (i) directly formed and released from the cells' plasma membrane e.g microparticles,[2] microvesicles,[3] or ectosomes,[4] (ii) with an endocytic origin and release in the extracellular media by exocytosis called exosomes.[5] (iii) that present several characteristics of exosomes but differ by certain biophysical properties, i.e. exosomes-like vesicles,[6,7] (iv) release by cells in apoptosis and called apoptotic vesicles.[8] Recently, a new type of EVs have been described in gastrointestinal stromal tumors called spheresomes.[9] All these vesicles types differ in their subcellular origin, their biophysical and/or biochemical properties, their receptors composition, and their content in soluble proteins and genetic material. As they contain some nanoliters of cytosol and expose at the outer space the same proteins than the parental cell, they are also considered as nanosized cells with a functional role in many biological processes. Among the different EVs, exosomes have been particularly studied since they have been shown to play a role in many physiological and pathological processes.[10-12] Exosomes are cup-shaped nanovesicles that represent a distinct class of membrane vesicles, with a density of 1.13–1.19 g/ml and a diameter of 50–200 nm. These vesicles form a bioactive cargo since they carry genetic material including DNA, mRNA and miRNA, and numerous proteins, notably heat shock proteins, known to play important roles in immunity and cancer.[10,13,14] The exosome, thanks to its a lipid bilayer, act like a nanoshuttle protecting these

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molecules from their degradation in the extracellular medium. In this review we provide a comprehensive overview of the interest of heat shock proteins contained in exosomes in cancer diagnosis and therapy.

Discovery of exosomes

The term "exosomes" was first used in 1981 by Trams *et al*. to appoint small vesicles secreted by several cell types in the extracellular media.[5] In 1983, Johnstone et Pan discovered with the help of electron microscopy that these vesicles derived from multivesicular bodies (MVBs) and have an endocytic origin.[15] At the time, exosomes generated a poor interest since they were considered as a mean to eliminate obsolete proteins.[16] But in 1996, Raposo *et al.* discovered for the first time that these nanovesicles secreted by antigen-presenting cells (APCs) bore functional peptide–MHC complexes.[17] This article opened a new field in the study of these interesting nanovesicles. Two years later, it was demonstrated the release of exosomes by dendritic cells (DCs) and the ability of tumor peptide-pulsed DC-derived exosomes to suppress growth tumor *in vivo*.[18] Following pioneer studies showing the potential role of exosomes in the regulation of immune responses, myriad of articles have been published related to the immune function of exosomes[10] and their role in cancer.[11] Furthermore, in addition to immune cells, many other cell types have been described as exosome secretory cells such as epithelial cells,[19] neurons[20] and tumor cells.[21]

Exosomes can be isolated from cell culture supernatants and can be found in numerous body fluids such as blood,[22,23] urine,[24] saliva,[25] bronchoalveolar fluid,[26] seminal fluid,[27] amniotic fluid,[28] breast milk,[29] tumor effusions[30] and cerebrospinal fluid.[31]

Biogenesis

The biogenesis of the exosome starts with the invagination of the plasma membrane leading to the endosome formation. Endosomes can differentiate in multi-vesicular bodies (MVBs), which are endocytic structures formed by the budding of an endosomal membrane into the lumen of the compartment.[32] This leads to the formation of small vesicles called intraluminal vesicles (ILVs), future exosomes. Then, the fusion of these MVBs with the plasma membrane provokes the release of the ILVs in extracellular space, and become exosomes (Fig. 1). Although the biological function of MVBs was

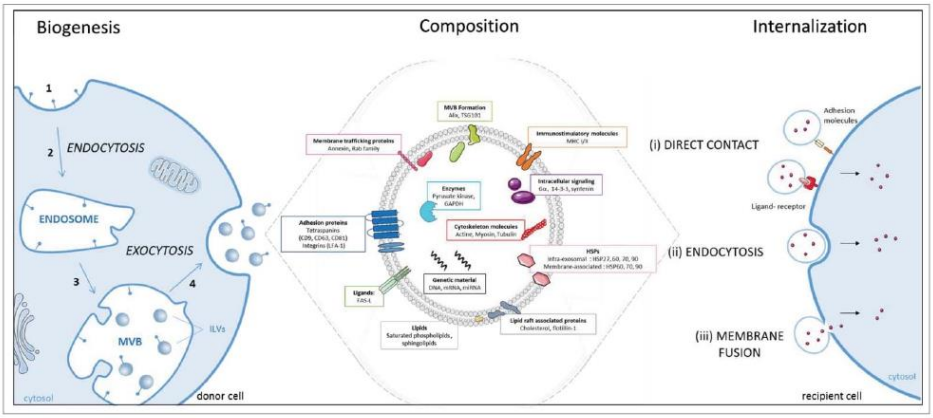

Figure 1. Scheme of exosomes biogenesis, composition and internalization. Biogenesis: the biogenesis of exosomes involves 4 different steps: (1) the membrane invagination; (2) endosome formation; (3) generation of the exosomes precursors, called intraluminal vesicles (ILVs), by inward budding of endosomes. These accumulations of ILVs is termed as multivesicular bodies (MVBs); (4) the fusion of MVBs with the plasma membrane release the ILVs in the extracellular space by exocytosis and become exosomes. Composition: Exosome are composed by different types of enzymes and proteins involved in: adhesion, traffic, intracellular signaling, immunostimulatory molecules, multivesicular body (MVB) formation and heat shock proteins (HSPs). Exosomes contain lipids such as (I) saturated phospholipids (phosphatidyl-ethanolamine, glycero-phospholipids, phosphatidyl-choline and phosphatidyL-serine) (ii) sphingolipids (ceramides), (iii) cholesterol. Finally, exosomes contain nucleic acids, including miRNA, mRNA, DNA and small non coding RNA (snRNA, tRNA). Internalization : The exosome may, (i) elicit transduction of the signal via intracellular signaling pathways by direct contact through adhesion molecules like integrin or through a ligand-receptor interaction, (ii) be endocytosed via phagocytosis, macropinocytosis or receptor-mediated endocytosis, or (iii) fusion with the plasma membrane and transfer its content into the cytoplasm of the recipient cell.

interpreted for many years to be a late step in the degradation pathway toward lysosomes, we now know that MVBs have an alternative fate participating in the exocytic fusion of their external membrane with the plasma membrane. This phenomenon allows the excretion of exosomes by exocytosis into the extracellular space. The mechanisms underlying the sorting of the intraluminal vesicles are not yet fully understood, but 2 ways of exosome sorting have been proposed, dependent or independent on Endosomal Sorting Complex Required for Transport (ESCRT) signals.[33] This complex consist of 4 soluble protein ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. ESCRT is involved in the process of membrane invagination to the formation of ILVs and in the selection of proteins integrating these vesicles.[34] Concerning ESCRT-independent signals that regulate exosomes secretion we can name the ceramide pathway,[35] intracellular Ca^{2+} levels,[36] p53 status,[37] Rab protein family,[38,39] Syndecan-Syntenin-ALIX proteins,[40] high level of heparanase[41] and pH.[42]

How the exosome penetrates into the recipient cell is still a debated issue. Three mechanisms have been proposed based on indirect evidences and in vitro studies: (i) direct contact between surface molecules of vesicles and cells, (ii) endocytosis of exosomes, and (iii) fusion between the membranes of the cell and the exosome.[10] Once the exosome penetrates into the host cell, its content is released in the plasma membrane or in the cytoplasm (Fig. 1).

Composition

The composition of exosomes allows their discrimination from other EVs' family members.[43,44] The exosome membrane composition is the same than that of the mother cell but present specific enrichments and they contain proteins, lipid and genetic material (Fig. 1). All exosome components described are listed in Exocarta website. Proteins present in exosomes include adhesion proteins such as tetraspanins (CD9, CD63, CD81) and integrins (LFA-1), immunostimulatory molecules (MHC I/II), cytoskeleton molecules (actin, myosin, tubulin), membrane trafficking proteins (Rab GTPases such as Rab 5 and annexin), proteins involved in MVB formation (ALIX, TSG101), intracellular signaling proteins (Gα, 14-3-3, syntenin), lipid raft associated proteins (flotillin-1), enzymes (pyruvate kinase, GAPDH), certain ligands such as FAS-L. Finally, several HSPs have been retrieved in exosomes lumen (HSP27, HSP60, HSP70, HSP90) and in exosome membrane (HSP70, HSP60 and HSP90). Some of these proteins are specifically enriched in exosomes compared to cell lysate and are classically used as exosome markers (CD9, CD63, CD81, ALIX, TSG101).[45] Nevertheless, very recently, Kowal et al. compared the composition of EVs subtypes and revealed that although exosomes are enriched in CD9, CD63 and CD81, only TSG101 allows to distinguish exosomes from other EVs subtypes.[46] Finally, numerous studies have revealed that some proteins within the exosomes are dependent on the cell type secreting them while others are independent from the parental cell.[47] These different types of proteins become incorporated into exosomes during exosome formation and serve as cargo for cell–cell communication. Besides, protein exosomes are enriched in lipids such as saturated phospholipids (i.e., phosphatidyl-ethanolamin, phosphatidyL-serin, phosphatidyl-choline), sphingolipids (e.g. ceramids), and cholesterol. These lipid compositions confer to exosomes an exceptional rigidity compared to a plasma membrane.[48] Additional components are found in exosomes including genetic materials such as mRNA (mRNA), transcripts, microRNA (miRNA), and small non coding RNA (snRNA, tRNA).[49]

Clinical interest of exosomes in cancer

Researches on exosomes have considerably increased over the past decade. Although different areas of research are interested in exosomes, most scientific publications are related to cancer. Exosomes have been reported to be involved in all stages in cancer development: (i) tumorigenic transformation, (ii) tumor growth, (iii) angiogenesis, (iv) modulation of immune responses, and (v) induction of mechanisms to acquire therapy resistance.[50-53] The impact of exosomes in clinical research is demonstrated by the fact that there are already 19 clinical trials ongoing (web site https://clinicaltrials.gov/). Among them, 13 involve the study of exosomes as cancer diagnosis biomarkers whereas the others use the exosomes for cancer therapy purposes. Thus, exosomes has emerged as potential biomarkers and therapeutic targets in cancer.

Exosomes as biomarkers

It is well established that the earlier the cancer is diagnosed, the better the survival rate. Although numerous works have been consecrated to early cancer diagnosis, today there is not yet a reliable detection non-invasive method. The main reasons for this are: first of all, in general there is a poor patients compliance, which make difficult to draw any conclusions from the clinical studies. For example, in France, in 2014, the participation rate for breast cancer screening was only of 52.1%.[54] Secondly, actual detection methods, mainly based on medical imaging, have the limitation of tumor detection at an

early stage. Finally, certain publications have shown that some imaging approaches can have undesirable side effects and favor the appearance of tumors. For instance, mammography has been related to cancer apparition -about 1 to 20 for 100 000 mammograms.[55] For all these reasons, it is necessary to develop more performing diagnosis methods. Exosomes appear to be powerful circulating biomarkers.[56-58] These vesicles, reported to be stable and biologically active in human blood plasma up to 3 months, can reveal potential diagnostic information through their examination in body fluids, known as liquid biopsies.[59] They are great potential tools for providing noninvasive, sensitive and economically justifiable new diagnosis methods in oncology.[60] The main advantage of quantifying tumor-derived exosomes compared to circulating tumor cells (CTCs) is that exosomes are found in large amounts compared to CTCs (e.g : $53.2 \pm 1.6 \times 10^8$ exosomes per 10^6 cells in the 24 h period, determined by Nanoparticle Tracking Analysis, Nanosight LM10,[61] Furthermore, exosomes can be quantified non-invasively in urines and other human fluids.

During the last years, improvement in some techniques like mass spectrometry has allowed to better study exosome protein content. First, several studies indicate that tumor derived exosomes carry more proteins than healthy donors-derived exosomes, particularly when compared to patients with an advanced stage disease.[62,63] In 2012, Peinado's team defined a melanoma-specific exosome signature that included tyrosinase-related protein-2 (TYRP2), very late antigen 4 (VLA-4), heat-shock-protein 70 (HSP70), an HSP90 isoform and the MET oncoprotein.[62] Furthermore, TrkB (Tropomyosin receptor kinase B) expression was detected in exosomes isolated from plasma of glioblastoma patients, suggesting that this receptor may be considered also as a new biomarker for glioblastoma diagnosis.[64] It was latter on reported that certain proteins were differentially expressed dependently on the melanoma cells from which the exosomes were analyzed, revealing a specific signature for metastatic cell lines.[65] In this way, in exosomes from patients with metastatic melanoma, MIA (Melanoma Inhibitory Activity) and S100B can be detected, therefore their quantification presents diagnostic and prognostic utility.[66] In 2015, Hoshino et al. revealed that specific integrin expression in exosomes could be used to predict organ-specific metastasis.[67] In Non Small Cell Lung Cancer (NSCLC), leucine-rich α-2-glycoprotein (LRG1) was found to be expressed at higher levels in urinary exosomes of NSCLC patients suggesting that LRG1 may be a candidate biomarker for non-invasive diagnosis of NSCLC in urine. (Li *et al.*,[68] 1) More recently, it has been determined a combination of several exosomal proteins (CD151, CD171 and tetraspanin 8) that could be used as a promising diagnostic tool of lung cancer independently of its stage and histology.[69] In acute myeloid leukemia, TGFβ1 expression seems to be useful to predict response to immunotherapy.[70] Finally, in urological malignancies, exosomes in the urine have been described as robust biomarkers and particularly those expressing survivin for early detection of prostate cancer.[71] Other proteins have been described as specific of cancer-derived exosomes compared to healthy donors and seem also candidates as cancer diagnosis tools. This is the case for Claudin, which is present only in exosomes derived from the plasma of women with ovarian cancer,[72] for Glypican-1 that appears to allow to distinguish an ovarian cancer with high specificity and sensitivity[56] or for CD9-CD147 that is embedded in colorectal cancer-derived exosomes.[73] It has also been reported that 80 percent of the exosomes isolated from NSCLC samples was positive for surface EGFR (epithelium growth factor receptor) by immune staining compared to only 2% of the exosomes in chronic inflammatory lung tissue.[74] Finally, as a general marker of cancer-derived exosomes, our team has recently proposed membrane HSP70 that is present in exosomes released by large panel of cancer cells but not by their normal counterparts[50]; see below.

New researches focus on miRNA potential because exosomes offer a miRNA protection from RNases contrary to free circulating miRNA. In 2007, Valadi *et al.* showed for the first time the transfer of functional miRNAs between 2 cells by means of exosomes.[75] miRNAs are a class of 21–25 small non coding but functional RNA that negatively regulates mRNA expression. These small non-coding RNAs plays important roles in cancer,[76] explaining why this discovery suggested a new regulatory role for exosomes in cancer. Today, numerous studies have identified different functional exosomal miRNAs and their role in cancer,[77,78] and proposed their use as diagnosis biomarkers.[79,80,81] For example, in lung cancer 2 miRNAs, miR-21 and miR-155 have been found to be significantly upregulated in recurrent tumors compared to primary tumors.

Exosomes as immunotherapy agents

Despite improvements in treatment and longer survival, cancer stays a principal cause of death in the world. Since the discovery of functional MHC-peptides complexes in DCs-derived exosomes,[18] many immune functions for exosomes have been described.[10] Researchers tend also to find a new way to modulate immune responses against cancer with the help of exosomes: it is called cell-free vaccines. A classical approach consists in loading exosomes derived from DCs with a tumor specific antigen to restore antitumor immunity. For example, André et al.

isolated exosomes from DCs following tumor peptide pulse and their administration in murine tumor models resulted in rejection of established tumors, an action mediated by T-cell activity.[82] It was later shown that vaccination with exosomes containing modified IL-2 could induce a significant regression of a pre-established tumor by targeting the antigen-specific Th1-polarized immune response and cytotoxic T lymphocytes (CTL).[83] More recently, it has been described an alternative approach to prepare exosomes GPI-IL-12 from fusion gene-modified renal cancer cells and to use them for immunization. This modified exosomes-based vaccine can induce an antigen-specific immune response and CTL more efficiently, resulting in more significant cytotoxic effects in vitro.[84]

Chaput *et al.* demonstrated that isolated DCs-derived exosomes pulsed with Mart1 (Melanoma antigen recognized by T-cells 1) peptides in vitro were able to activate CTL and in, combination with appropriate adjuvants, to induce an antitumor response.[85] In a sarcoma mice model, it was found that OVA (chicken egg ovalbumin) packaged-exosomes allowed a more efficient induction of antitumor immune responses than the native soluble OVA secreted form.[86]

Another way to modulate antitumor immune responses is to combine vaccination by exosomes with other molecules. It was found that ascite-derived exosomes combined to GM-CSF in the immunotherapy of colorectal cancer could induce an antitumor cytotoxic T lymphocyte response[87] whereas combined vaccination with tumor antigen loaded DC-derived exosomes with metronic cyclophosphamide, which inhibit Treg function and restore T and NK cell effector functions, could boost NK cell mediated antitumor immunity in lung cancer patients.[88] Finally, recent several studies have shown that HSP-exosomes can also modulate the immune system. This part is discussed in more detail below.

Exosomes as drug delivery cargos

From their characteristics and properties, exosomes have been used as natural drug delivery cargos. Indeed, exosomes offers several advantages: (i) from their composition, exosomes are capable to avoid immune response and are less immunogenic than any other drug delivery system,[89] (ii) exosomes can naturally and easily penetrate in a host cell by several means,[10] (iii) their nanometric size (50–200 nm) allows them to avoid phagocytosis by the circulating mononuclear phagocytic systems, and the easy extravasation through hyper-permeable blood vessels surrounding tumors, in order to reach tumor tissues[90,91] and (iv) exosomes' membrane protects their content from degradation and are very stable. Several means of modifying exosomes composition exists.[92]

The vast majority of exosome-based drug delivery works and reviews describe the therapeutic transfer of interfering RNAs like synthetic siRNAs or miRNAs and therefore will not be discussed here. We will focus on chemical compound, drugs and proteins.

In a zebrafish brain cancer model, exosome-delivered anticancer drugs through the blood brain barrier decreased tumor growth markers and so could be potentially used as a carrier for brain delivery of anticancer drugs.[93] In 2014, Pascucci *et al.* showed that Mesenchymal Stem Cells (MSC) could incorporate and deliver Paclitaxel to recipient cells through exosomes with increased anti-tumor effects.[94] This study suggests that MSC-derived exosomes could be a new strategy for drug delivery in cancer treatment. Exosomes have also been used as cargos of paclitaxel to increase the effectiveness of the treatment in prostate cancer cells.[95] It has also been shown the effectiveness of targeted exosome-encapsulated doxorubicin for integrin-positive breast cancer cells in inhibition of tumor growth.[96] Recently, Fuhrmann *et al.* found that exosomes loaded with hydrophilic porphyrins induced a stronger phototoxic effect than the free drug in a cancer cell model (Integrin-positive cancer cells).[97]

Zhang's research group used exosomes derived from different cell types to successfully delivered curcumin to activate myeloid cells, producing anti-inflammatory activity and apoptosis in monocytes.[98] Finally, genetically engineered exosomes expressing high levels of a suicide gene mRNA and protein-cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT) have been used to treat pre-established nerve sheath tumors (schwannomas) in an orthotopic mouse model and led to tumor regression.[99]

Exosomes seem to be new actors in theranostic oncology. In this context, recent studies have validated a major role for heat shock proteins in exosomes.

Heat shock proteins and exosomes

Heat shock proteins are stress proteins subdivided in several families according to their molecular weight: HSP110, HSP90, HSP70, HSP60 and small HSPs. These proteins, very well conserved during evolution, were first discovered in 1962. They represent about 2–3% of cellular proteins. In case of a cellular stress, several of these proteins are overexpressed. A wide variety of stress might induce HSPs expression such as hypoxia, infections, drugs and ischemia.[100] The induction of HSP genes require the activation and translocation to the nucleus of specific transcription factors called "Heat Shock Factors" (HSF). These HSF bind to DNA particular sequences

named "Heat Shock Elements" (HSE) in the promoter of HSP genes allowing their expression.[101]

HSPs have been retrieved in all cellular compartments including cytoplasm, nucleus, membrane, mitochondria or endoplasmic reticulum and act as molecular chaperones to maintain cellular homeostasis. HSPs allow the correct refolding of newly-synthesized proteins or incorrectly folded proteins, following physiological conditions or in response to stress.[102,103] If they can't refold correctly the abnormal protein, HSPs can facilitate their proteasomal degradation.[104] In case of a cell death stimulus, HSPs are overexpressed and have strong anti-apoptotic properties by associating to different key proteins of the apoptosis transduction signaling pathway.[105]

HSPs can also be extracellular (membrane-bound or free after secretion).[106,107] HSP27,[108] HSP70[109] and HSP90[110] have been found secreted in the extracellular media, and some of them, as already mentioned above, have been shown to be present in extracellular vesicles, notably in exosomes.[111-113]

HSPs have an important function in cancer by acting at different levels. First, they can promote tumor growth by stabilizing oncogenic proteins. For example, HSP90 can stabilize c-Src, STAT3, Raf-1 or HER2/neu.[114] Certain HSPs, mainly HSP70 and HSP27, can also increase the resistance to chemotherapy by inhibiting apoptosis.[115] Further, some HSPs can promote angiogenesis such as HSP70 and HSP90 that can sequester HIF-a, which is necessary for VEGF production.[116] HSP90 is also involved in the VEGF synthesis and may be a potential novel target for anti-angiogenic therapy.[117] Moreover, HSPs play a role in metastasis formation; some clinical studies have shown a correlation between the expression of HSP27 and/or HSP70 and the metastatic potential.[118-120] Finally, extracellular HSPs can have immunosuppressive functions. Indeed, HSP70 secreted by colorectal cancer cells can activate myeloid-derived suppressor cells and inhibit T cells activation.[121]

During the last few years there have been a growing interest in extracellular HSPs because of the increasing evidences of their role in the induction of innate immune responses with immunostimulatory or immunosuppressive effects, depending on the nature of the HSP, its localization and cell type.[122] Among them, exosomal HSPs seems to modulate the immune response and play anti-tumor functions.[13] This is the rational for the use of exosomal HSPs in cancer therapy and diagnosis.

Published data about extracellular HSPs can be confusing as the term "extracellular HSPs" is generally employed for both soluble, membrane-bound and exosomal HSPs. EV-associated HSPs are still quite new in the field of extracellular HSPs and therefore most papers do not unambiguously differentiate between the different forms of extracellular HSPs. Moreover, very often, researchers write about HSPs in "extracellular vesicles" without given any precision about which subtypes of vesicles they are analyzing. To overcome this problem, in this review, we will summarize mainly data about clearly established exosomal HSPs (i.e. studies in which HSPs are determined from previously isolated exosomes -from human body fluids or culture supernatants).

HSP-exosomes in cancer therapy

Several studies have shown that certain exosomal HSPs could modulate the immune system (Fig. 2). HSP70-exosomes could stimulate natural killer cells (NK) reactivity.[123] When preincubated with HSP70 surface-positive exosomes, NK cells initiated colon tumor cells apoptosis through granzyme B release.[123] It was later on discovered that extracellular HSP70 could also activate macrophages and that this immune modulator effect depended on the ability of HSP70, present on the cell surface, to translocate into the plasma membrane.[124] It was suggested that HSP70, release through exosomes derived from stressed cells, constitute a form of intercellular communication in order to inform macrophages and to induce innate immune responses.[125] These studies suggested that exosomal HSPs could be used in cancer therapy. In 2006, Chen al. tested the vaccination with exosomes presenting HSP60 and HSP90, derived from lymphoma cells. Researchers found an increase in the antitumor immune response involving the induction of IFN production and the activation/maturation of dendritic cells.[126] Several years later, myeloma cell derived exosomes were genetically modified to express endogenous P1A tumor antigen and a transgenic form of membrane-bound HSP70. These HSP70-modified exosomes were able to stimulate in vitro DC maturation more efficiently. The researchers used them as a vaccine and found that they stimulate type 1 CD4(+) helper T (Th1) cell responses, P1A-specific CD8(+) CTL responses and antitumor immunity.[127] More recently, Li-Hong et al. demonstrated that exosomes derived from resistant anticancer drug-treated Hepatocellular Carcinoma (HCC) cells conferred a higher antitumor response by inducing HSP-specific NK cell responses *in vitro* and suggested HSP-bearing exosomes could be used as an efficient vaccine for hepatocellular carcinoma immunotherapy.[128]

In apparent contrast with these results, we discovered an immunosuppressive function of HSP70 at the surface of exosomes. We have shown that all cancer cells analyzed so far have the ability to secrete exosomes with HSP70 in their membrane while normal "non cancerous" cells do not. These tumor-derived exosomes, through membrane-anchored HSP70, can activate Myeloid Derived Suppressor Cells (MDSCs),[121,50] which are abundant cells in a cancer context that restrain antitumor immunity and promote

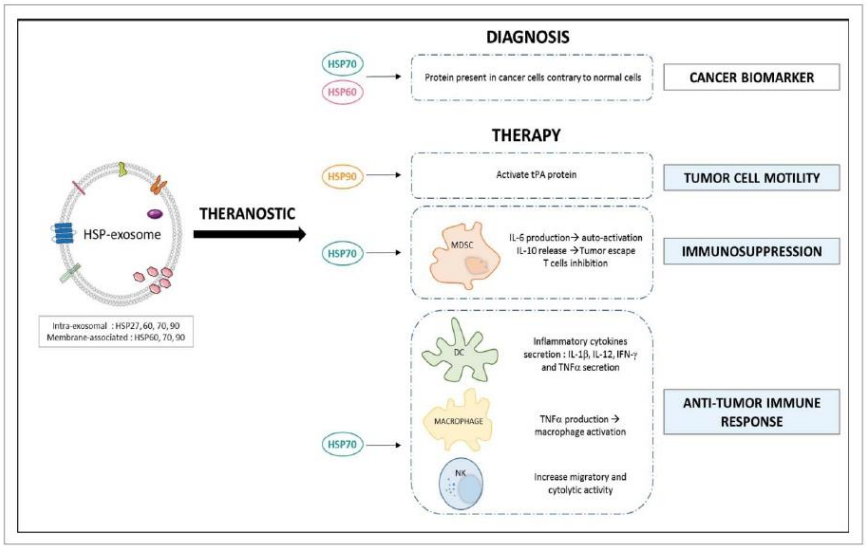

Figure 2. Exosomal heat shock proteins: theranostic oncology tools. HSP60 and HSP70 are proposed as potential cancer biomarkers because of their presence only in exosomes derived from cancer cells. Exosomal HSP70 can act in 2 different ways in the modulation of the immune system. Indeed, it can play an immunosuppressive role through the activation of MDSCs that block the anti-tumor response. But it can have an opposite effect by activating immune cells such as macrophages, dendritic cells (DC) or natural killer cells (NK) which may lead to anti-tumor response. Furthermore, HSP90 may represent a therapeutic target because of their ability to respectively increase tumor cell motility (activation of plasmin tPA).

tumor expansion. At the molecular level, the extracellular domain of membrane HSP70 binds to the Toll-Like Receptor 2 at the surface of MDSCs thus activating them. This interaction triggers NF-kB signaling pathway allowing the expression of the inflammatory cytokine IL-6, which binds to its receptor IL-6R in an autocrine manner. This interaction leads to STAT3 phosphorylation via JAK2 pathway, activating survival genes in MDSCs that could exert their immunosuppressive functions.

Recently, our team has confirmed the release of HSP70-exosomes by cancer cells and their ability to activate MDSC in a small cohort of colon cancer patients. Further, we have developed a peptide aptamer (A8) that binds to the extracellular domain of membrane-bound HSP70, called "TKD."[129] Membrane HSP70 binds with much higher affinity to A8 than to the TLR2 receptor in the MDSC. As a result, A8 block the capacity of these tumor-derived exosomes to activate MDSC. Thereby, in vivo and in vitro, A8 induce the development of an efficient anti-tumor immune response that was associated to an inhibition of MDSC.[50] In line with our results proposing an HSP70 inhibitor -A8- as an agent that can boost the anti-cancer immune response, HSP90-exosomes have been described as to be involved in the activation of plasmin and cancer cells' motility in several cancer models. Thus, targeting HSP90 could also represent a way to limit tumor invasion by inhibiting a growing number of proteins that are involved in tumor cell motility.[130]

HSP-exosomes in cancer diagnosis

The detection and quantification of exosomal HSPs can provide useful information for establishing new circulating and non-invasive biomarkers (Fig. 2). Our team suggests the use of HSP70-exosomes as a cancer marker because they seem a general feature of cancer cells (but not of "normal" non-cancerous cells) and we have demonstrated that they can be measured in large amounts in biological fluids from cancer patients but not from healthy individuals where they are hardly detected.[50] We have patented an interference biolayer protocol to easily capture HSP70-exosomes isolated from human fluids using as a high affinity ligand our peptide aptamer A8 (WO2015/189395[131]). To move beyond the proof of principle that these tumor-derived exosomes (HSP70-exosomes) can be quantified and might be interesting to follow up cancer patients, we have started a prospective study with the anticancer Center Georges-François Leclerc (CGFL, Dijon, France) in breast, ovarian and

lung cancer aiming at determining whether the presence of HSP70-exosomes is predictive of the patients' outcome and whether their detection precedes CTCs and the apparition of metastases.

Finally, HSP60 have also been localized at the membrane of exosomes isolated from blood patients suffering from large bowel cancer, before surgery, but was absent after surgery and in healthy controls. Because of its presence in tumor cells but not in healthy cells, HSP60-exosomes seem to be also interesting biomarkers in cancer, at least for large bowel cancer diagnosis.[132]

Concluding remarks

In conclusion, there are no doubts that even if there are still many questions remaining to be answered, the relatively young field of exosomes in cancer is gaining greater interest within the scientific and medical communities. There are 2 main limitations in the discussion of the works presented in this review. The first and most important is the lack of standardized protocols for isolation of tumor-derived exosomes; the second is the still partial understanding of the mechanisms involving exosomes functions in cancer. Indeed, there is a Janus faced implication of exosomes in cancer biology explained by the fact that exosomes can transfer both tumor-promoting molecules (e.g.,: oncoproteins) and tumor suppressors and can either induce or suppress an immune response. We believed these debated issues could be solved with more precise protocols to isolate cancer exosomes and taking into account the *in vivo* cancer environmental context.

The available data on exosomes strongly suggest that these diamonds in the rough might represent a revolution in cancer diagnosis and toward a more personalized medicine. Exosomes can be a fingerprint of the parental cell type and of its status. Moreover, they are abundant in body fluids such as blood and urine, therefore representing a precious biomedical tool for non-invasive approaches in cancer diagnosis and cancer patients' follow up. Furthermore, as nanoshuttles of biomarkers and/or anti-tumor drugs, exosomes open new avenues for the clinical management of cancer.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ANNEXE 6

Liste des publications et des participations à un congrès

Liste des publications

Monitoring HSP70 exosomes in cancer patients' follow up: a clinical prospective pilot study
GAËTAN CHANTELOUP, Marine Cordonnier, Nicolas Isambert, Aurélie Bertaut, Alice Hervieu, Audrey Hennequin, Maxime Luu, Sylvie Zanetta, Bruno Coudert, Leila Bengrine, Isabelle Desmoulins, Laure Favier, Aurélie Lagrange, Pierre-Benoit Pages, Ivan Gutierrez, Jeanine Lherminier, Laure Avoscan, Clémentine Jankowski, Cédric Rébé, Angélique Chevriaux, Marie-Martine Padeano, Charles Coutant, Sylvain Ladoire, Sylvain Causeret, Laurent Arnould, Céline Charon-Barra, Vanessa Cottet, Julie Blanc, Christine Binquet, Marc Bardou, Carmen Garrido & Jessica Gobbo , **J Extracell Vesicles,** 2020 May 20.

Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients
Cordonnier M*, Nardin C*, CHANTELOUP G, Derangere V, Algros M, Arnould L, Garrido C**, Aubin F**, Gobbo J**. **J Extracell Vesicles**, 2020 Jan 7.

Membrane-anchored heat-shock protein 70 (Hsp70) in Cancer
Elmallah MIY, Cordonnier M, Vautrot V, CHANTELOUP G, Garrido C, Gobbo J. **Cancer Lett**, 2019 Oct 25.

Exosomal miRNA: Small Molecules, Big Impact in Colorectal Cancer
Vautrot V, CHANTELOUP G, Elmallah M, Cordonnier M, Aubin F, Garrido C, Gobbo J. **J Oncol**, 2019 Oct 13

Exosomal HSP70 for Monitoring of Frontotemporal Dementia and Alzheimer's disease: Clinical and FDG-PET Correlation.
CHANTELOUP G*, Cordonnier M*, Moreno-Ramos T, Pytel V, Matías-Guiu J, Gobbo J, Cabrera-Martín MN, Gómez-Pinedo U, Garrido C, Matías-Guiu JA. **J Alzheimers Dis**. 2019.

Increased levels of IL-17a exosomes in psoriasis
Jacquin-Porretaz C, Cordonnier M, Nardin C, Boullerot L, CHANTELOUP G, Vautrot V, Adotevi O, Garrido C, Gobbo J, Aubin F. **ActaDV**, 2019.

HSP110 translocates to the nucleus upon genotoxic chemotherapy and promotes DNA repair in colorectal cancer cells.
Causse S.Z., Marcion G., CHANTELOUP G., Boudesco C., Grigorash B.B., Uyanik B., Douhard R., Dias A., Dumetier B., Dondaine L., Gozzi G., Moussay E., Paggetti J., Mirjolet C, de Thonel A., Dubrez L., Demidov O.N., Gobbo J., Garrido C. **Oncogene**, 2019.

The vesicular transfer of CLIC1 from glioblastoma to microvascular endothelial cells requires TRPM7.
Thuringer D, CHANTELOUP G, Wrinckler P, Garrido C, **Oncotarget**, 2018.

HSP27 is a partner of JAK2-STAT5 and a potential therapeutic target in myelofibrosis.
Sevin M, Kubovcakova L, Pernet N, Causse S, Vitte F, Villeval JL, Lacout C, Cordonnier M, Rodrigues-Lima F, CHANTELOUP G, Mosca M, Chrétien ML, Bastie JN, Audia S, Sagot P, Ramla S, Martin L, Gleave M, Mezger V, Skoda R, I Plo, Garrido C, F Girodon, A de Thonel. **Nat Commun**. 2018 Apr 12.

Modulation of the inwardly rectifying potassium channel Kir4.1 by the pro-invasive miR-5096 in glioblastoma cells.
Thuringer D, CHANTELOUP G, Boucher J, Pernet N, Boudesco C, Jego G, Chatelier A, Bois P, Gobbo J, Cronier L, Solary E, Garrido C. **Oncotarget**, 2017 Jun 6.

Exosomes in cancer theranostic: Diamonds in the rough.
Cordonnier M*, CHANTELOUP G*****, Isambert N, Seigneuric R, Fumoleau P, Garrido C, Gobbo J**. Cell Adh Migr**, 2017 Mar 4.

Visualization of RNA-Quadruplexes in Live Cells.
Laguerre A, Hukezalie K, Winckler P, Katranji F, CHANTELOUP G, Pirrotta M, Perrier-Cornet JM, Wong JM, Monchaud D**. J Am Chem Soc**, 2015 Jul 8.

Liste des participations à un congrès

Communication orale :

Monitoring HSP70 exosomes in cancer patients' follow up: a clinical prospective pilot study, Tumor Liquid Biopsy Symposium, Nancy, 2017.

Exosomal HSP70 for Monitoring of Frontotemporal Dementia and Alzheimer's disease: Clinical and FDG-PET Correlation, CALM, Montpellier, 2019.

Communication affichée :

Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients, ISEV, Tokyo, 2019.

Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients, ASCO, Chicago, 2019.

Monitoring HSP70 exosomes in cancer patients' follow up: a clinical prospective pilot study, OncoTrans, Besançon, 2016.

Résumé

Le cancer est aujourd'hui une maladie qui fait malheureusement partie de notre quotidien, et bien qu'il soit de mieux en mieux traité, de plus en plus de gens en sont atteints. La recherche s'oriente sur deux pans : la thérapie et le diagnostic. Les nouvelles thérapies présentent des résultats spectaculaires jamais atteints à ce jour, mais l'amélioration du diagnostic peut aussi sauver des vies. Il est établi que plus un cancer est diagnostiqué tôt, plus le patient a de chances de guérir. Idem pour le suivi de la maladie. Dans ce contexte de précision et de précocité, la biopsie liquide émerge et possède un avenir très prometteur. Elle consiste en l'étude d'analytes présents dans les fluides corporels, particulièrement la circulation sanguine. On y retrouve principalement, mais pas exclusivement, l'ADN tumoral circulant, les cellules tumorales circulantes (CTCs) et les exosomes.

Ce manuscrit a pour objectif de replacer mon travail de recherche, qui porte sur les exosomes, dans le contexte de la biopsie liquide, afin de laisser libre court aux comparaisons et à la compréhension de leur potentiel diagnostic. Les exosomes sont des nanovésicules libérées par les cellules dans le sang. Elles contiennent du matériel génétique, des lipides et des protéines. Une étape clé pour leur utilisation en tant que biomarqueur est de différencier les exosomes dérivés de tumeur de ceux dérivés d'autres cellules de l'organisme.

La protéine de stress Heat shock protein-70 (HSP70) a été décrite comme étant surexprimée dans les cellules cancéreuses et associée à un mauvais pronostic. Nous avons précédemment démontré que seuls les exosomes dérivés de cellules cancéreuses portaient HSP70 à la membrane. Dans ce travail, nous avons ouvert une étude clinique pilote prospective incluant des patients atteints d'un cancer du sein et du poumon afin de déterminer s'il était possible de détecter et quantifier les HSP70-exosomes dans le sang de patients atteints de tumeurs solides malignes.

Nous avons montré que le taux de HSP70 dans les exosomes, contrairement à la forme soluble, reflétait le contenu en HSP70 dans la biopsie tumorale. Le taux de HSP70-exosomes circulants est augmenté chez les patients atteints d'un cancer à un stade métastatique comparé aux non-métastatique et aux donneurs sains. Nous avons ensuite démontré que les niveaux de HSP70-exosomes étaient corrélés au statut de la maladie, et étaient potentiellement de meilleurs marqueurs que les CTCs. Enfin, nous avons indiqué que le taux de HSP70-exosomes étaient inversement corrélés à la réponse au traitement, et, par conséquent, que le suivi du taux de HSP70 dans les exosomes pourrait être utile dans la prédiction de la réponse au traitement.