Université du Québec
Institut National de la Recherche Scientifique
Institut Armand-Frappier

EXPRESSION ET FONCTIONS DES GALECTINES DANS LE CANCER

Par

Marilyne Labrie, M.Sc.

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Président du jury et examinateur interne	Pascale Duplay, Ph.D. INRS-Institut Armand-Frappier
Examinateur externe	Dominique Trudel, MD Ph.D. FRCP(c) CRCHUM
Examinateur externe	Carlos Reyes-Moreno, Ph.D. UQTR
Directeur de recherche	Yves St-Pierre, Ph.D. INRS-Institut Armand-Frappier

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RÉSUMÉ

Dans une ère où la médecine personnalisée prend de plus en plus d'ampleur, il est devenu primordial de mieux comprendre les différents évènements moléculaires qui régulent la progression tumorale. Depuis plusieurs années, les galectines sont au centre d'un intérêt grandissant pour la découverte de biomarqueurs exploitables. De par leur capacité à lier une grande variété de molécules glycosylées, ces lectines animales régulent différents aspects de la progression tumorale, dont la résistance à la chimiothérapie, l'immunoévasion et le potentiel métastatique. Puisque leur expression est souvent dérégulée dans plusieurs types de cancer, il est clair qu'une meilleure caractérisation de leur expression et de leurs fonctions permettra de développer de nouveaux outils pour le diagnostic, le pronostic et le traitement des patients atteints du cancer.

L'objectif de ce projet de thèse était, dans un premier temps, de déterminer l'expression et les fonctions de la galectine-7 dans le cancer de la prostate et de l'ovaire. Dans un deuxième temps, nous avons étudié la possibilité d'utiliser une signature de galectines comme biomarqueur et cible thérapeutique dans le cancer ovarien.

Pour ce faire, nous avons utilisé des micromatrices tissulaires construites à partir de tissus provenant de patients atteints de cancer. Nous avons d'abord observé que l'expression de la galectine-7 est réprimée dans le cancer de la prostate. Avec l'aide de modèles cellulaires *in vitro* utilisant les cellules DU-145, nous avons ensuite mis en évidence une activité anti-tumorale de la galectine-7 dans le cancer de la prostate. Nous avons ensuite étudié les fonctions CRD-dépendantes de la galectine-7 à l'aide d'un vecteur d'expression de la galectine-7 dont la séquence codant pour le CRD a été muté. Nous avons établi que la fonction pro-apoptotique de la galectine-7 est indépendante de sa localisation mitochondriale et nucléaire et qu'il s'agit d'une fonction CRD-indépendante. À l'opposé, la réduction du potentiel invasif est un évènement CRD-dépendant. Enfin, avec l'aide d'un modèle pré-clinique murin, nous avons pu constater que la présence de la galectine-7 réduit la croissance tumorale, alors que la galectine-7 dont le CRD est muté a une fonction inverse et stimule la croissance tumorale. Nos résultats suggèrent donc que les interactions CRD-indépendantes de la galectine-7 régulent un phénotype pro-tumoral.

Dans le cas du cancer ovarien, nous avons démontré que la présence de galectine-7 est associée à des tumeurs de haut grade et aux métastases, ainsi qu'à un plus haut taux de mortalité. Avec l'aide de modèles *in vitro*, nous avons établi que la galectine-7 favorise le potentiel invasif des cellules cancéreuses de l'ovaire en augmentant la motilité cellulaire et la production de la métalloprotéinase de la matrice 9. Nous avons également mis en évidence

l'activité immunosuppressive de la galectine-7 via sa capacité à induire l'apoptose des cellules immunitaires, dont les monocytes et les lymphocytes T CD4+ et T CD8+.

Dans un dernier temps, en utilisant des micromatrices tissulaires comprenant des échantillons de tumeurs ovariennes de sous-type séreux de haut grade, nous avons identifié une signature prédictive constituée de différentes galectines pour les patientes atteintes de cancer ovarien. Plus précisément, nous avons observé que la galectine-1 stromale est associée à la récidive des patientes, alors que la présence de puncta péri-nucléaires de galectine-9 prédit un plus haut taux de mortalité. La présence de galectine-8 épithéliale, quant à elle, est associée à la résistance à la chimiothérapie. Plus important encore, cette signature de galectines permet d'établir un pronostic pour les patientes qui sont négatives pour CA125, le seul biomarqueur utilisé au niveau clinique pour le cancer de l'ovaire.

En conclusion, notre étude a permis de caractériser les fonctions anti- et pro-tumorales de la galectine-7 dans les cancers de la prostate et de l'ovaire, respectivement. Cette étude est la première à démontrer que la perturbation du CRD de la galectine-7 peut provoquer un changement phénotypique d'anti- à pro-tumoral. Cela amène une réflexion sérieuse sur l'utilisation d'inhibiteurs de galectines qui ciblent le CRD de ces protéines. Nos recherches ont aussi permis de définir une signature d'expression des galectines qui permettrait d'établir le pronostic des patientes atteintes du cancer ovarien. Nos résultats valident l'hypothèse que les galectines peuvent servir de biomarqueurs et cibles thérapeutiques pour différents cancers et justifie la poursuite de nos recherches dans ce domaine.

Mots clés: cancer de la prostate, cancer ovarien, galectines, galectine-7, domaine de liaison aux sucres (CRD), apoptose, potentiel invasif, immunoévasion, biomarqueur exploitable

ABSTRACT

In an era where there is an emerging interest in personalized medicine, it becomes essential to have a better understanding of the different molecular events regulating cancer progression. For these reasons, attempts to discover new actionable biomarkers had put the galectins at the center of attention. Members of this group of lectins have the ability to bind a large variety of glycosylated molecules and to regulate different aspects of tumor progression such as chemoresistance, immune evasion and spread of metastatic cells. Because their expression is often deregulated in cancer cells, it is becoming clear that a better understanding of their functions may lead to the development of new tools for the diagnosis, prognosis and treatment of cancer patients.

The objectives of this doctoral project were to determine the expression and functions of galectin-7 in prostate and ovarian cancer and to define a galectin expression signature in ovarian cancer that could be used as an actionable biomarker. For this purpose, we used tissue microarrays constructed from cancer patients' tissue samples and showed that galectin-7 expression was abrogated in prostate cancer tissues. These results are consistent with galectin-7 anti-tumoral functions obtained using *in vitro* experiments with DU-145 prostate cancer cells. Using a mutant form of galectin-7 with a non-functional carbohydrate recognition domain (CRD), we further established that the pro-apoptotic function of galectin-7 is independent of its mitochondrial and nuclear localization and independent of its CRD activity. On the other hand, we found that the reduced invasive behavior of galectin-7 expressing cells is a CRD-dependent event. Finally, using a preclinical murine model, we observed that galectin-7 reduces the tumor growth, while the CRD-defective galectin-7 had the opposite effect. Our results suggest that galectin-7 CRD-independent interactions regulate a pro-tumoral phenotype.

In ovarian cancer, we demonstrated that galectin-7 expression is associated with higher grade tumors, metastasis and to a shorter survival. With an *in vitro* model, we established that galectin-7 stimulates the invasive potential of ovarian cancer cells by inducing motility as well as matrix metalloproteinase 9 expression. We also found that galectin-7 has an immunosuppressive activity by inducing apoptosis of immune cells such as monocytes and CD4+ and CD8+ T cells.

Finally, using tissue microarrays constructed from ovarian high grade serous carcinoma samples, we identified a galectin signature that could predict the outcome of ovarian cancer

patients. More specifically, we found that stromal galectin-1 was associated with a shorter disease-free survival and galectin-9 perinuclear puncta were associated with a shorter overall survival. Also, epithelial galectin-8 is associated with chemoresistance. More importantly, this galectin signature could predict the outcome of CA125 negative patients. CA125 is the only clinically available biomarker for ovarian cancer.

In conclusion, we characterized the anti- and pro-tumoral functions of galectin-7 in prostate and ovarian cancer, respectively. This study is the first to demonstrate that the CRD is the phenotypical switch between the pro- and anti-tumoral functions of galectin-7. This finding raises some questions regarding the use of inhibitors that target galectins CRD. Our research also helps to define a novel galectin expression signature that might help ovarian cancer patients' management. They further validate the hypothesis that galectins can be use as actionable biomarkers for different cancer types.

Key words: prostate cancer, ovarian cancer, galectins, galectin-7, CRD, apoptosis, invasive potential, immune escape, actionable biomarker

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LISTE DES ABRÉVIATIONS

APS	Antigène prostate spécifique
AR	Récepteur des androgènes
Bcl-2	*B-cell lymphoma 2*
BRCA	*Breast cancer antigen*
C/EBPβ	*CCAAT/enhancer-binding protein beta*
CA125	*Cancer antigen 125*
CD	Cluster de différenciation
CFG	*Consortium for functional glycomics*
CRD	Domaine de liaison aux sucres
CTLA-4	*Cytotoxic T-lymphocyte antigen 4*
DHT	Dihydrotestostérone
EGR	*Early growth response protein 1*
EOC	Cancer épithélial de l'ovaire
ERK	*Extracellular signal-regulated kinases*
FGFR	Récepteur de facteurs de croissance de fibroblastes
FIGO	Fédération internationale de gynécologie et d'obstétrique
Ga	Gallium
Gal	Galectine
Gemin	*Gem-associated protein*
HE4	Protéine épididimaire humaine 4
HGSC	Cancer séreux de haut grade
hK	Kallikréines
IHC	Immunihistochimie
IL	Interleukine
INFγ	Interféron gamma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRM	Imagerie par résonnance magnétique
JNK	*c-jun N-terminal kinases*
LacNAc	N-acetyl lactosamine
LH-RH	Hormone de libération des gonadotrophines hypophysaires
MAPK	*Mitogen-activated protein kinase*
miRNA	microARN
MMP	Métalloprotéinase de la matrice
PARP	Polymérase poly(ADP-ribose)
PC3	Antigène 3 du cancer prostatique
Pca	Prostate cancer
PD-1	*Programmed cell death 1*
PD-L1	*Programmed cell death-ligand 1*
PI3K	Phosphoinositide 3-kinase
PMSA	Prostate membrane specific antigen
PTEN	*Phosphatase and tensin homolog*
qRT-PCR	*Quantitative Reverse transcription polymerase chain reaction*
RANK	*Receptor activator of nuclear factor kappa-B*

RIPA	*Radio-immunoprecipitation assay*
RMI	Risque de malignité
ROMA	Algorithme du risque de malignité de la tumeur ovarienne
RTPU	Résection transurétrale de la prostate
SMAD4	*Mothers against decapentaplegic homolog 4*
SRD5A2	Stéroïde 5-alpha réductase de type II
SSPA	*Stringent starvation protein A*
TEP	Tomographie par émission de positrons
TH1	*Type 1 T helper*
TMA	Micromatrice tissulaire
TMPRSS2	*Transmembrane protease, serine 2*
TNM	Taille, ganglions lymphatiques, métastases
Treg	T régulatrice
VEGF	Facteur de croissance de l’endothélium
VEGFR	Récepteur de facteurs de croissance de l'endothélium
VIH-1	Virus de l’immunodéficience humaine 1
Wt	*Wild type*

CHAPITRE 1

INTRODUCTION

1 LES GALECTINES

Lors de la synthèse protéique, on estime qu'environ 70 % des protéines humaines sont modifiées par la glycosylation [1]. La majorité des protéines glycosylées sont retrouvées à la surface des cellules, dans l'environnement extracellulaire ou encore dans des organelles intracellulaires [2]. Plusieurs protéines, telles que les galectines, sont spécialisées dans la reconnaissance de molécules glycosylées. Il est maintenant bien reconnu que la glycosylation est un événement essentiel à la régulation de plusieurs processus physiologiques. Sa dérégulation ou celle de protéines qui ont une affinité pour des glycans peuvent mener à des pathologies, dont le cancer [3].

1.1 Structure et classification

Jusqu'à aujourd'hui, 16 galectines (galectines-1 à -16) ont été caractérisées et 12 d'entre elles sont exprimées chez l'humain [4-6]. Les galectines-5 et -6 sont uniquement exprimées chez le rat et la souris, respectivement, alors que les galectines-11 et -15 sont exclusivement retrouvées chez les ruminants [4-6]. Les galectines ont en commun un domaine de liaison aux sucres (CRD) formé d'environ 130 acides aminés hautement conservés [7, 8]. Le CRD leur permet de se lier à des molécules glycosylées qui contiennent des résidus de sucres composés de N-acetyl lactosamine (LacNAc). Les galectines se lient donc à des molécules glycosylées contenant des N- ou O-glycans [8-11]. Malgré l'homologie fortement conservée de la séquence du CRD, chaque galectine a des préférences pour certains sucres et bien qu'elles partagent un grand nombre de ligands, ces fines spécificités leur assurent des interactions distinctes [12, 13].

De façon générale, les galectines sont classées selon des critères structuraux (Figure 1). On retrouve ainsi les galectines « prototypes » (galectine-1, -2, -7, -10, -13, -14 et -16) constituées d'un seul CRD et capables de former des homodimères [14]. Les galectines avec « répétitions en tandem » (galectine-4, -8, -9 et -12) possèdent quant à elles deux CRD reliés par une chaîne peptidique [14]. Enfin, la troisième classe de galectines, dites « chimériques », comprend un seul membre, la galectine-3. Cette protéine ne possède qu'un CRD et détient une élongation peptidique non-lectine en N-terminal qui lui permet de s'oligomériser et former des pentamères [14].

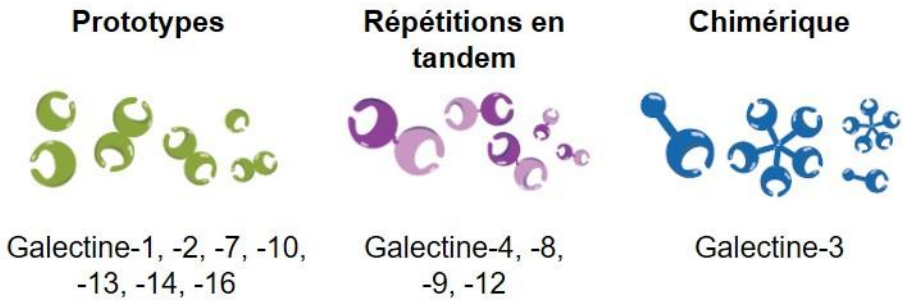

Figure 1 Classification des galectines. Schématisation des trois classes de galectines: prototypes, chimérique et répétitions en tandem. Modifié de [14].

1.2 Expression et fonctions physiologiques

1.2.1 Distribution tissulaire et cellulaire

Les galectines sont exprimées dans la plupart des tissus, bien que le « répertoire » des galectines (galectinome) varie considérablement d'un tissu à l'autre (Tableau 1).

Tableau 1 Distribution tissulaire des galectines. Adaptation de [15].

Galectines	Tissus
Galectine-1	Tissu adipeux, moelle osseuse, système nerveux central, glandes endocrines, endothélium, systèmes reproducteurs mâle et femelle, organes lymphatiques, placenta, système respiratoire, peau, muscle lisse
Galectine-2	Sang, moelle osseuse, système digestif, cellules immunitaires, organes lymphatiques, placenta, système urinaire
Galectine-3	Tissu adipeux, moelle osseuse, système nerveux central, système digestif, glandes endocrines, endothélium, systèmes reproducteurs mâle et femelle, muscles cardiaques, organes lymphatiques, cellules immunitaires, placenta, système respiratoire, peau, muscle lisse, système urinaire
Galectine-4	Système digestif, système reproducteur mâle, peau
Galectine-7	Peau, système digestif, système reproducteur femelle, cœur, organes lymphatiques
Galectine-8	Moelle osseuse, système digestif, glandes endocrines, système reproducteur mâle et femelle, cellules immunitaires, organes lymphatiques, placenta, système urinaire
Galectine-9	Tissu adipeux, moelle osseuse, système digestif, glandes endocrines, organes lymphatiques, placenta, système respiratoire, peau, muscles lisses
Galectine-10	Moelle osseuse, cellules immunitaires, organes lymphatiques
Galectine-12	Tissu adipeux, moelle osseuse, système reproducteur femelle, cellules immunitaires
Galectine-13	Placenta
Galectine-14	Placenta
Galectine-16	Placenta

Les galectines-1 et -3 sont celles qui semblent avoir la plus grande distribution tissulaire. On retrouve la galectine-1 dans les cellules épithéliales et le stroma, incluant les fibroblastes et les cellules endothéliales, de la majorité des tissus [15, 16]. La galectine-3 se retrouve aussi dans les cellules épithéliales et le stroma de plusieurs organes [15, 16]. Les galectines-2 et -4 ont un profil d'expression plus restreint, étant principalement retrouvées dans les cellules

du tube digestif [15, 16]. Pour ce qui est de la galectine-7, elle est presque exclusivement exprimée dans les épithéliums stratifiés, alors que la galectine-8 est fortement exprimée dans les cellules endothéliales de plusieurs organes [16]. La galectine-9 a une distribution plus globale, étant exprimée dans les cellules épithéliales et le stroma de plusieurs organes et elle est présente dans plusieurs types de cellules du système immunitaire [15, 16]. Quant à la galectine-10, elle est retrouvée dans les éosinophiles et les basophiles, alors que la galectine-12 est exprimée dans les adipocytes [15, 16]. Enfin, les galectines-13, -14 et -16 sont surtout reconnues pour leur expression dans les tissus formant le placenta [15]. Il est fort probable que ces dernières soient exprimées dans d'autres tissus, mais les études à ce sujet sont rares.

L'expression des galectines est régulée par plusieurs facteurs tels que des cytokines ou des facteurs de croissance. Par exemple, il a été établi que l'expression de la galectine-1 est induite par les cytokines inflammatoires IL-17, TNF-α et l'INF-γ, alors que l'expression de la galectine-3 peut être réprimée par l'EGF [17, 18]. On peut aussi observer une modulation de l'expression de certaines galectines lors de plusieurs processus cellulaires, dont l'inflammation, le stress oxydatif, l'apoptose ou l'hypoxie. Il a d'ailleurs été démontré que l'inflammation cause une augmentation de l'expression des galectines-1, -3, -9 et -10, alors que l'hypoxie induit l'expression des galectines-1 et -3 (Revue dans [19]).

Au niveau de leur compartimentalisation cellulaire, les galectines peuvent se retrouver pratiquement n'importe où. Elles peuvent être sécrétées par une voie d'exocytose non classique indépendante du réticulum endoplasmique et de l'appareil de Golgi [20]. Une fois dans l'environnement extracellulaire, elles peuvent se lier à la surface des cellules sur des récepteurs membranaires, se loger dans la matrice extracellulaire par la liaison à des glycoprotéines ou encore, être relâchées dans la circulation sanguine [20, 21]. Au niveau intracellulaire, les galectines sont généralement cytoplasmiques et/ou nucléaires et peuvent être associées à plusieurs structures intracellulaires, dont les autophagosomes, les lysosomes, les mitochondries et les endosomes [22-25]. Les mécanismes régulant la compartimentalisation des galectines sont encore méconnus. Cependant, il semblerait que certains mécanismes cellulaires puissent provoquer un changement de localisation des galectines. Par exemple, il a été démontré que la galectine-2 transloque au noyau de fibroblastes lors d'une exposition à des rayons UV [19].

1.2.2 Partenaires d'interaction

Historiquement, on connait les galectines pour leur capacité à lier de façon CRD-dépendante des récepteurs glycosylés présents à la surface des cellules immunitaires. Cependant, au cours des dernières années, de nombreuses études ont mis en évidence des interactions avec des protéines intracellulaires et extracellulaires qui sont indépendantes du CRD [26, 27]. Le tableau 2 décrit plusieurs partenaires d'interactions intracellulaires et extracellulaires des galectines. Ces études apportent non seulement un changement radical dans notre façon de voir les interactions médiées par les galectines, mais nous obligent à remettre en question nos stratégies d'inhibition de leurs fonctions qui sont presque exclusivement concentrées sur le CRD.

Tableau 2 Partenaires d'interaction des galectines. Principaux ligands intracellulaires et extracellulaires des galectines [28-38].

Galectine	Partenaires intracellulaires	Partenaires extracellulaires
Galectine-1	H-RAS, Gemin4, complexe Gemin2, protocadherine-24, actine monomérique, LAMP-1	CA125, CD3, CD7, CD29, CD45, CD43, fibronectine, lamine, Mac-2
Galectine-2	LTA	CD29
Galectine-3	ATP-synthase, protocadherine-24, CD95, nucléine, synexine, CBP70, β-caténine/TCF, Axine/ β-caténine/APC, TTF-1, K-Ras, Bcl-2, Alix/AIP-1, gemin4, complexe gemin2, Chrp, LAMP-1	CD7, CD29, CD45, CD71, CD98, CD66, fibronectine, lamine, Mac-2
Galectine-4	β-caténine/APC/Axine	CD3
Galectine-7	Bcl2, Smad3	CD44
Galectine-8	LRP1, MRC2, uPAR	CD44, podoplanine, CD166
Galectine-9	C/EBPβ	CD44, P4HB, TIM3
Galectine-10	Lisophospholipases	

Une caractéristique importante qui définit la majorité des fonctions extracellulaires des galectines est leur capacité à former des treillis moléculaires. Puisque les galectines prototypes peuvent former des homodimères, que la galectine-3 s'oligomérise et que les galectines en répétitions en tandem possèdent deux CRD, elles ont toutes la capacité de lier simultanément plusieurs récepteurs glycosylés et ainsi former une structure ressemblant à celle d'un treillis (Figure 2) [39, 40]. La formation de cette structure a plusieurs conséquences fonctionnelles, dont la stabilisation de récepteurs membranaires, l'induction de voies de signalisation intracellulaires et la modulation des interactions cellule-cellule et cellule-matrice extracellulaire [29, 41]. La matrice extracellulaire est d'ailleurs un composant riche en glycoprotéines telles que la fibronectine et la lamine, qui permet d'augmenter la concentration locale des galectines [41, 42].

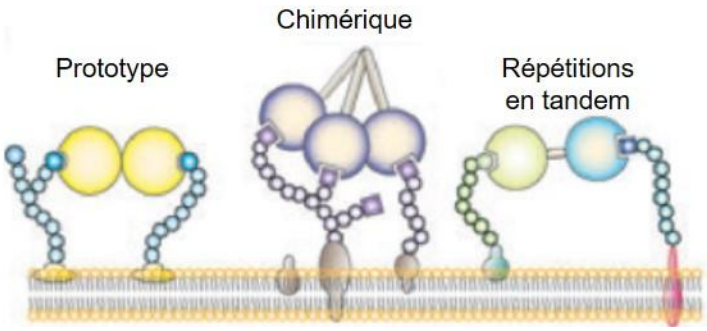

Figure 2 Treillis moléculaires formés de galectines et de ligands glycosylés. En se liant à des récepteurs glycosylés, les galectines prototypes, chimériques et répétitions en tandem forment des treillis moléculaires. Adaptation de [21].

Les interactions CRD-indépendantes, quant à elles, ont été négligées par la communauté scientifique et demeurent encore relativement peu connues. La majorité de ces interactions ont été caractérisées au niveau intracellulaire. Par exemple, il a été démontré que les galectines-3 et -7 peuvent lier directement la protéine anti-apoptotique Bcl-2 (B-cell lymphoma 2), indépendamment du CRD [43, 44]. D'autres études ont également démontré que les galectines-1 et -3 interagissent avec Gemin4 (Gem-associated protein 4) et Gemin2 sans la contribution, du moins directe, du CRD. [45-47]. Il existe aussi des interactions CRD-indépendantes extracellulaires, la plus connue étant la liaison de la galectine-1 au pré-récepteur antigénique des lymphocytes B [48].

1.2.3 Rôles physiologiques

Fonctions intracellulaires

Au niveau intracellulaire, les galectines ont plusieurs rôles physiologiques. La régulation du cycle cellulaire et de l'apoptose sont parmi les rôles des galectines qui ont été les mieux caractérisés. Par exemple, il a été démontré que l'expression intracellulaire de la galectine-3 augmente la prolifération de lymphocytes T activés et les protège contre l'apoptose induite via Fas [49, 50]. La galectine-12 semble aussi impliquée dans la régulation du cycle cellulaire puisqu'elle cause l'arrêt du cycle des cellules HeLa en phase G1 [51]. La galectine-7 a souvent été associée à l'apoptose. Elle est induite par le facteur de transcription p53 lorsque des kératinocytes sont exposés à des rayons UVB [52]. Dans des conditions pro-apoptotiques, la présence de la galectine-7 est associée à une activation de la voie JNK (c-jun N-terminal kinases), une voie de signalisation pro-apoptotique [53].

Une autre fonction des galectines est leur capacité à réguler certaines voies de signalisation cellulaires en interagissant directement avec certaines protéines qui font partie de la cascade de signalisation. Par exemple, les galectines-1 et -3 régulent les voies H-Ras et K-Ras, respectivement. En stabilisant les protéines Ras à la membrane cellulaire, les galectines activent la voie de signalisation, menant à la transformation cellulaire [54, 55]. Il a aussi été démontré que les galectine-1 et -3 interfèrent avec la localisation de la β-caténine via leurs interactions avec la protocadherine-24. Cela a pour conséquence la modulation de la voie de signalisation Wnt/β-caténine [56].

La régulation de l'épissage nucléaire de pré-ARNm est aussi une fonction majeure des galectines-1 et -3 qui interagissent avec les protéines Gemin2 et Gemin4 [45]. La répression de l'expression des galectines-1 et -3 a permis de démontrer qu'elles sont essentielles au bon fonctionnement de la machinerie de l'épissage. Cependant, elles semblent avoir un rôle redondant puisqu'il semble que la présence de l'une ou l'autre de ces galectines soit suffisante pour un épissage normal [57].

Fonctions extracellulaires

Parmi les fonctions extracellulaires des galectines, les mieux connues sont l'adhésion et la motilité, la protection contre les infections et la régulation du système immunitaire. Elles jouent également un rôle important dans l'homéostasie tissulaire. Ainsi, plusieurs galectines sont exprimées par les cellules épithéliales et participent à la réépithélialisation du tissu [58]. En fait, la galectine-3 serait impliquée dans la migration des cellules épithéliales de la cornée, de l'intestin et de la peau, alors que les galectines-2 et -4 pourraient promouvoir la réépithélialisation de l'intestin [58]. La galectine-7, quant à elle, serait nécessaire à la migration de cellules de la cornée, de la peau, des reins et de l'utérus [58]. La fonction des galectines dans la réépithélialisation s'explique entre autres par leur capacité à moduler les interactions cellules-cellules et cellules-matrice extracellulaire par la formation de treillis moléculaires [58]. De plus, la liaison des galectines aux intégrines présentes à la surface des cellules épithéliales induit une activation de voies de signalisation, telle que la voie α3β1-intégrines-Rac1, menant à une augmentation de la motilité par la formation de lamellipode [59].

Les galectines ont récemment été caractérisées comme étant des détecteurs d'infections et sont même parfois considérées comme des alarmines [60]. Il semblerait que les galectines puissent servir de récepteur de reconnaissance de motifs moléculaires en se liant

directement à certaines protéines glycosylées associées aux virus, aux bactéries, aux protistes et au fungus [60]. Par exemple, il a été démontré que la galectine-1 se lie à l'enveloppe du virus Niah, empêchant sa fusion avec les cellules hôtes [61]. La galectine-3, quant à elle, peut se lier de façon CRD-dépendante sur les lipopolysaccharides de la bactérie *K. pneumoniae* ou encore d'une façon CRD-indépendante via son domaine N-terminal, à la bactérie *S. enterica* [62]. À l'inverse, les galectines ne sont pas toujours des outils efficaces contre les infections. Dans certains cas, les pathogènes peuvent se servir des galectines afin d'infecter l'hôte. C'est le cas du parasite *Trypanosoma cruzi* qui présente en sa surface plusieurs protéines glycosylées. Les galectines se lient à ces glycoprotéines, favorisant ensuite l'interaction entre le parasite et les cellules hôtes [63]. Le VIH-1 (virus de l'immunodéficience humaine 1) se sert aussi des galectines afin de déjouer l'hôte. Les galectine-1 et -9 facilitent l'attachement du virus à la surface des cellules, favorisant ainsi l'infection [33, 60].

La fonction des galectines qui a été la plus étudiée est sans aucun doute la modulation de la réponse immunitaire. En se liant aux récepteurs membranaires glycosylés présents à la surface des cellules immunitaires, elles stabilisent les récepteurs en perturbant leur motilité latérale et leur recyclage, ce qui a des conséquences directes sur les voies de signalisation intracellulaires associées à ces récepteurs [29]. Ceci aura pour effet, par exemple, d'altérer la survie cellulaire, le chimiotactisme, la différenciation et le profil des cytokines sécrétées [15]. La figure 3 décrit les principales fonctions des galectines dans la régulation du système immunitaire innée et adaptative. En fait, il faut comprendre que chaque type cellulaire répond différemment à la présence de galectines à cause du type de récepteurs exprimés, le niveau et le type de glycosylation, et les galectines impliquées [15, 29]. Le meilleur exemple qui souligne l'importance des galectines dans la régulation du système immunitaire est sans aucun doute leur implication dans l'immunotolérance materno-fœtale. Pendant la grossesse, les galectines-1, -8, -9, -13, -14 et -16 induisent l'immunotolérance du fœtus en inhibant la prolifération des cellules T, en induisant l'apoptose des cellules T activées et en stimulant la production de cellules dendritiques tolérogéniques [15]. De fait, plus de la moitié des galectines (galectines-1, -2, -3, -4, -8, -9, -13, -14 et -16) sont exprimées à l'interface fœto-maternelle [15, 64]. Leur distribution tissulaire et leur niveau d'expression varient en fonction du stade de développement fœtal, ce qui supporte l'hypothèse qu'elles ont des fonctions distinctes [15]. Ainsi, les galectines-1, -3 et -9 faciliteraient l'implantation de l'embryon, alors que les galectines-1, -3, -8 et -13 seraient impliquées dans la réorganisation de la matrice extracellulaire afin de permettre l'invasion du trophoblaste. Quant aux galectines-1 et -13, elles seraient responsables d'assurer l'angiogenèse via l'augmentation de la production de

facteurs angiogéniques [15]. La régulation de l'expression des galectines semble d'ailleurs essentielle pour le bon déroulement de la grossesse puisqu'une dérégulation de l'expression des galectines-1, -3 et -13 est associée à la préclampsie [15].

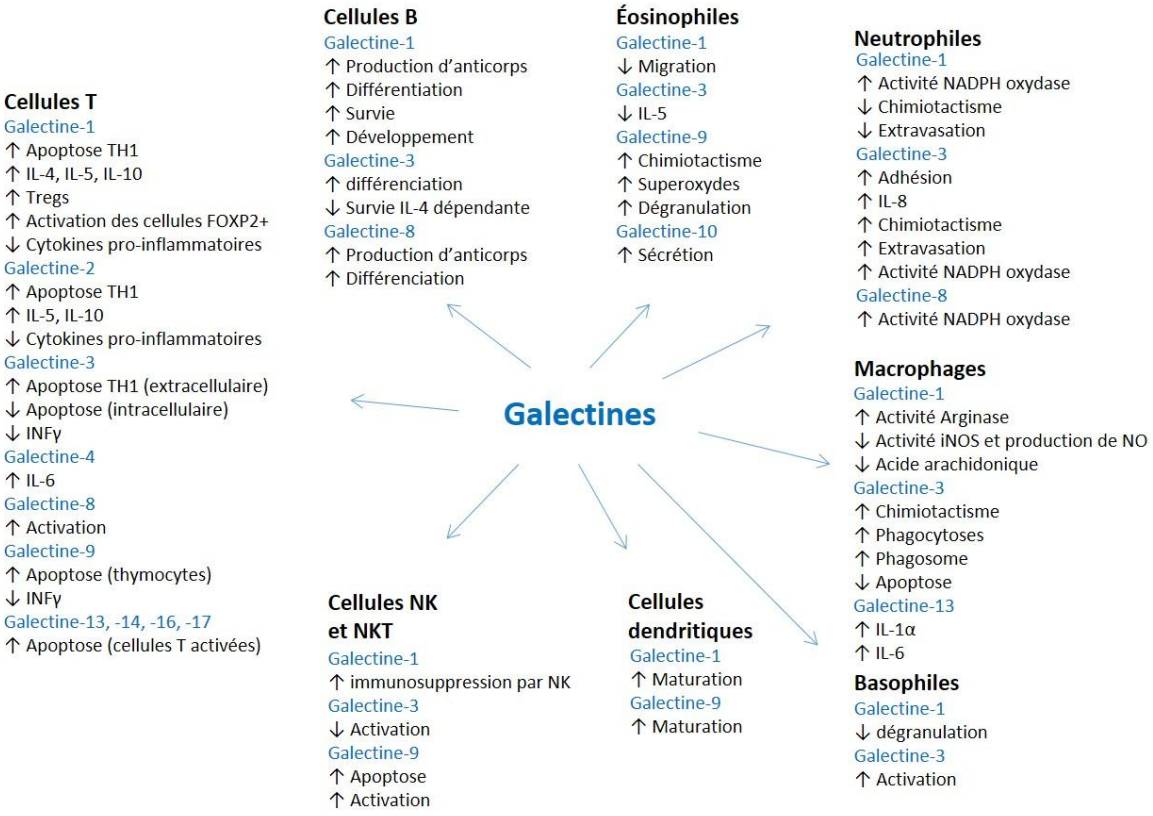

Figure 3 Modulation des fonctions du système immunitaire par les galectines. Conséquence de la présence de galectines sur les différents types de cellules immunitaires. Adaptation de [15].

1.3 Les galectines dans le cancer

Il est maintenant bien établi que l'expression des galectines est très souvent dérégulée dans les cellules cancéreuses. Il n'est pas rare d'observer la répression ou la surexpression de certaines galectines ou encore d'observer une variation de leur distribution intracellulaire ou tissulaire [27]. Cette dérégulation peut influencer positivement ou négativement la progression tumorale et il existe de plus en plus de preuves démontrant l'importance de la localisation cellulaire et tissulaire des galectines [27, 65]. Le tableau 3 décrit l'association entre l'expression des galectines et leur effet sur la progression de différents types de cancer [27].

Tableau 3 Expression des galectines dans le cancer. Association entre la présence de galectines et l'inhibition ou la stimulation de la progression de différents cancers. Adaptation de [27].

Galectine	Inhibition de la progression	Stimulation de la progression
Galectine-1		Thyroïde, prostate, sein, col de l'utérus, poumon, ovaire, gliome, lymphome B, foie, pancréas
Galectine-2		Sein, colon
Galectine-3	Gliome	Colon, sein, prostate, thyroïde, foie, poumon, lymphome B, leucémie myéloïde, mélanome, vessie, ovaire, pancréas, estomac, langue, rein
Galectine-4	Colorectal, pancréas	
Galectine-7	Neuroblastome, col de l'utérus, colorectal, estomac	Sein, lymphome, ovaire, urothélial, mélanome,
Galectine-8	Colon	Glioblastome
Galectine-9	Mélanome, colon, vésicule biliaire, estomac	
Galectine-12	Col de l'utérus, leucémie	

1.3.1 Fonctions dans la progression tumorale

Historiquement, les galectines ont souvent été associées à la modulation de la progression tumorale. La figure 4 résume les différentes fonctions pro- ou anti-tumorales de ces protéines. Un aspect intéressant de la biologie des galectines est qu'un même membre de la famille affiche parfois des fonctions inverses selon le type de cancer dans lequel il est exprimé, sa localisation cellulaire et la disponibilité de ses partenaires d'interaction (revue dans [27]). Cela ajoute une complexité à l'étude des galectines et justifie le besoin d'approfondir nos connaissances sur cette famille de protéines dans différents cancers.

Fonctions intracellulaires

Au niveau intracellulaire, les galectines participent à plusieurs aspects de la progression tumorale, dont la transformation cellulaire, la prolifération et l'apoptose. Les galectines-1 et -3 sont reconnues pour induire la transformation cellulaire. Des expériences menées sur les cellules 293T ont démontré que la galectine-1 stabilise H-Ras à la membrane cellulaire interne et active la voie de signalisation ERK (Extracellular signal-regulated kinases), menant à l'induction de gènes de survie et de prolifération cellulaire [54]. Des résultats similaires ont été obtenus avec la galectine-3 et K-RAS, qui mènent à l'activation de la voie PI3K (Phosphoinositide 3-kinase) [66].

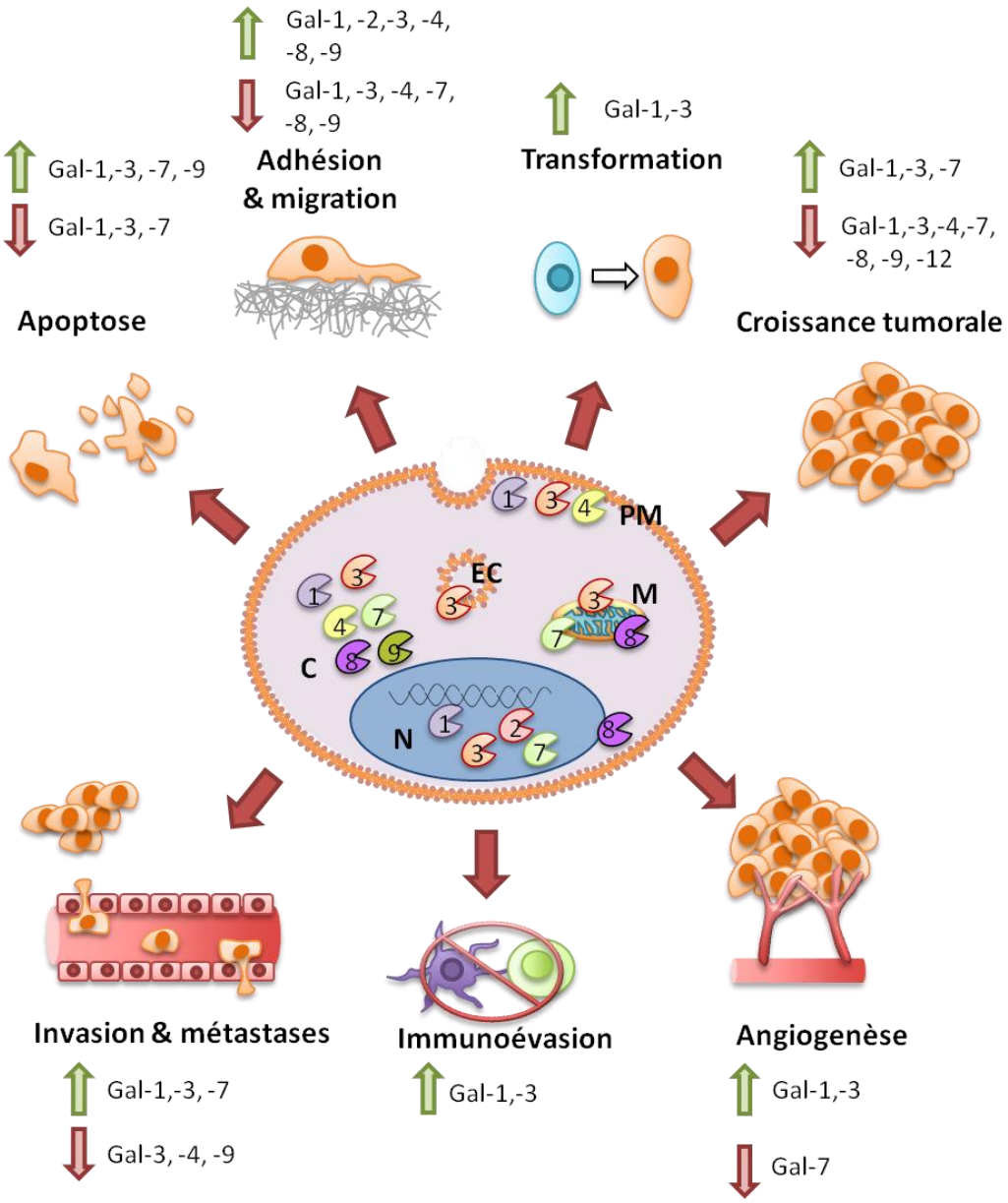

Figure 4 Fonctions des galectines dans le cancer. Localisation intracellulaire et implication des galectines dans la progression tumorale. Les flèches rouges et vertes indiquent respectivement une induction et une répression des processus biologiques spécifiés. Adaptation de [27].

Au niveau de la régulation du cycle cellulaire, les galectines peuvent moduler positivement ou négativement l'expression de différentes protéines clés, dont les cyclines A, D1 et E, p21 et p27 [67-69]. Une même galectine peut afficher des fonctions antiprolifératives dans un cancer et stimuler la prolifération cellulaire dans un autre. La galectine-7 en est un bon exemple. Elle réduit la croissance des neuroblastomes et des cellules des cancers colorectaux et gastriques, alors qu'elle est associée à la prolifération des cellules du cancer ovarien [27].

La fonction intracellulaire des galectines qui a été la mieux caractérisée jusqu'à maintenant est la modulation de l'apoptose. Les galectines peuvent sensibiliser les cellules cancéreuses à l'apoptose induite par des agents chimiothérapeutiques ou encore favoriser la résistance à la chimiothérapie. Les galectines-3 et -7 constituent les meilleurs exemples de galectines qui possèdent un double rôle à cet égard. La galectine-3 a une fonction anti-apoptotique dans le cancer du sein, de la thyroïde et colorectal et une fonction pro-apoptotique dans certains lymphomes B [27]. Plusieurs groupes se sont intéressés aux mécanismes moléculaires impliqués dans la régulation de l'apoptose par la galectine-3. Il a été démontré qu'elle comporte en sa structure une séquence homologue à celle de certains membres de la famille Bcl-2. Il s'agit de la séquence NWGR qui se trouve dans la partie C-terminale de la galectine-3. La galectine-3 interagit avec la protéine Bcl-2 via ce domaine ce qui lui confère des propriétés anti-apoptotiques dans les cellules du cancer du sein BT-549 [43, 50]. D'autre part, Califice *et al.* ont établi que la localisation intracellulaire de la galectine-3 dans le cancer de la prostate détermine ses fonctions pro- et anti-tumorale [65]. La protéine nucléaire est associée à une sensibilisation à l'apoptose, alors que la protéine cytoplasmique induit la résistance à la chimiothérapie [65]. Pour ce qui est de la galectine-7, elle a une fonction anti-apoptotique dans le cancer du sein et le mélanome et elle a une fonction pro-apoptotique dans le cancer cervical, colorectal et urothélial [27]. Les mécanismes moléculaires sous-jacents sont peu connus, mais il a été démontré que tout comme la galectine-3, la galectine-7 peut aussi interagir avec Bcl-2 [44]. Notre équipe a récemment mis en évidence que la galectine-7 a une fonction anti-apoptotique dans le cancer du sein, possiblement en favorisant la dégradation de p53 par le protéasome [24]. Des études utilisant une galectine-7 avec un CRD non fonctionnel ont aussi démontré que sa fonction anti-apoptotique est indépendante de son CRD et que la localisation mitochondriale ou nucléaire n'est pas essentielle à cette fonction [24]. Des études plus approfondies seront nécessaires afin de déterminer si les fonctions anti-apoptotiques de la galectine-7 sont également indépendantes du CRD et de la localisation mitochondriale ou nucléaire.

Fonctions extracellulaires

La réponse immunitaire anti-tumorale est essentielle au combat contre le cancer. Lors de la réponse anti-tumorale adaptative, les lymphocytes de type TH1 (*type 1 T helper*) et TH17 sont activés par les cellules dendritiques et facilitent l'élimination des cellules cancéreuses par la sécrétion de cytokines pro-inflammatoires (INFγ, IL-17, etc.) qui stimulent le recrutement de macrophages [70, 71]. Les lymphocytes T CD8+, quant à eux, éliminent les cellules cancéreuses via leur activité cytotoxique [70, 71]. À l'opposé, les lymphocytes de type TH2 induisent une réponse humorale, inefficace contre les cellules cancéreuses, alors

que les cellules T régulatrices (Treg) induisent la tolérance immunitaire via la sécrétion de TGFβ, inhibant la réponse de type TH1 [70, 71]. Plusieurs études ont démontré la corrélation entre la présence de cellules T infiltrant la tumeur et le pronostic des patients [72-74]. Dans plusieurs types de cancer, on peut observer qu'une infiltration de lymphocytes T CD8 et l'absence de cellules Treg corrèlent avec une meilleure survie des patients, alors qu'une réponse de type TH2 et l'expansion des cellules Treg sont associées à un mauvais pronostic [72-74].

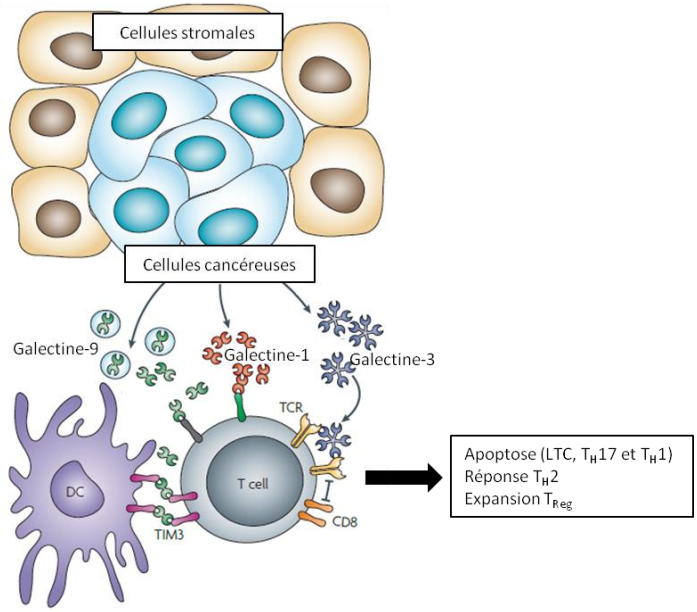

Figure 5 Immunoévasion des cellules cancéreuses induite par les galectines. Schématisation de l'influence des galectines péritumorales sur la réponse anti-tumorale (adaptation de [29]).

Puisqu'elles peuvent être exprimées et sécrétées par les cellules cancéreuses ainsi que les cellules stromales, une grande variété de galectines peut se retrouver dans l'environnement péritumoral [29]. Il a été démontré que les galectines peuvent affecter différents aspects de la réponse anti-tumorale, dont la signalisation et l'activation des lymphocytes T, leur survie, ainsi que le profil de cytokines qui est sécrété. Dans plusieurs cas, la présence de galectines a été associée à l'immunoévasion via l'induction de l'apoptose des lymphocytes T CD8+, TH1 et TH17, à l'induction d'une réponse de type TH2 et à l'expansion des cellules Treg (Figure 5) (Revue dans [29]).

Il a été démontré que les niveaux de glycosylation des récepteurs présents à la surface des cellules immunitaires changent drastiquement pendant leur maturation, activation et différenciation [75, 76]. Cela permet donc de réguler leur susceptibilité aux galectines. Par exemple, il a été démontré que la galectine-1 induit l'apoptose des cellules de type TH1 via

la ségrégation des récepteurs CD45 et CD3 ainsi que CD43 et CD7, alors que les cellules de type TH2 sont résistantes à l'apoptose induite par la galectine-1 [77]. Dans un contexte tumoral, il y aura donc une diminution de production d'interféron gamma (INFγ) et une augmentation des niveaux d'interleukine 4 (IL-4), IL-5 et IL-10. Ce profil de cytokines favorise la tolérance immunitaire en bloquant la réponse de type TH1, en inhibant les fonctions des cellules présentatrices d'antigènes et en induisant une réponse Treg [78]. La galectine-9 a aussi une fonction pro-apoptotique via sa liaison à Tim-3 sur les lymphocytes de types TH1 et les lymphocytes T CD8+ [79].

Les galectines présentes dans l'environnement péri-tumoral sont impliquées dans d'autres aspects de la progression du cancer, dont l'angiogenèse et le potentiel invasif des cellules tumorales [27]. L'angiogenèse consiste en la production de nouveaux vaisseaux sanguins. Ce processus est essentiel à la croissance tumorale puisqu'il permet l'apport en oxygène et en nutriments. De plus, la présence de vaisseaux sanguins facilite la dissémination des cellules cancéreuses vers d'autres sites (formation de métastases) [80]. Quant au potentiel invasif, il consiste en la capacité des cellules cancéreuses à digérer la matrice extracellulaire par des enzymes spécialisées telles que les métalloprotéinases de la matrice (MMP) et se déplacer (motilité) vers des sites secondaires. La régulation de l'adhésion aux tissus est également un aspect important qui permettra aux cellules cancéreuses de se détacher de la tumeur primaire et de coloniser un nouveau tissu [81].

La présence de galectines dans la matrice extracellulaire peut favoriser le chimiotactisme et stimuler la migration et la prolifération des cellules endothéliales. Cela aura pour conséquence l'induction de l'angiogenèse. Ce mécanisme CRD-dépendant a été bien caractérisé dans un modèle murin du cancer du sein où la présence de galectine-3 a été associée à une plus grande irrigation de la tumeur [82]. Les galectines peuvent également inhiber l'angiogenèse. C'est le cas de la galectine-7 dans le cancer du côlon. Nous ne savons toutefois pas par quel mécanisme elle exerce cet effet [27, 83]. Dans le cas du potentiel invasif, les galectines peuvent se lier à plusieurs récepteurs membranaires, ainsi qu'à des glycoprotéines de la matrice extracellulaire. Ces interactions sont donc importantes afin de moduler les interactions cellule-cellule et cellule-matrice extracellulaire [27]. Les galectines ont aussi la possibilité de moduler l'expression de protéases essentielles à la digestion de la matrice extracellulaire. Par exemple, notre équipe a démontré que la galectine-7 stimule la tumorigenèse des lymphomes par l'augmentation de l'expression de MMP-9. Park *et al*. ont aussi démontré que la présence ectopique de galectine-7 stimule la

production de MMP-9 par les cellules HeLa via la voie de signalisation p38-MAPK (mitogen-activated protein kinase), favorisant le déplacement des cellules au travers du matrigel [84, 85].

En conclusion, les galectines sont impliquées dans une multitude de mécanismes pro- et anti-tumoraux. Une même galectine peut afficher des fonctions inverses dépendamment du type cellulaire, de sa localisation intra ou extracellulaire et de la disponibilité de ses partenaires d'interaction. Il est fort probable que les galectines aient des fonctions redondantes, complémentaires, synergiques ou opposées. Puisqu'une tumeur exprime généralement plus d'une galectine, plusieurs croient qu'il serait possible de déterminer des signatures d'expression de galectines comme biomarqueur et cible thérapeutique. Cependant, devant la complexité des mécanismes cellulaires régulés par les galectines, il est primordial de mieux comprendre les fonctions individuelles de chaque galectine avant de considérer les utiliser comme cible thérapeutique.

2 LE CANCER DE LA PROSTATE

Le cancer de la prostate est le cancer le plus fréquent chez les hommes. En fait, on estime qu'environ un homme sur sept en Amérique du Nord en développera un au cours de sa vie. Ce cancer se traite relativement bien, avec un taux de survie d'environ 95 % pendant les cinq années qui suivent la détection de la maladie. Malheureusement, son taux d'incidence élevé fait en sorte que le cancer de la prostate est la deuxième cause de mortalité associée au cancer chez l'homme [86]. De plus, certains cas plus rares de cancer prostatiques sont métastatiques et résistants à la castration chimique. Les hommes qui en sont atteints ont peu de chances de survie puisqu'il n'existe pas de traitements efficaces contre ces cancers agressifs [87]. Il peut s'agir de formes rares de cancers, tel que le carcinome à cellules basale, reconnus pour leur phénotype agressif [88]. Il peut aussi s'agir de cancers prostatiques initialement peu agressifs qui ont subi une accumulation d'altérations génétiques les rendant métastatiques et résistants aux androgènes [89].

2.1 La prostate

La prostate fait partie de l'appareil reproducteur masculin. Il s'agit d'une glande qui a pour principale fonction la production de liquide séminal, un composant du sperme. Composé

d’une cinquantaine de glandes tubulo-alvéolaires ramifiées, la prostate est subdivisée en trois zones : périphérique, centrale et de transition (Figure 6) [90].

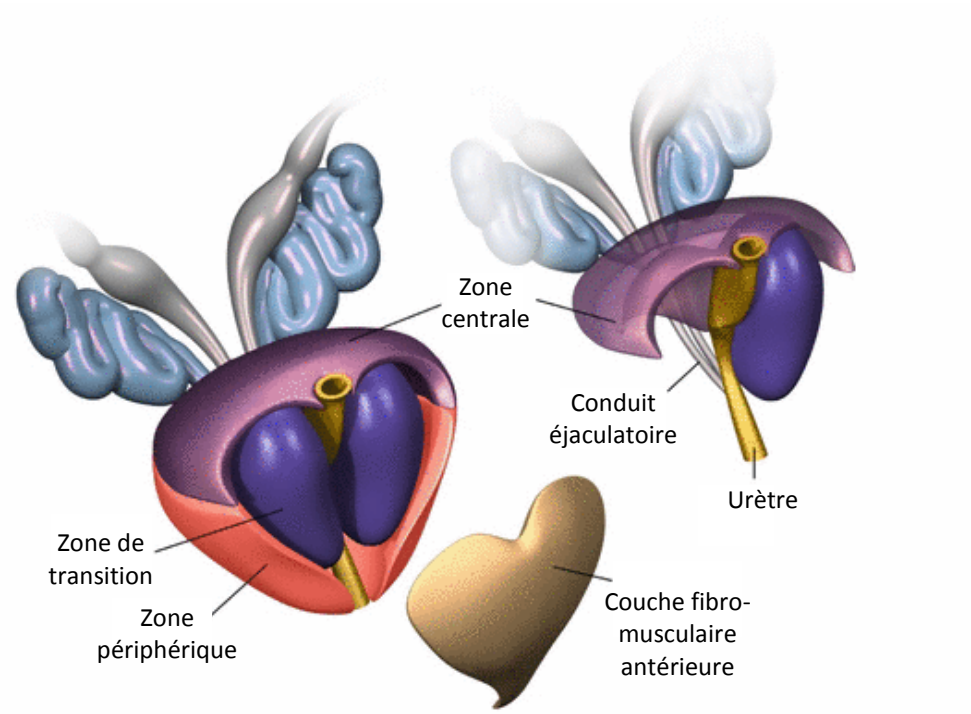

Figure 6 Structure de la prostate. Schématisation de la prostate et des tissus avoisinants. Adaptation de [90].

Alors que le stroma contient majoritairement des fibroblastes, des cellules musculaires lisses, des cellules endothéliales et de la matrice extracellulaire, l’épithélium glandulaire est composé de différents types cellulaires, dont des cellules luminales sécrétrices et des cellules basales. D’autres types cellulaires sont présents en moins grande quantité, tels que des cellules souches, neuroendocrines et neuronales (Figure 7).

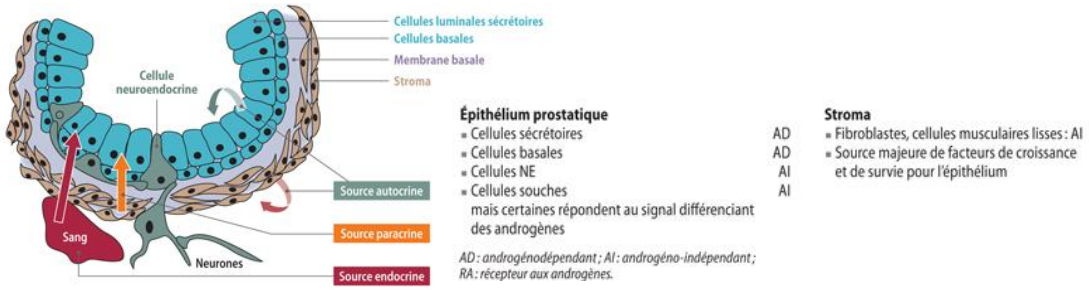

Figure 7 Histologie de la prostate. Schématisation d’une glande prostatique et des différents types cellulaires qui la composent. Adaptation de [91].

Les hommes peuvent être atteints par certaines affections bénignes de la prostate, telles que la prostatite, caractérisée par une infection ou une inflammation de la prostate, ou encore l'hyperplasie bénigne, qui consiste en une surcroissance des cellules de la prostate [86]. Bien qu'inconfortables, ces affections n'augmentent pas les risques de développer un cancer de la prostate. Il existe cependant des états précancéreux qui demandent une surveillance régulière, telle que la néoplasie intraépithéliale de la prostate qui se caractérise par une prolifération des cellules épithéliales bordant les canaux et acini prostatiques [86].

2.2 Classification

La classification du cancer de la prostate est une étape essentielle pour le clinicien afin de déterminer le type de traitement que nécessite le patient. Dans un premier temps, il est essentiel de déterminer le type histologique de la tumeur et sa sensibilité aux androgènes. Ensuite, il faut établir le score de Gleason et le stade TNM (taille, ganglions lymphatiques, métastases). Toutes ces informations permettront de déterminer si le patient a un cancer de la prostate de haut risque. Le tableau 4 décrit les caractéristiques associées à des patients ayant un cancer avec risques faibles, modérés ou élevés.

Tableau 4 : Facteurs permettant d'évaluer le niveau de risque associé au cancer prostatique [92, 93]

Risque	Score de Gleason	Niveau d'APS	Stadification T
Faible	6	Inférieur à 10 ng/ml	T1 à T2a
Modéré	7	Entre 10 et 20 ng/ml	T2b
Élevé	8 à 10	Plus de 20 ng/ml	T2c, T3 ou T4

2.2.1 Types histologiques

Il existe différents types de carcinomes prostatiques et dans 95 % des cas il s'agit d'adénocarcinome, c'est-à-dire une tumeur qui a pris naissance dans les cellules glandulaires de la prostate [86]. Environ 70 % des cancers de la prostate se développent dans la zone périphérique, 20 % dans la zone de transition et seulement 10 % dans la zone centrale de la prostate [90]. Le type histologique le plus fréquent est l'adénocarcinome acinaire. Les adénocarcinomes ont un phénotype s'appariant aux cellules luminales. Cela suggère donc que les adénocarcinomes proviennent de cellules luminales ou encore, de cellules basales qui ont acquis des caractéristiques de cellules luminales [94]. L'origine du

cancer de la prostate est encore aujourd'hui un sujet controversé. Auparavant, il était considéré que le cancer de la prostate prenait naissance à partir de cellules luminales, puisqu'il avait été noté que les cellules basales sont absentes des tumeurs prostatiques [95]. Cependant, plusieurs évidences indiquent maintenant que les cellules basales pourraient aussi être à l'origine du cancer prostatique. Par exemple, il a été démontré que l'expression de c-myc ou ERG et l'activation de la voie AKT dans des cellules basales permettait de former des tumeurs in vivo [96, 97]. Lawson et al. ont d'ailleurs démontré qu'il était possible de stimuler la formation de tumeurs par des cellules souches/basales via différentes techniques, dont la surexpression d'EGR1, la stimulation de la voie FGF ou l'activation de la voie AKT [98]. Ce qui est d'autant plus intéressant est que ces tumeurs qui prennent naissance à partir de cellules basales affichent un phénotype ressemblant aux adénocarcinomes acinaires agressifs androgènes-indépendants [96, 98].

2.2.2 Sensibilité aux androgènes

Les adénocarcinomes peuvent être hormono-dépendants ou hormono-indépendants. Chez l'homme, la testostérone est principalement produite par les cellules de Leydig dans les testicules et les glandes surrénales. La production de testostérone est stimulée par l'hormone de libération des gonadotrophines hypophysaires (LH-RH) qui est produite par l'hypothalamus. Lorsque la testostérone diffuse dans le cytoplasme des cellules luminales de la prostate, elle est rapidement convertie en dihydrotestostérone (DHT) par l'enzyme stéroïde 5-alpha réductase de type II (SRD5A2) [99]. Lorsque le récepteur des androgènes (AR) lie la DHT, le complexe récepteur/ligand transloque dans le noyau des cellules et active la transcription de plusieurs gènes impliqués dans la survie, la prolifération et la synthèse protéique et lipidique [100]. Les adénocarcinomes hormono-dépendants nécessitent donc la présence de testostérone et de son dérivé DHT pour survivre et proliférer. Dans plusieurs cas, les adénocarcinomes ont des voies de signalisation altérées ou encore des mutations qui leur permettent d'être hormono-résistants (Figure 8) [101]. On considère qu'un cancer est hormono-résistant lorsqu'on détecte une augmentation du niveau de l'APS, une progression de la maladie pré-existante ou l'apparition de nouvelles métastases chez un patient castré chimiquement [102].

Plusieurs évènements peuvent provoquer l'hormono-résistance (Figure 8). Premièrement, une amplification/surexpression du gène codant pour le AR augmente la sensibilité de la voie de signalisation, alors qu'une mutation au niveau de ce gène peut augmenter son affinité pour d'autres ligands tels que la progestérone, l'hydrocortisone et l'œstradiol [91, 101]. Puisque l'activité transcriptionnelle d'AR est régulée par des co-activateurs et des co-

répresseurs, tels que la famille SRC et FKBP52, une dérégulation de ces derniers peut causer une activation inappropriée d'AR [94, 99, 101]. Une dérégulation des modifications post-transcriptionnelles d'AR, la présence de facteurs de croissance et cytokines ou une dérégulation des voies de signalisation de kinases peuvent mener à l'activation d'AR [101]. Une production anormalement élevée d'androgènes par les glandes surrénales causée par une dérégulation de la stéroïdogenèse peut aussi activer AR [101]. Finalement, l'épissage alternatif d'AR peut produire des variants constitutivement actifs [101]. Tous ces événements ont le potentiel d'augmenter l'agressivité du cancer prostatique.

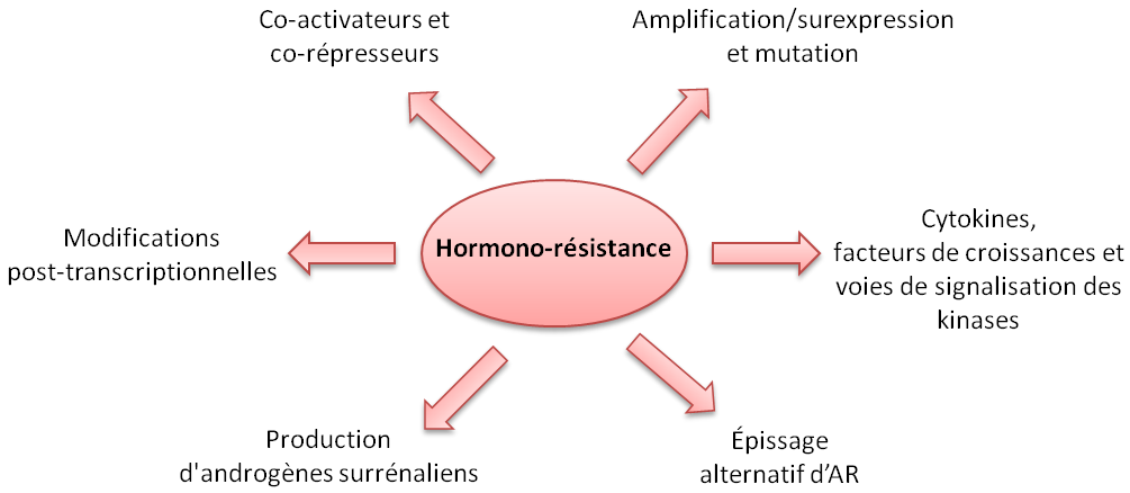

Figure 8 Mécanismes moléculaires d'hormono-résistance dans le cancer de la prostate. Illustration des différents mécanismes moléculaires qui mènent à l'hormono-résistance.

2.2.3 Score de Gleason

Le score de Gleason est un système utilisé pour caractériser le degré d'agressivité des adénocarcinomes de la prostate. Il est basé sur le niveau de différenciation de la tumeur ainsi que sur la croissance des cellules cancéreuses [86]. Cette classification s'échelonne sur cinq grades, mais seuls les grades 3 à 5 sont attribués au cancer. Le grade 3, un bas grade, ressemble beaucoup au tissu normal. Il représente une tumeur bien différenciée ayant une croissance cellulaire lente. À l'inverse, le grade 5, un haut grade, représente une tumeur peu différenciée et associée à une forte croissance cellulaire (Figure 9) [103]. Les adénocarcinomes sont généralement multifocaux, c'est-à-dire qu'il y a souvent un nodule primaire, puis des nodules secondaires. Afin d'avoir une meilleure caractérisation du phénotype tumoral et de déterminer si le patient est à haut risque, les cliniciens utilisent le score de Gleason, qui consiste en l'addition des deux grades les plus représentés dans le tissu cancéreux. Un score de Gleason de 6 est généralement associé à un faible risque,

alors que le score 7 et les scores 8 à 10 sont associés à un risque modéré et élevé, respectivement [92].

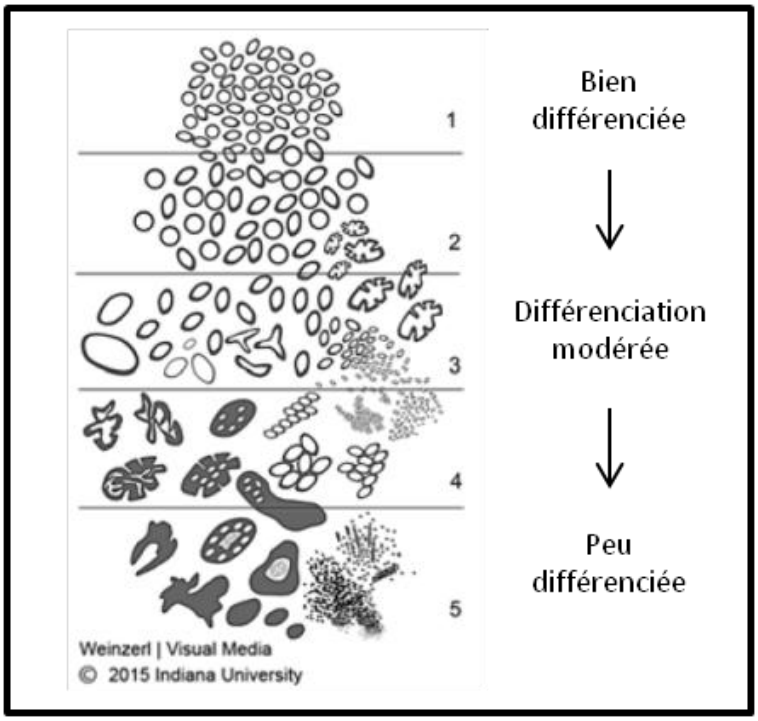

Figure 9 Classification histologique des adénocarcinomes de la prostate. Modification 2014 par l'ISUP (International Society of Urological Pathology) de l'échelle de Gleason. Adaptation de [103].

2.2.4 Stadification TNM

Le cancer de la prostate est également classé selon le stade de la tumeur par le système de stadification TNM. Le tableau 5 décrit les différents niveaux de ce système de classification.

Tableau 5 Stadification clinique du cancer de la prostate selon la société canadienne du cancer. Adapté de [86].

Classification	Code	Caractéristiques
Tumeur primitive (T)	TX	Évaluation impossible
	T0	Aucun signe de tumeur
	T1	Tumeur indétectable par imagerie ou palpation
	T1a	Détecté par hasard au cours d'une résection transurétrale. Cellules cancéreuses représentant 5 % ou moins du tissu retiré
	T1b	Détecté par hasard au cours d'une résection transurétrale. Cellules cancéreuses représentant plus de 5 % du tissu retiré
	T1c	Détectée en raison d'un taux élevé d'APS
	T2	Tumeur limitée à la prostate
	T2a	Tumeur atteignant la moitié d'un lobe ou moins
	T2b	Tumeur atteignant plus de la moitié d'un lobe, mais pas les deux lobes
	T2c	Tumeur atteignant les deux lobes
	T3	Propagation de la tumeur hors de la prostate
	T3a	Propagation hors de la prostate ou au col de la vessie
	T3b	Propagation à une vésicule séminale ou aux deux
	T4	Tumeur attachée ou propagée à des structures avoisinantes: rectum, paroi pelvienne, vessie
Ganglions lymphatiques régionaux (N)	NX	Évaluation impossible
	N0	Aucune métastase dans les ganglions lymphatiques régionaux
	N1	Présence de métastases dans les ganglions lymphatiques régionaux
Métastases à distance (M)	M0	Aucune métastase à distance
	M1	Présence de métastases à distance
	M1a	Métastases dans les ganglions lymphatiques non régionaux
	M1b	Métastases aux os
	M1c	Métastases à d'autres emplacements – poumon, foie, rein

2.2.5 Développement de nouveaux outils

Les outils et les modèles de classification et de prédiction pour le cancer de la prostate ne permettent pas toujours d'identifier les patients à haut risque. En fait, il est parfois difficile pour le pathologiste d'évaluer la progression tumorale en se basant sur les analyses qui ont été effectuées par le clinicien [105]. Les techniques d'imageries actuelles, scintigraphie osseuse, résonnance magnétique (IRM) ou tomodensitométrie, ne permettent pas toujours de repérer les métastases et c'est une des raisons qui font en sorte que le diagnostic est parfois imprécis [106]. Plusieurs équipes tentent de développer de nouvelles techniques, pour la plupart basées sur l'imagerie. Par exemple, plusieurs ont récemment testé un système d'imagerie en tomographie par émission de positrons (TEP), basée sur un inhibiteur de l'antigène prostatique membrane spécifique (PMSA) couplé au gallium-68 (68-Ga-PMSA) (revue dans [106]). PMSA est une protéine transmembranaire principalement exprimée par les cellules de la prostate et elle est souvent surexprimée dans les cancers hormono-indépendants [106]. En fait, on estime que son expression est généralement de 100 à 1000

fois plus élevée dans les cellules cancéreuses de la prostate que dans les tissus sains [106]. Cela fait donc de cette protéine une cible parfaite pour le TEP. Les résultats de diverses études ont d'ailleurs démontré que l'utilisation du 68-Ga-PMSA-TEMP en combinaison avec l'IRM permettait de mieux caractériser la localisation des tumeurs primaires au niveau de la prostate et offrirait une meilleure sensibilité pour la détection des métastases [106]. Ainsi, le 68-Ga-PMSA-TEMP semble être un outil intéressant qui permettra, dans le futur, de mieux classifier les cancers prostatiques et identifier les patients à haut risque.

2.3 Traitements

Le traitement qui sera choisi pour un patient atteint du cancer prostatique dépend de plusieurs facteurs dont le type histologique, le stade et le grade, la présence de métastases et l'état général du patient [86]. Les différents traitements comprennent une surveillance active, la chirurgie, la radiothérapie, l'hormonothérapie, la chimiothérapie, la thérapie biologique et l'utilisation de biphosphonates.

Surveillance active : La surveillance active sert à observer la progression de la maladie afin de décider du meilleur moment pour commencer un traitement. Ce suivi comprend généralement une surveillance par toucher rectal, la mesure du niveau sérique de l'APS, une biopsie, une imagerie par résonnance magnétique de la prostate et une scintigraphie osseuse ou une radiographie pulmonaire selon les symptômes du patient. On effectue généralement une surveillance active pour les patients qui ont un cancer de faible risque. On peut également opter pour cette méthode selon l'espérance de vie du patient, sa condition générale de santé ainsi que ses préférences quant au traitement [86, 107].

Chirurgie : Lorsque la tumeur est localisée, une des options à envisager est de la retirer via la prostatectomie radicale. Dans les situations où le cancer est plus avancé, il est possible de dégager l'obstruction des voies urinaires via une résection transurétrale de la prostate (RTUP). Enfin, une lymphadénectomie servira à vérifier si le cancer s'est disséminé aux ganglions pelviens [86].

Radiothérapie : On peut utiliser la radiothérapie comme traitement principal ou encore comme adjuvant lors du traitement initial ou pour traiter une récidive. Lorsqu'une tumeur localisée est retirée par prostatectomie radicale, la radiothérapie est souvent utilisée afin d'éliminer les risques de récidive. Elle sert aussi à réduire la taille de tumeurs invasives. Il existe deux options : la radiothérapie externe et la curiethérapie. La radiothérapie externe consiste à envoyer des radiations vers la tumeur et certains tissus avoisinants, alors que la curiethérapie utilise des implants radioactifs qui sont placés directement dans la prostate, par chirurgie. Dans certains cas lorsque le cancer est hormono-résistant et qu'il s'est propagé

aux os, il est possible d'effectuer une radiothérapie systémique en administrant un isotope tel que le dichlorure de radium 223 [86, 108].

Hormonothérapie : L'hormonothérapie a pour objectif l'inhibition de la voie des androgènes et est généralement prescrite aux patients ayant un cancer métastatique. Plusieurs classes de médicaments peuvent être utilisées, telles que des analogues ou des antagonistes de LH-RH, des anti-androgènes et des œstrogènes [86, 109].

Chimiothérapie : La chimiothérapie est généralement offerte aux patients qui ont un cancer métastatique et/ou hormono-résistant. Les taxanes sont les agents chimiothérapeutiques les plus utilisés pour le traitement du cancer de la prostate à haut risque. Il s'agit de molécules stabilisatrices des microtubules. Le docétaxel et le cabazitaxel sont les deux taxanes les plus utilisés au niveau clinique. Il existe d'autres options pour les patients qui ne répondent pas aux taxanes. Par exemple, il est possible d'utiliser le mitoxantrone, un inhibiteur de la topoisomérase de type II [86, 109-111].

Thérapie biologique : Il s'agit d'un traitement qui a pour objectif de réduire les risques de complications osseuses dues au cancer métastatique et aussi prévenir l'ostéoporose qui peut être causée par l'hormonothérapie. Le Denosumab, un anticorps monoclonal dirigé contre le ligand RANK (receptor activator of nuclear factor kappa-B), est le plus souvent utilisé [86, 112].

Biphosphonates : Ce traitement systémique à base d'acide zolédronique sert à renforcer les os [86, 113].

Malgré le fait que les tumeurs localisées de bas stade se traitent relativement bien, certains cancers de la prostate sont métastatiques et résistants à la castration. Il s'agit généralement de cancers qui répondent aux traitements initiaux, mais développent par la suite une résistance à la chimiothérapie et à l'hormonothérapie [87]. C'est pourquoi il est essentiel de développer de nouveaux traitements contre ces cancers. Au cours des dernières années, un intérêt particulier a été porté au développement de traitements immunothérapeutiques. Il en existe cinq classes : les vaccins, les inhibiteurs de «checkpoint» immunologiques, les virus oncolytiques, les adjuvants et le transfert adoptif de cellules [114].

Les vaccins thérapeutiques : Ceux-ci ont pour objectif l'induction d'une réponse immunitaire dirigée contre les cellules cancéreuses en ciblant un antigène spécifique ou associé aux cellules cancéreuses. Le cancer de la prostate présente plusieurs antigènes intéressants, tels que l'APS, la phosphatase acide prostatique et certains antigènes

membranaires spécifiques à la prostate [87]. Sipuleucel-T est un exemple de vaccin thérapeutique qui a récemment été approuvé aux États-Unis, suite à un essai clinique de niveau III. Ce vaccin induit une réponse immunitaire contre la phosphatase acide prostatique et il a été démontré qu'il permet d'augmenter la survie des patients ayant un cancer métastatique hormono-résistant [17]. D'autres vaccins sont actuellement à l'étude, c'est le cas du PROSTAVAC, un vaccin qui cible l'APS. Des résultats prometteurs indiquent que ce traitement permettrait lui aussi d'augmenter la survie des patients [115]. Les patients avec un cancer de bas stade répondent mieux aux vaccins thérapeutiques que les patients avec une maladie plus avancée. Il semblerait que ce type de traitement ait une plus grande efficacité lorsque combiné avec un autre type d'immunothérapie telle que l'utilisation d'inhibiteurs de «checkpoint» immunologique [116]. Cette combinaison offrirait de meilleures chances de survie pour les patients atteints d'un cancer métastatique résistant à la castration.

Les inhibiteurs du «checkpoint» immunologiques : Ils ont pour objectif l'activation ou l'augmentation de la réponse immunitaire anticancéreuse via la modulation de molécules l'inhibitrice de l'activité des cellules T, tels que les récepteurs CTLA-4, PD-1 et PD-L1. Il existe de nombreux traitements en développement dont l'Ipilimumab, un anticorps qui cible l'antigène CTLA-4 (*cytotoxic T-lymphocyte antigen 4*), le Pembrolizumab, un anticorps anti-PD-1 (*programmed cell death 1*), et l'Atezolizumab, un anticorps anti-PD-L1 (*programmed cell death-ligand 1*) [18, 19]. Tout comme pour les vaccins thérapeutiques, il semblerait que les inhibiteurs du «checkpoint» immunologiques soient plus efficaces en combinaison avec un deuxième traitement. C'est pourquoi plusieurs études s'intéressent à combiner un traitement cytotoxique aux traitements d'immunothérapie. La réponse immunitaire qui en résulte pourrait ainsi être augmentée [87].

Les virus oncolytiques : Ces derniers sont des virus modifiés qui infectent et tuent les cellules cancéreuses, tout en induisant une réponse immunitaire contre le cancer. ProstAtak est actuellement à l'étude dans un essai clinique de phase III. Il s'agit d'un virus de la vaccine qui a été modifié et inactivé et qui infecte les cellules cancéreuses. Ce virus contient un gène qui sensibilise les cellules cancéreuses à l'apoptose induite par le Valacyclovir, un agent antiviral développé initialement contre les virus herpes [114].

Les adjuvants : Ceux-ci sont utilisés afin de stimuler la réponse immunitaire et peuvent être utilisés seuls ou en combinaisons avec d'autres agents d'immunothérapie. Un exemple d'adjuvant est l'Indoximod, un inhibiteur de la voie de l'indoleamine 2,3-dioxygenase, qui est utilisé en combinaison avec le Sipuleucel-T dans un essai clinique de phase II [114].

Le transfert adoptif de cellules : Lors de cette approche, les cellules T du patient sont isolées, modifiées génétiquement ou traitées afin d'augmenter leur pouvoir anti-tumoral, puis réintroduites dans le patient [114].

2.4 Biomarqueurs

Utilisé depuis 1986, l'APS est le biomarqueur le plus utilisé au niveau clinique pour le cancer de la prostate. L'APS est une sérine protéase qui fait partie de la famille des kallikréines (hK). L'APS est anormalement exprimé par les cellules cancéreuses de la prostate et son expression peut être détectée dans le sang des patients [117]. Ainsi, la mesure du niveau d'APS sérique peut servir à détecter le cancer de la prostate, évaluer son niveau d'agressivité et faire le suivi tumoral pendant et après le traitement. Les principaux avantages de l'APS sont qu'il est spécifique à la prostate et les tests de détection sont efficaces et peu coûteux, ce qui en fait un bon candidat pour des examens de routine [118]. Malheureusement, comme tout biomarqueur, APS n'est pas infaillible. Les principaux problèmes associés à l'utilisation d'APS comme biomarqueur sont que son expression n'est pas exclusive au cancer et que les tests de dépistage manquent de sensibilité pour la détection des cancers en stade précoce. De plus, le niveau d'APS sérique peut être altéré par plusieurs facteurs, dont certains médicaments, la manipulation urologique et la présence d'une prostatite [118, 119]. Ainsi, les tests peuvent mener à de faux résultats. Le plus grand enjeu concernant l'utilisation d'APS comme biomarqueur est que les faux positifs mènent à de nombreuses biopsies inutiles. D'ailleurs, on estime que seulement 25 % des patients qui ont un niveau d'APS légèrement au-dessus de la normale ont un cancer de la prostate [120]. Chaque biopsie est associée à des coûts supplémentaires pour le système de la santé et peut entraîner des conséquences pour la santé du patient [120].

L'utilisation de différents biomarqueurs serait potentiellement beaucoup plus efficace pour le diagnostic du cancer de la prostate, l'identification des cas qui sont à haut risque ainsi que pour le suivi du traitement. Le tableau 6 décrit plusieurs biomarqueurs qui sont présentement à l'étude. Différents types d'échantillons peuvent être utilisés : le sang, l'urine ou des tissus tumoraux. Pour ce qui est des tests sériques, le test le plus fréquent est celui d'APS. Cependant, il a été démontré que d'autres membres de la famille des kallikréines, telle que hK2, peuvent augmenter considérablement la valeur prédictive d'APS [120, 121]. Pour ce qui est des biomarqueurs urinaires, l'ARNm de l'antigène 3 du cancer prostatique (PC3) et l'ARNm codant pour la protéine de fusion TMPRSS2:EGR (*transmembrane protease, serine 2 : early growth response protein 1*) sont parmi les biomarqueurs les plus développés. En

fait, on estime que la détection de ces deux ARNm en plus de la détection sérique d'APS permettrait d'atteindre une spécificité de 90 % et une sensibilité de 80 % pour le diagnostic du cancer prostatique. L'avantage de ces tests est qu'ils sont rapides et relativement peu coûteux [122]. Enfin, lorsque le cancer de la prostate a été détecté, il est possible d'utiliser des biomarqueurs tissulaires afin d'établir un pronostic. Par exemple, la détection de Ki67 par immunohistochimie (IHC) permet de déterminer le taux de prolifération des cellules tumorales et permet ainsi de déterminer si le patient est à haut risque [123]. Tout comme pour le diagnostic du cancer prostatique, l'établissement d'un pronostic peut être facilité par l'utilisation simultanée de plusieurs biomarqueurs. C'est entre autres ce que suggère Ding *et al.* par la détection par IHC de PTEN (*phosphatase and tensin homolog*), SMAD4 (*mothers against decapentaplegic homolog 4*), Cycline D1 et SSPA (*stringent starvation protein A*). Ce test permettrait de déterminer les risques de récidives [124].

Tableau 6 Biomarqueurs potentiel pour la détection et le pronostic du cancer de la prostate.

Type d'échantillon	Biomarqueur	Méthode de détection	Références
Sérum	ASP	ELISA	[117]
	hK2	ELISA	[120, 121]
	miR-221	qRT-PCR	[120, 126-128]
Urine	PCA3	RT-qPCR	[122, 131]
	TMPRSS2-ERG	RT-qPCR	[122, 132]
	SPINK1	RT-qPCR	[133]
	GSTP1^{MET}	PCR méthylation spécifique	[134]
	RARB ^{MET}	PCR méthylation spécifique	[134]
	APC ^{MET}	PCR méthylation spécifique	[134]
Tumeur	Ki67	IHC	[123]
	PTEN	IHC, FISH	[135, 136]
	TMPRSS2-ERG	FISH	[137, 138]
	MYC	FISH	[139]
	TP53	IHC	[140]
	SMAD4	IHC	[124]
	Cycline D1	IHC	[141]
	SPP1	IHC	[141]
	miR-221	qRT-PCR	[119, 125]

MET : hyperméthylation

Depuis quelques années émerge un intérêt particulier pour l'utilisation de microARN (miRNA) comme biomarqueurs. En effet, certains miRNA peuvent être détectés dans les fluides corporels ainsi que dans les échantillons de tumeurs. De plus, ils peuvent être quantifiés par diverses méthodes telles que l'hybridation *in situ*, le séquençage de nouvelle génération, la qRT-PCR (*quantitative Reverse transcription polymerase chain reaction*) ainsi

que l'utilisation de micromatrices [118, 123]. Par exemple, miR-221 augmente dans le sang de patients atteints du cancer de la prostate comparativement à des donneurs sains. Ce miRNA est reconnu pour cibler *p27kip1* et moduler le cycle cellulaire ainsi que la voie de signalisation de l'androgène [119, 120, 125-128]. Il serait également possible d'utiliser une signature de miRNA composé de miR-141 et miR-375, permettant d'identifier les patients dont le cancer s'est répandu et de déterminer si le stade et le score de Gleason sont élevés [121, 123, 129, 130].

En résumé, le test de détection d'APS est actuellement le test le plus utilisé par les cliniciens. Bien que spécifique aux cellules de la prostate, APS peut être anormalement élevé dans des situations où il n'y a pas de cancer de la prostate, menant à des biopsies inutiles. De plus, le test n'est pas suffisamment sensible pour les patients qui sont atteints d'un cancer de bas stade. Ainsi, il y a un besoin de développer de nouveaux tests qui seront plus spécifiques et plus sensibles. Jusqu'à maintenant, tout semble indiquer que l'utilisation simultanée de plusieurs biomarqueurs serait une bonne alternative.

2.5 Expression et fonction des galectines

Il est maintenant clair que le microenvironnement tumoral joue un rôle clé dans le cancer. Sa composition aura un impact direct sur la réponse immunitaire anti-tumorale. Il est reconnu que les galectines ont la capacité de remodeler le microenvironnement et peuvent perturber l'équilibre de la réponse immunitaire. De plus, l'expression des galectines est souvent modulée dans les cellules cancéreuses, influençant la croissance tumorale, la sensibilité à l'apoptose, l'angiogenèse et le potentiel invasif des cellules cancéreuses. C'est pour ces raisons que plusieurs groupes de recherche se sont intéressés à l'expression et à la fonction des galectines dans le cancer de la prostate.

Dans le tissu normal, il a été démontré que les cellules épithéliales ont des niveaux détectables d'ARNm codant pour galectines-1, -3, -4, -7, -8 et -9 [142]. Au niveau protéique, seules les galectines-1 et -3 ont été étudiées. Il a été démontré que la galectine-1 est fortement exprimée dans le stroma alors que la galectine-3 est retrouvée principalement dans le noyau des cellules épithéliales [143]. Pour ce qui est du cancer prostatique, l'expression et la localisation de plusieurs galectines sont altérées. Il a été noté que l'expression de la galectine-1 augmente pendant la progression tumorale, alors que l'expression des galectines-3, -4, -9 et -12 diminue [144]. Pour ce qui est de la galectine-8, deux groupes de recherches s'entendent à dire que son expression reste inchangée pendant

la progression tumorale, alors que Su *et al.* affirment que son expression augmente dans les carcinomes comparativement aux lésions bénignes et au tissu normal [144-146].

La présence de galectines peut influencer la progression tumorale de différentes façons (Tableau 7). Tout d'abord, la galectine-1 peut être exprimée par les cellules cancéreuses, le stroma péritumoral et les cellules endothéliales qui irriguent la tumeur. Sa présence est associée à un mauvais pronostic [143, 144, 147, 148]. En effet, il semblerait que la galectine-1 induise l'angiogenèse et l'immunosuppression [144, 149]. Pour ce qui est de la galectine-3, il a été démontré que son expression est réprimée dans les cancers hormono-indépendants [150]. Lorsqu'elle est exprimée, sa distribution est cytoplasmique plutôt que nucléaire. Des études ont d'ailleurs prouvé que la galectine-3 nucléaire a des fonctions anti-tumorales alors que la galectine-3 cytoplasmique est pro-tumorale [65]. Enfin, pour ce qui est de la galectine-8, Gopalkrishnan *et al.* ont démontré que cette protéine inhibe la formation de colonies sur *soft agar* [151].

Tableau 7 Fonctions des galectines dans le cancer de la prostate.

Galectines	Fonctions	Références
Galectine-1	Augmente l'adhésion à la lamine et à la fibronectine	[152]
	Induit l'arrêt de croissance des cellules LNCaP	[152]
	Induit l'apoptose des cellules LNCaP	[152]
	Réduit la migration transendothéliale des lymphocytes T	[144]
	Stimule l'angiogenèse	[144]
Galectin-3	Augmente le potentiel invasif	[65, 153]
	Augmente la croissance tumorale	[65, 153]
	Augmente l'angiogenèse	[154]
	Effet anti-apoptotique	[65, 153]
Galectine-8	Inhibe la formation de colonies sur *soft agar*	[151]

En conclusion, plusieurs groupes de recherche s'intéressent à l'utilisation d'inhibiteur de galectines pour le traitement du cancer. Des inhibiteurs qui ciblent leur CRD auraient le potentiel de favoriser la réponse immunitaire anti-tumorale et ainsi réduire la progression du cancer [108, 109, 155, 156]. L'expression de plusieurs galectines semble modulée dans les tumeurs prostatiques et leur présence peut perturber la progression tumorale. Cependant, plusieurs autres galectines telles que les galectines-2, -7, -10, -13, -14 et -16 pourraient aussi être impliquées. Celles-ci devront être étudiées afin de déterminer si l'utilisation d'inhibiteurs pourrait être envisagée afin de traiter les patients atteints du cancer de la prostate. De plus, les inhibiteurs qui ciblent le CRD des galectines sont peu spécifiques et pourraient inhiber une galectine aux fonctions anti-tumorales. C'est pourquoi il est important de bien comprendre l'implication des différentes galectines dans la progression tumorale de la prostate et de vérifier les fonctions pro- ou anti-tumorales de chacune d'entre-elles. Il a été

noté que l'ARNm de la galectine-7 est détecté dans le tissu normal de la prostate, mais aucune étude n'a porté sur son expression protéique et sur sa fonction. Il est donc possible que celle-ci soit impliquée dans la progression de ce cancer.

3 LE CANCER DE L'OVAIRE

Le cancer de l'ovaire est le deuxième cancer gynécologique le plus fréquent chez la femme et il est le plus meurtrier [86]. Puisqu'il s'agit d'un cancer indolore et difficile à détecter, la majorité des cas sont diagnostiqués à un stade avancé. Bien qu'une grande proportion des patientes répondent aux traitements initiaux, la plupart développeront éventuellement une résistance à la chimiothérapie. En fait, on estime qu'environ 80 % des patientes auront une récidive suite aux traitements initiaux et que plus de la moitié d'entre elles en mourront dans une période de cinq ans suivant le diagnostic [114]. Il existe donc un besoin urgent de développer de nouveaux outils pour le diagnostic, l'établissement d'un pronostic et le traitement des femmes atteintes d'un cancer ovarien.

3.1 Développement et progression tumorale

L'ovaire a pour principales fonctions la maturation des ovules et leur relâchement vers les trompes de Fallope ainsi que la production d'hormones sexuelles (œstrogènes et progestérone) [86]. L'ovaire est composé d'une monocouche de cellules épithéliales et est séparé en deux zones : une zone périphérique corticale, qui contient les follicules ovariens, et une zone centrale médullaire, qui contient des nerfs, des vaisseaux sanguins et lymphatiques.

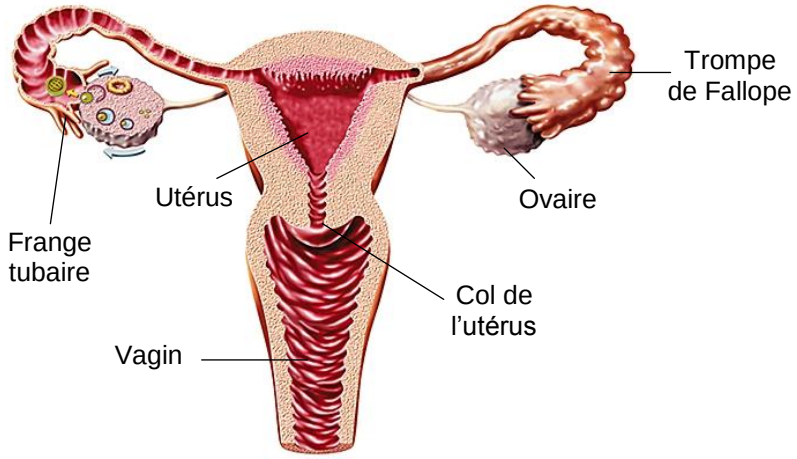

Figure 10 Anatomie de l'appareil reproducteur féminin. Schématisation des différentes composantes de l'appareil reproducteur féminin (adaptation de [158]).

Environ 10 % de tous les cancers épithéliaux de l'ovaire sont héréditaires. C'est le cas notamment des mutations dans les gènes *BRCA1* (*breast cancer antigen 1*) et *BRCA2* impliqués dans la réparation de l'ADN. Ces mutations prédisposent au développement du cancer de l'ovaire [159]. D'ailleurs, afin de réduire leurs risques de développer un cancer, on recommande généralement aux femmes portant ces mutations de procéder à une ovariectomie, une hystérectomie et une tubectomie dès qu'elles ne désirent plus porter d'enfants. Pour les autres cas de cancers ovariens, divers événements moléculaires peuvent causer la transformation des cellules cancéreuses. Dans tous les cas, la progression du cancer de l'ovaire est unique et ne peut être comparée aux autres types de cancers. Dans les premières étapes de la tumorigenèse ovarienne, la tumeur se développe à la surface de l'ovaire puis forme des métastases par une expansion directe vers des sites adjacents [160]. Une transition épithéliale à mésenchymale permet ensuite aux cellules cancéreuses de se détacher de la tumeur et de coloniser des tissus éloignés [161, 162]. Il est à noter que les métastases distantes, hors de la cavité péritonéale, sont rares. Il semblerait que la dissémination des cellules cancéreuses se fasse de façon passive via les fluides présents dans la cavité péritonéale [163]. Une grande proportion des patientes développe également des ascites qui s'accumulent dans la cavité péritonéale. On y retrouve fréquemment des cellules cancéreuses libres, favorisant la dissémination du cancer [160].

3.2 Classification du cancer ovarien

Le cancer de l'ovaire est reconnu pour être très hétérogène. On utilise généralement la classification histologique et le stade de la FIGO (fédération internationale de gynécologie et d'obstétrique) pour établir le pronostic d'une patiente atteinte de ce cancer. La classification histologique se base sur la morphologie des tumeurs, alors que le stade de la FIGO se base sur une stadification TNM et la propagation du cancer.

3.2.1 Classification histologique

Il existe plusieurs sous-types histologiques du cancer ovarien et chacun d'entre eux a un profil moléculaire distinct. Ces diversités moléculaires peuvent être expliquées par les différents types cellulaires précurseurs de ce cancer. Selon l'hypothèse actuelle, les cellules épithéliales des trompes de Fallope seraient à l'origine de la majorité des cancers ovariens [136]. Le tableau 8 résume l'origine et le profil moléculaire des sous-types histologiques les plus fréquents.

Tableau 8 Classification histologique du cancer ovarien. Origine et profil moléculaire des sous-types histologiques les plus fréquents [136].

Sous-type histologique	Origine	Profil moléculaire
Séreux de haut grade	Trompes de Fallope	*TP53, BRCA1/2, NF1, RB1, DK12, PI3K/RAS/NOTCH/FOXM1*
Séreux de bas grade	Trompes de Fallope	*BRAF, KRAS, NRAS, ERBB2*
Mucineux	Non-müllérienne	*KRAS, HER2*
Endométrioïde	Endométriose	*ARID1A, PIK3CA, PTEN, PPP2R1α*
À cellules claires	Endométriose	*ARID1A, PIK3CA, PTEN, CTNNB1, PPP2R1α*
Stromal et des cordons sexuels	Non-épithéliale	*FOXL2 (Granulosa) ou DICER1 (Sertoli-Leydig)*

On estime qu'environ 90 % des cancers de l'ovaire sont d'origine épithéliale et que la majorité d'entre eux sont des cancers de type séreux. Au niveau morphologique, ces tumeurs sont généralement bien différenciées et d'apparence glandulaire ou papillaire, ressemblantes à la surface épithéliale des trompes de Fallope [163]. Il existe deux types de cancer séreux : de haut grade et de bas grade. Le cancer ovarien de haut grade est de loin le plus fréquemment diagnostiqué. Ce sous-type histologique est caractérisé par une forte instabilité chromosomale, une dérégulation de la voie PI3K/RAS/NOTCH/FOXM1 ainsi que des mutations dans les gènes *TP53, BRCA1/2, NF1, RB1* et *CDK12* [136]. Le sous-type séreux de bas grade est quant à lui caractérisé par des mutations de *BRAF, KRAS, NRAS* et *ERBB2* [136]. Plusieurs évidences indiquent que les cancers séreux proviennent de cellules de trompes de Fallope. En fait, les cancers séreux de haut grade proviendraient de tumeurs tubaires qui s'implanteraient dans l'ovaire alors que les cancers séreux de bas grade proviendraient d'un kyste, d'origine tubaire, qui se formerait à la surface de l'ovaire suite à l'ovulation [136].

Les cancers endométrioïdes et à cellules claires représentent de 15 à 20 % des cancers ovariens épithéliaux. Au niveau morphologique, le cancer de type endométrioïde a une organisation semblable à celle des carcinomes de l'endomètre [163]. Le cancer ovarien à cellules claires, quant à lui, est caractérisé par des cellules ayant un cytoplasme clair, riche en glycogène et a une structure glandulaire. Il peut ressembler au cancer de type séreux ou encore au carcinome de l'endomètre [163-165]. Ces deux sous-types histologiques ont un profil moléculaire similaire, avec des altérations d'*ARID1A, PIK3CA, PTEN,* et *PPP2R1α*. Les cancers à cellules claires ont également des mutations du gène *CTNNB1*, codant pour la β-caténine [163]. Pour ce qui est de l'origine de ces deux sous-types, l'hypothèse actuelle est qu'ils proviendraient d'une endométriose. Les cellules de l'endomètre s'implanteraient dans l'ovaire suite à des menstruations rétrogrades [136].

Pour ce qui est du sous-type mucineux, il s'agit d'un cancer peu fréquent (3 % des cancers ovariens épithéliaux). L'organisation des cellules cancéreuses de ce sous-type ressemble généralement aux glandes endocervicales ou encore à l'épithélium gastro-intestinal [163]. Au niveau moléculaire, ce cancer est caractérisé par des mutations de KRAS et ERBB2 [163]. Les cellules cancéreuses de ce sous-type histologique seraient d'origine non müllérienne [136].

Finalement, pour ce qui est des cancers ovariens d'origine non épithéliale, ils sont très rares et donc peu connus. Il existe différents profils moléculaires, dont des mutations dans les gènes *FOXL2* ou *DICER1* [163]. Ces cancers peuvent émerger des différents types cellulaires qui composent le stroma, dont des cellules germinales ou des fibroblastes [136].

3.2.2 Stade de la FIGO

Afin de caractériser la progression tumorale, le stade de la FIGO est la classification la plus répandue auprès des cliniciens. Ce système de classification a été instauré par la fédération internationale de gynécologie et d'obstétrique et s'échelonne sur quatre stades. Un bas stade consiste en un cancer localisé alors qu'un stade élevé représente un cancer qui s'est propagé. Le tableau 9 résume les différentes caractéristiques associées à chacun des stades.

Tableau 9 Classification selon le stade de la FIGO. Adaptation de [86].

Stade FIGO	TNM	Caractéristiques
IA	T1aN0M0	Un seul ovaire est atteint et la capsule de l'ovaire est intacte
IB	T1bN0M0	Les deux ovaires sont atteints et les capsules sont intactes
IC	T1cN0M0	Un ou deux ovaires atteints. La tumeur peut avoir traversé la capsule ou s'être propagée à la surface de l'ovaire. Il peut y avoir des cellules cancéreuses dans l'ascite ou les lavages péritonéaux.
IIA	T2aN0M0	La tumeur s'est propagée à l'utérus ou aux trompes de Fallope
IIB	T2bN0M0	La tumeur s'est propagée à d'autres organes du bassin
IIC	T2cN0M0	La tumeur s'est propagée à tous les organes du bassin et il y a des cellules cancéreuses dans l'ascite ou les lavages péritonéaux
IIIA	T3aN0M0	Présence de petites métastases dans le péritoine, hors du bassin
IIIB	T3bN0M0	Présence de métastases péritonéales de 2 cm ou moins
IIIC	T3cN0M0 ou T3cN1M0	Présence de métastases péritonéales de plus de 2 cm et le cancer peut avoir atteint les ganglions lymphatiques régionaux.
IV	T1 à T3, N0 ou N1, M1	Le cancer s'est propagé à des organes éloignés

3.2.3 Modèle de classification dualiste

Historiquement, tous les cancers de l'ovaire étaient traités de la même façon sans égard aux sous-types histologiques. Il est maintenant clair que le cancer de l'ovaire est une maladie hétérogène et que chaque sous-type a une origine et un profil moléculaire distinct. Ce qui apporte donc l'hypothèse que chaque sous-type devrait être traité de façon personnalisée. Afin de faciliter la personnalisation des traitements offerts aux patientes atteintes du cancer ovarien, Shih *et al.* avaient proposé en 2004 un modèle dualiste pour la classification du cancer de l'ovaire [166]. Ce modèle a récemment été révisé afin d'inclure les dernières découvertes concernant la tumorigenèse de l'ovaire [167]. Ce modèle sépare en deux familles (type I et type II) tous les cancers épithéliaux de l'ovaire. Il prend en compte le tissu d'origine de la tumeur ainsi que ses caractéristiques moléculaires et morphologiques. Le tableau 10 résume les différents sous-types présents dans chacune de ces familles.

Tableau 10 Modèle dualiste de classification du cancer de l'ovaire. Modèle séparant différents sous-types histologiques en deux groupes. Adaptation de [167].

Type	Tissu d'origine	Sous-type histologique
I	Endométriose	Endométrioïde
		À cellules claires
		Seromucineux
	Trompes de Fallope	Séreux de bas grade
	Cellules germinales	Mucineux
	Cellules transitionnelles	Mucineux
		De Brenner
II	Trompes de Fallope	Séreux de haut grade
		Carcinosarcome
		Indifférencié

Les tumeurs faisant partie du type I sont considérées moins agressives et comptent pour seulement 10 % des mortalités associées au cancer de l'ovaire. Il s'agirait de tumeurs de bas stade et de bas grade, ayant un faible index de prolifération, mais qui répondent peu à la chimiothérapie. La progression des cancers de cette classe est lente, ce qui permettrait une meilleure survie malgré la faible réponse à la chimiothérapie [167]. Au niveau moléculaire, les tumeurs de type I se caractérisent par l'activation des voies ERRB2/KRAS/BRAF/MEK, PI3K/AKT et Wnt ainsi que l'inactivation de la voie PTEN et de remodelage de la chromatine via ARID1A [167]. Pour ce qui est des tumeurs faisant partie du type II, ce sont des cancers généralement avancés et de haut grade avec un fort index de prolifération cellulaire. Ce groupe est caractérisé par une forte instabilité chromosomale, une mutation de *TP53* et des défaillances au niveau des mécanismes de réparation de l'ADN par recombinaison homologue. La réponse initiale à la chimiothérapie est généralement bonne, mais les taux de

récidive sont très élevés et le taux de survie associé à ce groupe est bas [167].Ce nouveau système de classification n'est pas utilisé au niveau clinique. Mais il est de plus en plus reconnu par la communauté scientifique. Il permettra sans doute de mieux comprendre la tumorigenèse de l'ovaire et ainsi de mieux adapter les traitements contre ce cancer.

3.3 Traitements

Les traitements contre le cancer ovarien sont peu nombreux et font principalement appel à la chirurgie et la chimiothérapie. Pour des femmes qui ne désirent plus porter d'enfants, on pratique généralement une hystérectomie totale et une salpingo-ovariectomie bilatérale, c'est-à-dire le retrait de l'utérus, des trompes de Fallope ainsi que des ovaires. Pour ce qui est de la chimiothérapie, l'utilisation de taxol (paclitaxel) et/ou de platine (carboplatine) est généralement privilégiée. Les agents chimiothérapeutiques peuvent être appliqués de façon locale (intrapéritonéal) ou systémique (intraveineux). La radiothérapie, qui est rarement prescrite, peut aussi servir de traitement principal, de traitement complémentaire à la chimiothérapie ou encore d'outil palliatif afin de réduire la taille des tumeurs de stade avancé[86, 168-173].

Maintenant qu'il a été établi que les différents sous-types histologiques ont des comportements cliniques distincts, plusieurs traitements personnalisés sont en développement. En fait, il semblerait que cibler certains aspects moléculaires d'un type particulier de cancer ovarien permet de réduire la toxicité du traitement tout en augmentant son efficacité. Le tableau 11 résume différents traitements qui sont actuellement à l'étude. Un intérêt particulier est porté à l'angiogenèse via l'inhibition de molécules clés telles que le facteur de croissance de l'endothélium (VEGF) et son récepteur (VEGFR) et le récepteur de facteurs de croissance de fibroblastes (FGFR). Il a d'ailleurs récemment été démontré que le Bevacizumab, un anticorps monoclonal dirigé contre VEGF, augmente la survie des patientes et réduit le taux de récidive lorsqu'il est utilisé en combinaison avec le paclitaxel et la carboplatine [124]. Il s'agit donc d'une alternative intéressante qui prend en compte l'importance de l'environnement péritumoral.

Les inhibiteurs de la polymérase poly(ADP-ribose) (PARP) suscitent également beaucoup d'intérêt. Les membres de la famille PARP sont impliqués dans la réparation de simples brins d'ADN. Ainsi, pour des cellules cancéreuses qui sont déficientes en mécanismes de réparation de l'ADN double brin par recombinaison homologue, le bon fonctionnement de PARP est essentiel à leur survie. L'utilisation d'inhibiteur de PARP permet donc d'éliminer

ces cellules cancéreuses [135, 140]. L'Olaparib est un inhibiteur de PARP qui a récemment été approuvé en Europe et aux États-Unis pour le traitement des femmes ayant un cancer avec des mutations des gènes *BRCA1/2*. Jusqu'à maintenant, les résultats obtenus sont encourageants, d'autant plus qu'il a été démontré que près de 50 % des cancers séreux de haut grade ont des altérations dans les mécanismes de réparation de l'ADN par recombinaison homologue [135].

Tableau 11 Traitements expérimentaux contre le cancer ovarien. Différents traitements utilisés dans des essais cliniques pour le traitement du cancer de l'ovaire [124, 174].

Cellules cibles	Cibles moléculaires	Traitements
Endothéliales	VEGF	Bevacizumab
		VEGF trap
	VEGFR	Pazopanib
		Sunitinib
		Nintedanib
		Brivanib
	FGFR	Nintedanib
		Brivanib
	PDGFR	Pazopanib
		Nintedanib
		Sunitinib
	VDA	Combretastatin
		Ombrabulin
	Récepteur Tie2/angiopoiétine	AMG 386
	MET	Cabozantinib
Cancéreuses	PI3X/AKT/mTOR	MK-2206
		AZD2014
	Récepteur de folate	Farletuzumab
		Vintafolide
	EGFR/HER2/HER3	Erlotinib
		Trastuzumab
		Pertuzumab
		MM-121
	IGFR	AMG 479
		Linsitinib
	Cycle cellulaire	Alisertib
		MK-1775
	Src	Saracatinib
	Ras/Raf/MEK	Selumetinib
		Trametinib
		MEK162
	PARP	Olaparib
		Rucaparib
		Veliparib
		Niraparib
		BMN-673
Lymphocytes T	CTLA-4	Ipilimumab
	PD1	Nivolumab
		Pembrolizumab

Il est maintenant clair que le microenvironnement tumoral et le système immunitaire jouent un rôle important dans la progression du cancer ovarien [89]. En effet, il a été démontré que l'infiltration de cellules T CD8+ dans la tumeur corrèle directement avec la survie des patientes, alors que la présence de cellules Treg ou une réponse immunitaire de type TH2 sont associées à un mauvais pronostic [92, 175-177]. De plus, plusieurs mécanismes d'immunoévasion ont été percés à ce jour. Par exemple, la surexpression de PD-L1 par les cellules cancéreuses ou l'expression du récepteur de l'endothéline B sont associées à une diminution de l'infiltration lymphocytaire [165, 178]. De plus, il a été démontré que le microenvironnement tumoral contient plusieurs molécules immunosuppressives, dont IL-10 et TGF-β (revue dans [89]). Comme pour plusieurs autres types de cancer, les thérapies immunologiques sont présentement à l'étude pour le traitement du cancer de l'ovaire. Les études cliniques sont peu avancées en comparaison avec celles effectuées pour d'autres cancers. Cependant, certains inhibiteurs de «checkpoint» immunologique tels que des anticorps anti-CTLA-4 (Ipilimumab) ou encore des inhibiteurs de l'axe PD1/PD-L1 (Nivolumab) ont récemment été utilisés dans des essais cliniques de niveaux I et II [89, 174]. De plus, plusieurs vaccins thérapeutiques sont en cours de développement [89, 174]. Il est encore trop tôt pour savoir si l'immunothérapie est une option efficace contre le cancer ovarien, mais les résultats préliminaires de plusieurs études semblent indiquer qu'elle pourrait aider à réduire les risques de récidive ainsi que le taux de mortalité [89, 174].

3.4 Biomarqueurs

Comme il est difficile de détecter un cancer ovarien et que l'ovaire est difficilement atteignable pour le prélèvement d'un échantillon de tissu, l'utilisation de biomarqueurs sériques semble la meilleure solution pour détecter ou encore établir un pronostic pour le cancer ovarien. Jusqu'à aujourd'hui, un seul biomarqueur est utilisé en milieu clinique. Il s'agit de l'antigène tumoral 125 (CA125), une glycoprotéine qui est sécrétée par les cellules cancéreuses de l'ovaire et se retrouve dans la circulation sanguine. On estime que CA125 est élevée dans environ 85 % des cancers de types séreux et près de 80 % des cancers de stade avancé [164]. Malheureusement, l'utilisation de CA125 pour la détection du cancer ovarien est inappropriée puisque 50 % des patientes qui ont un cancer de bas stade ne surexpriment pas la protéine [164]. De plus, certaines conditions telles que l'endométriose ou la présence de tumeurs bénignes mènent à la surexpression de CA125, causant de faux résultats positifs [172, 179]. Ainsi, son utilisation se limite au suivi de la maladie pendant et après le traitement. Afin d'augmenter la sensibilité et la spécificité du CA125, il a été proposé de combiner la valeur sérique de CA125 à l'imagerie par ultrasons. En effet, un algorithme prenant en compte ces deux paramètres permettrait d'obtenir un index de risque de

malignité (RMI). Cet algorithme devient de plus en plus utilisé au niveau clinique pour la détection du cancer ovarien. Il permet d'obtenir une sensibilité et une spécificité de 78 % et 87 %, respectivement [173]. Cependant, malgré l'avancement qui a été fait avec le RMI, il y toujours un problème au niveau de la détection des cancers de bas stade. C'est pourquoi de nombreux efforts sont déployés dans l'établissement de nouveaux biomarqueurs.

Au niveau des tests sériques, la protéine épididimaire humaine 4 (HE4) est le biomarqueur le plus développé après CA125. Il s'agit d'une glycoprotéine dont l'expression augmente dans plus de 90 % des cas de cancer ovarien. En fait, la sensibilité et la spécificité de HE4 semblent supérieures à celles de CA125 [169, 170, 179]. Malheureusement, HE4 varie avec l'âge des femmes, causant donc de possibles faux positifs. Une meilleure détermination des seuils de HE4 est nécessaire afin de l'utiliser de routine en milieu clinique [164]. Pour améliorer la sensibilité et la spécificité de HE4, l'algorithme du risque de malignité de la tumeur ovarienne (ROMA) a été proposé. Il s'agit de combiner les valeurs de CA125 et de HE4 et de prendre en compte le statut de ménopause de la patiente [170]. Malheureusement, les études qui ont été effectuées sur cette méthode sont très hétérogènes et il est encore difficile de déterminer si l'algorithme ROMA sera utile au niveau clinique [180-182].

D'autres tests, tel que Ova1, ont été commercialisés aux États-Unis dans les dernières années. Ova1 est un test sérique basé sur la présence de cinq molécules : CA125, β-microglobuline, apolipoprotéine A1, préalbumine et transferrine. Lors d'un cancer de l'ovaire, les niveaux de CA125 et de β-microglobuline augmentent alors que l'expression de l'apolipoprotéine A1, la préalbumine et la transferrine diminuent. Ainsi, la mesure de ces cinq protéines sériques permet de détecter la présence du cancer ovarien. Malheureusement, bien que la sensibilité de ce test soit de 96 %, sa spécificité n'est que de 51 % (revue dans [164]). Ceci indique donc que dans plusieurs cas, de faux résultats positifs sont obtenus. Ainsi, des efforts supplémentaires seront nécessaires afin de déterminer quelle combinaison de biomarqueurs permet la détection du cancer ovarien.

Actuellement, plusieurs groupes de recherche s'intéressent à la présence de miRNA dans le sang des patientes. Des études effectuées sur le tissu cancéreux de l'ovaire avaient démontré que la présence de miRNA spécifiques permettait d'établir un diagnostic de cancer ovarien et de prédire le pronostic des patientes [183, 184]. L'avantage d'étudier les miRNA est qu'ils sont aussi retrouvés dans la circulation sanguine, souvent dans des exosomes ou

des microvésicules [185, 186]. Il semblait donc pertinent d'étudier leur utilité comme biomarqueurs sériques. Tout d'abord, il a été démontré que la famille miR-200 (miR-200a, b et c) augmente dans le sang de patientes atteintes du cancer comparativement aux témoins en santé et aux femmes ayant une maladie bénigne de l'ovaire [187, 188]. Il semblerait que cette famille de miRNA pourrait servir au diagnostic et au suivi pendant et après le traitement des patientes [189]. D'autres miRNA ont aussi suscité de l'intérêt. Par exemple, il a été démontré que l'expression de miR-21, miR-92 et miR-93 augmente dans le sang de patientes atteintes du cancer de l'ovaire. Non seulement ces miRNA permettraient de diagnostiquer le cancer chez les patientes CA125 négatives, mais ils pourraient aussi servir de cibles thérapeutiques puisqu'ils sont considérés comme étant des oncomirs [183, 190, 191]. Les résultats préliminaires obtenus dans les différentes études sur le miRNA sont encourageants, mais des études plus approfondies seront nécessaires afin de déterminer si ceux-ci pourraient être utilisés au niveau clinique. Dans tous les cas, il semblerait que la meilleure façon d'augmenter la sensibilité et la spécificité des tests de détections du cancer de l'ovaire soit de combiner la présence de plusieurs biomarqueurs et d'établir des algorithmes basés sur l'âge des patientes, le statut de ménopause et la présence des biomarqueurs spécifiques.

3.5 Les galectines dans le cancer ovarien

Jusqu'à maintenant, peu de recherches ont porté sur l'expression et la fonction des galectines dans le cancer de l'ovaire. Seuls les galectines-1, -3, -4 et -7 ont été étudiées. Le tableau 12 résume les différentes fonctions des galectines dans ce cancer.

Tableau 12 Fonctions des galectines dans le cancer de l'ovaire.

Galectines	Fonctions	Références
1	Stimule la prolifération cellulaire	[192]
	Augmente le potentiel invasif	[192]
	Induit la résistance à l'apoptose	[193, 194]
3	Réduit la prolifération cellulaire	[195]
	Induit la résistance à l'apoptose	[195-197]
	Stimule l'angiogenèse	[197]
	Augmente le potentiel invasif	[198]
7	Stimule la prolifération cellulaire	[199]

La galectine-1 est celle qui a suscité le plus d'intérêt. On estime que 95 % des carcinomes de l'ovaire ont une surexpression du gène qui code pour la galectine-1 comparativement à l'ovaire normal. Cette surexpression est associée à la présence de la protéine dans le cytoplasme des cellules cancéreuses et dans le stroma péritumoral [200]. Par l'utilisation de

micromatrice tissulaire, Kim et al. ont démontré que la présence de galectine-1 dans l'environnement péritumoral est associée à un plus haut taux de récidive [192]. Che *et al.* ont démontré que l'expression de la galectine-1 dans le stroma péritumoral est associée à un stade FIGO plus élevé, à un plus haut taux de récidive et à la présence de métastases, alors que l'expression de la galectine-1 dans les cellules cancéreuses ne permet pas de prédire le pronostic [201]. En analysant le sérum des patientes, il a été possible de déterminer que la galectine-1 est retrouvée en plus forte concentration chez les patientes qui ont des métastases et qui sont CA125-positives. Cependant, la galectine-1 sérique ne permet pas de diagnostiquer le cancer ovarien et n'est pas associée au stade de la FIGO [201].

Au niveau fonctionnel, la galectine-1 favoriserait la progression tumorale en induisant la prolifération cellulaire, la résistance à l'apoptose et le potentiel invasif des cellules cancéreuses [192-194, 202]. Au niveau moléculaire, il a été démontré que la répression de la galectine-1 diminue l'activité de la voie de signalisation H-Ras/Raf-1/ERK ainsi que l'expression de MMP-9 et de c-jun [194]. Un autre aspect intéressant de la galectine-1 est sa capacité à se lier à CA125 soluble et membranaire. Il n'est pas surprenant qu'une galectine se lie à CA125 puisqu'il s'agit d'une glycoprotéine. Cependant, il a été démontré que des cellules exprimant CA125 à leur surface ont dix fois plus de galectine-1 liée à la membrane [203]. Des études supplémentaires seront nécessaires afin de déterminer si cela affecte la progression tumorale.

Les études concernant la galectine-3 sont peu homogènes. En effet, Kim *et al.* ont démontré que l'expression de la galectine-3 augmente dans les tissus malins comparativement aux tissus sains [196]. Deux autres groupes de recherche, quant à eux, ont établi que l'expression de la galectine-3 est réduite dans les tissus cancéreux comparativement aux tissus sains [204, 205]. Lee *et al.* pour leur part, prétendent que les niveaux protéiques de la galectine-3 ne sont pas modulés pendant la progression tumorale [196]. L'hétérogénéité de ces études pourrait être expliquée par les différentes cohortes de patientes qui ont été étudiées. Il est d'ailleurs possible que la galectine-3 se comporte différemment en fonction des sous-types histologiques. Alors qu'il est difficile de prédire les niveaux d'expression de la galectine-3 en fonction de la progression tumorale, plusieurs ont démontré que sa présence est associée à un mauvais pronostic. En effet, la présence de galectine-3 dans le sang de patientes est associée à la présence de métastases et son expression dans les tumeurs est associée à un plus haut taux de récidive [196, 206]. Dans une étude qui a porté sur le sous-type de cellules claires, Min *et al.* ont démontré que la présence de galectine-3 augmente

dans 80 % des cas et qu'elle est associée au stade de la FIGO III et IV ainsi qu'à une augmentation du marqueur de prolifération cellulaire Ki67 [207]. Au niveau fonctionnel, par des expériences *in vitro* et *in vivo*, il a été démontré que la galectine-3 stimule le potentiel invasif et l'angiogenèse, réduit la prolifération cellulaire et mène à la résistance à la chimiothérapie [195-198]. Les mécanismes moléculaires sous-jacents sont encore méconnus, mais il semblerait que la galectine-3 puisse activer la voie de NF-κB [198].

Il existe peu d'information concernant l'expression et la fonction des autres galectines dans le cancer ovarien. Dans le cas de la galectine-4, elle serait exclusivement exprimée dans le sous-type histologique mucineux [208]. Bien qu'on ne sache pas si cette galectine module la tumorigenèse ovarienne, son expression semble augmenter pendant la progression tumorale [208]. Pour ce qui est de la galectine-7, Kim *et al.* ont démontré que son expression augmente dans les cancers de l'ovaire et que la présence de la protéine est associée à un plus haut taux de mortalité [199]. Par des expériences *in vitro*, ils ont également démontré que la galectine-7 module la prolifération cellulaire [199].

Un des plus grands défis pour la gestion des patientes atteintes du cancer de l'ovaire est le manque de traitements personnalisés et de biomarqueurs qui sont sensibles et spécifiques. Or, étant donné leur rôle clé dans la réponse immunitaire et la régulation de l'apoptose, de plus en plus d'études démontrent que les galectines ont un grand potentiel en tant que biomarqueurs et cibles thérapeutiques [209-212]. Leur rôle en tant qu'alarmines et leur implication dans la protection du fœtus contre la réponse immunitaire maternelle en sont des exemples éloquents [15]. Il serait donc pertinent d'approfondir les recherches sur l'expression et la fonction des galectines dans le cancer ovarien puisqu'elles pourraient potentiellement servir de biomarqueurs et cibles thérapeutiques.

HYPOTHÈSES ET OBJECTIFS DU PROJET DE THÈSE

Mise en contexte : Des observations antérieures effectuées dans notre laboratoire avaient mis en évidence une dérégulation de l'expression de la galectine-7 dans les cancers de la prostate et de l'ovaire (Annexe I). Les mécanismes d'action de la galectine-7 dans ces cancers sont cependant inconnus. De plus, il existe plusieurs indications que la mesure de l'expression de l'ensemble des galectines peut servir d'outil de pronostic et que certaines galectines seraient de bonnes cibles thérapeutiques pour plusieurs maladies, dont le cancer. Alors qu'une signature des galectines prédictives du pronostic a déjà été définie pour le cancer de la prostate, l'expression des galectines dans le cancer de l'ovaire et leur possible association au pronostic sont encore méconnues.

But de l'étude : Déterminer l'expression et les fonctions de la galectine-7 dans les cancers prostatiques et ovariens et établir une signature d'expression des galectines, prédictive du pronostic, dans le cancer de l'ovaire.

Hypothèses:

- La galectine-7 a une fonction anti-tumorale dans le cancer de la prostate et pro-tumorale dans le cancer de l'ovaire.
- Les interactions CRD-dépendantes et CRD-indépendantes de la galectine-7 peuvent influencer la progression tumorale de façon différente.
- Les galectines ont une expression distincte dans le cancer de l'ovaire et leur distribution cellulaire et tissulaire est importante pour leurs fonctions.
- Les galectines peuvent servir d'outils de pronostic dans le cancer ovarien.

Objectifs:

- Étudier le rôle de la galectine-7 dans la progression du cancer de la prostate en portant une attention particulière à sa fonction dans l'apoptose, le potentiel invasif et la croissance tumorale.
- Déterminer l'importance des interactions CRD-dépendantes et CRD-indépendantes de la galectine-7 lors de la progression tumorale.
- Définir le rôle de la galectine-7 dans la progression du cancer de l'ovaire, plus précisément dans le potentiel invasif et dans l'immunoévasion.
- Établir une signature de galectines dans les cancers ovariens séreux de haut grade.

CHAPITRE 2

UNE MUTATION DANS LE DOMAINE DE LIAISON AU SUCRE DE LA GALECTINE-7 INVERSE SA FONCTION DANS LE CANCER DE LA PROSTATE

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RÉSUMÉ

L'observation que la galectine-7 (gal-7) est spécifiquement exprimée dans les cellules myoépithéliales (basales) de la glande mammaire nous a mené à vouloir déterminer si cette protéine est exprimée dans les cellules basales d'autres tissus. Puisque les glandes mammaires et prostatiques sont similaires et puisque les cellules basales ont un rôle important dans le cancer de la prostate, nous avons examiné la distribution tissulaire de gal-7 dans la prostate humaine. À l'aide de micromatrices tissulaires, nous avons découvert que malgré une présence marquée de gal-7 dans les cellules basales de la prostate normale, son niveau d'expression est réduit dans les cellules cancéreuses prostatiques. L'expression *de novo* de gal-7 dans les cellules cancéreuses de la prostate augmente la sensibilité à l'apoptose en réponse à l'étoposide et à la cisplatine. L'utilisation d'une forme mutée de gal-7 (R74S) dont le domaine de liaison au glucide (CRD) est perturbé, a démontré que la fonction pro-apoptotique de gal-7 est CRD-indépendante et ne nécessite pas la localisation mitochondriale ou nucléaire de la protéine. Cependant, le CRD est indispensable pour l'inhibition du potentiel invasif des cellules cancéreuses de la prostate par gal-7. *In vivo*, la surexpression de gal-7 dans les cellules du cancer de la prostate a causé une légère réduction significative de la croissance tumorale. Cependant, en comparaison au témoin, la forme mutée de gal-7 a causé une augmentation significative de la croissance tumorale. Ensemble, ces résultats suggèrent que gal-7 réduit l'agressivité des cellules cancéreuses de la prostate et qu'une perturbation de l'activité de son CRD inverse sa fonction anti-tumorale. Cette activité CRD-indépendante change le paradigme de notre compréhension de la fonction des galectines. Le modèle R74S sera utile afin de distinguer les fonctions CRD-dépendantes et CRD-indépendantes de gal-7 dans la progression tumorale.

CONTRIBUTION DES AUTEURS

Yves St-Pierre et moi avons décidé du plan expérimental. J'ai réalisé l'ensemble des expériences de cet article et participé à chacune d'entre elles. Andrée-Anne Grosset et Louis Gaboury ont participé au marquage immunohistochimique des micromatrices tissulaires et à leur évaluation. Maria Vladoiu, Bruno G Leclerc et John Stagg ont participé aux expériences *in vivo*. Yves St-Pierre et moi avons écrit l'article et tous les auteurs ont révisé et approuvé le manuscrit final.

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RESEARCH ARTICLE

A Mutation in the Carbohydrate Recognition Domain Drives a Phenotypic Switch in the Role of Galectin-7 in Prostate Cancer

Marilyne Labrie[1], Maria Vladoiu[1], Bruno G. Leclerc[2], Andrée-Anne Grosset[1,3], Louis Gaboury[3], John Stagg[2], Yves St-Pierre[1]*

1 INRS-Institut Armand-Frappier, Laval, Québec, Canada, **2** Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montréal, Québec, Canada, **3** Institut de Recherche en Immunologie et Cancérologie, Montréal, Québec, Canada

* yves.st-pierre@iaf.inrs.ca

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Abstract

The observation that galectin-7 (gal-7) is specifically expressed in mammary myoepithelial (basal) cells prompted us to investigate whether this protein is expressed in the basal cells of other tissues. Given that breast and prostate cancer have remarkable underlying biological similarities and given the important roles of basal cells in prostate cancer, we examined the expression patterns and role of gal-7 in human prostate cancer. Using tissue microarray, we found that although gal-7 is readily expressed in basal cells in normal prostate tissue, it is downregulated in prostate cancer (PCa) cells. *De novo* expression of gal-7 in prostate cancer cells increases their sensitivity to apoptosis in response to etoposide and cisplatin. The assessment of a carbohydrate-recognition domain (CRD)-defective mutant form of gal-7 (R7S) showed that the ability of this protein to modulate apoptosis was independent of its CRD activity. This activity was also independent of its ability to translocate to the mitochondrial and nuclear compartments. However, CRD activity was necessary to inhibit the invasive behaviors of prostate cancer cells. *In vivo*, gal-7 overexpression in PCa cells led to a modest yet significant reduction in tumor size, while its CRD-defective mutant form significantly increased tumor growth compared to controls. Taken together, these results suggest that although *de novo* expression of gal-7 may be an interesting means of increasing the tumorigenic phenotypes of PCa cells, alterations in the CRD activity of this protein drive a phenotypic switch in its role in PCa cells. This CRD-independent activity represents a paradigm shift in our understanding of the functions of galectin. The R74S model will be useful to distinguish CRD-dependent and CRD-independent functions of gal-7 in cancer progression.

Introduction

Galectin-7 (gal-7) is a p53-induced gene that is mainly expressed in stratified epithelial cells [1, 2]. Its expression can also be induced by other transcription factors, including mutant

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forms of p53 and CCAAT/enhancer-binding protein beta (C/EBPβ) [3, 4]. Its expression is also regulated by epigenetic mechanisms, including DNA methylation [5, 6]. Its role in UVB-induced keratinocyte apoptosis [7] and in re-epithelialization of corneal wounds [8] support the idea that gal-7 is important for maintaining homeostasis in epithelial cells. Unsuprisingly, a number of studies have shown that dysregulation of gal-7 expression has a strong effect on the progression of multiple types of cancers of epithelial origin. In mammary tissues, for example, gal-7 is specifically expressed in myoepithelial (basal) cells, and its overexpression in breast cancer tissues correlates with resistance to apoptosis and the spread of metastasis to the bone and lung [9]. Overexpression of gal-7 is also associated with poor survival in patients with epithelial ovarian cancer [6, 10] and with malignancy in patients with squamous cell carcinoma of the tongue [11]. These associations between abnormally high levels of gal-7 and poor prognosis are also present in esophageal and hypopharyngeal squamous cell carcinomas [12, 13]. However, as a number of studies have shown, gal-7, similar to other galectins, plays a dual role in cancer and can have a protective role in certain cases, most notably by increasing the sensitivity of cancer cells to pro-apoptotic stimuli and by reducing cell growth and angiogenesis. These activities have been relatively well documented in gastric, urothelial, and colon cancers, as well as in cervical squamous carcinoma [6, 14, 15]. In fact, the observations that genetically engineered cervical, gastric and colon cancer cells overexpressing gal-7 fail to induce gastric tumors in xenografted mice suggest that epigenetic drugs or gal-7-specific gene therapy could be used to suppress the development of specific types of cancer [6, 14, 15]. Given the increasing popularity of epigenetic treatments for cancer, it is thus imperative to determine whether gal-7 has a pro- or anti-tumor function in any given type of cancer, most notably those of epithelial origin.

The various roles of gal-7 in cancers of epithelial origin are currently unclear and may be associated with a variety of factors. One must first consider the importance of the subcellular compartmentalization of gal-7, which has been found in the cytosolic, mitochondrial, and nuclear compartments [15–17]. Gal-3, for example, is able to induce resistance to apoptosis, and this activity depends on its translocation from the cytosol to the mitochondria [18]. Whether such intracellular compartimentalization is also important for gal-7 to regulate apoptosis is unknown. Alternatively, the dual role of gal-7 may depend on its binding partners because it is well known to bind glycosylated proteins via its carbohydrate-recognition domain (CRD). There are increasing indications, however, that galectins also interact with non-glycosylated proteins in a CRD-independent manner [19]. This observation has been well documented for intracellular galectins. The most important feature of intracellular galectins may be their ability to directly bind Bcl-2 family members via a CRD-independent interaction. This activity has been shown for many galectins, including gal-7 [16]. The galectin/Bcl-2 interaction shifts the balance of activity between pro- and anti-apoptotic members of the Bcl-2 family to regulate apoptosis [16, 20, 21]. Other CRD-independent functions of galectins include RNA processing in the nucleus [22] and the regulation of cell cycle progression [23]. All of these CRD-independent functions rely on protein-protein interactions. In fact, certain galectins, such as gal-10, harbor markedly low affinities for galactosides and are believed to act mainly through other factors [24]. These CRD-independent functions represent a paradigm shift in our understanding of galectin function and in the development of galectin-specific inhibitors.

The observation that gal-7 is specifically expressed in epithelial cells, particularly in mammary myoepithelial (basal) cells (but not in luminal cells), prompted us to investigate whether it is expressed in the basal cells of other tissues. Given that breast and prostate cancer have remarkable underlying biological similarities [25] and given the important role of basal cells in prostate cancer [26], we studied the expression pattern and role of gal-7 in human prostate cancer.

Materials and Methods

Cell lines and animals

PC3 [27] and DU-145 [28] cell lines were a generous gift from Dr. Benoit Ochietti (McGill University, Montréal, QC), and the LNCaP cells [29] were kindly provided by Dr. Thomas Sandersons (INRS-Institut Armand-Frappier, Laval, QC. The HACAT cell line [30] was provided by Dr. Thierry Magnaldo (Génétique et Physiopathologie des Cancers Épidermiques, Faculté de Médecine, Nice, France). All cell lines used in this study were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The DU-145 and HACAT cell lines were maintained in Dulbecco's modified Eagle's medium. The PC3 and LNCaP cell lines were maintained in an F-12k nutrient mixture and RPMI 1640 medium, respectively. Culture media were supplemented with 10% (v/v] fetal bovine serum, 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer, and 1 mmol/L sodium pyruvate. All cell culture products were purchased from Life Technologies (Burlington, ON, Canada). NOD/SCID mice were obtained from The Jackson Laboratory. Animals were housed under sterile conditions with *ad libitum* access to food and water. All animal studies were approved by the Institutional Animal Care and Use Committee of the Centre de Recherche du Centre Hospitalier de l'Université de Montréal

Immunohistochemistry

Tissue microarrays were used to assay 32 tissue samples of prostate adenocarcinoma, 20 hyperplasia, 5 saccular ectasia and 3 cancer-adjacent normal prostate tissues (US Biomax, Rockville, MD, USA and LifeSpan BioSciences, Seattle, WA, USA). Immunostaining reactions for gal-7 were performed using a Discovery XT automated immunostainer (Ventana Medical Systems). Deparaffinized sections were incubated in cell conditioning solution, pH 8.0 (Ventana Medical Systems), for antigen retrieval and then stained for 60 min with an anti-human gal-7 polyclonal antibody (R&D Systems, Minneapolis, MN, USA) diluted 1:150. The slides were counterstained with hematoxylin. The sections were scanned at a high resolution using a Nanozoomer Digital Pathology scanner (Hamamatsu, Bridgewater, NJ).

Immunofluorescence

Cells were fixed in 3% (w/v) paraformaldehyde for 15 min, permeabilized in 0.1% (v\v) PBS/Triton X-100 for 5 min and blocked overnight at 4°C in 1% (w/v). PBS/BSA (PBA). A goat anti-human gal-7 (diluted 1:750) primary antibody and rabbit anti-goat Alexa Fluor 488 (diluted 1:500) secondary antibody were used (Life Technologies). Filamentous actin was stained with Alexa Fluor 594-conjugated phalloidin (1:500 dilution; Life Technologies). All antisera were diluted in 1% (w/v) PBA, and all washing steps were performed with PBS. Nuclei were stained with ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). Cells were visualized under a Carl Zeiss LSM780 confocal microscope, and digitized images were generated using Carl Zeiss ZEN software (Zeiss, Jena, Germany).

RNA isolation and RT-PCR

Total cellular RNA was isolated from cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 μg of cellular RNA in a total reaction volume of 20 μL using Omniscript reverse transcriptase (QIAGEN, Mississauga, ON, Canada). After reverse transcription, human *gal-7* (gene ID 3963, sense primer: 5'-TCC CAA TGC CAG CAG GTT CCA TGT-3' and antisense primer: 5'-GAA GCC GTC GTC TGA CGC GAT GAT-3') and *GAPDH* (gene ID 2597, sense primer: 5'-CGG AGT CAA CGG

ATT TGG TCG TAT-3' and antisense primer: 5'-CAG AAG TGG TGG TAC CTC TTC CGA-3') cDNAs were amplified under the following conditions: 94°C for 3 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 seconds, and 72°C for 40 seconds and then a final extension step at 72°C for 10 min. PCR was performed in a thermal cycler (MJ Research, Watertown, MA). The amplified products were analyzed on 1% (w/v) agarose gels by electrophoresis followed by gel staining with SYBR Safe (Life Technologies).

Generation of stable transfectants expressing gal-7

To obtain stable DU-145 transfectants expressing gal-7^{wt} or gal-7^{R74S}, cDNAs encoding the wild-type or mutated (R74S) human gal-7 gene were cloned into the Srα eukaryotic expression vector (kindly provided by Dr. François Denis) as previously described [17] Control cells were generated using an empty Srα vector. Transfections were performed with Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies). After 48 h of culturing, transfected cells were allowed to grow in complete medium containing 2 μg/ml puromycin. Individual colonies were expanded, and gal-7 expression was monitored by western blot analysis. A minimum of two clones of each type was used to confirm the results.

Western blot analysis

For whole cell extracts, cells were homogenized and resuspended in radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Ottawa, ON, Canada) containing a cocktail of protease inhibitors (Roche, Mississauga, ON, Canada). Mitochondria and nuclei were isolated using a mitochondrial isolation kit (Thermos Scientific) and nuclear extraction kit (Sigma-Aldrich, Oakville, ON, Canada), respectively, according to the manufacturers' instructions. Equal amounts of whole-cell, cytoplasmic, mitochondrial or nuclear extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada). The membranes were first blocked with 5% (w/v) milk in PBS/0.5% Tween 20 (v/v) for 60 min at room temperature and subsequently blotted overnight in a solution containing 3% PBA, 0.5% Tween 20 and the following antibodies: a goat anti-mouse gal-7 polyclonal antibody (diluted 1:1000; R&D Systems), a rabbit anti-poly(ADP-ribose) polymerase (Parp)-1 (p25) polyclonal antibody (1:5000; Epitomics, Burlingame, CA, USA), a mouse anti-β-actin (1:20000; Sigma-Aldrich), a rabbit anti-COX IV (1:1000; Cell Signaling Technology, Beverly, MA, USA), a rabbit anti-tubulin (1:1000; Cell Signaling Technology) or a mouse anti-lamin A/C (1:1000; Cell Signaling Technology) antibody. Secondary antibodies included horseradish peroxidase-conjugated donkey anti-rabbit (GE Healthcare, Baie-d'Urfé, QC, Canada), donkey anti-goat (R&D Systems) or sheep anti-mouse (GE Healthcare) IgG. Detection was performed by the enhanced chemiluminescence method (GE Healthcare).

Electron microscopy

Cells were fixed in a 0.1% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde solution and embedded in Spurr's resin. Ultrathin sections were placed on nickel grids and incubated in sodium metaperiodate. Samples were then blocked in 1% PBA for 5 min and incubated for 60 min with a goat anti-human gal-7 polyclonal antibody (1:150) followed by incubation with a rabbit anti-goat 10-nm gold-conjugated secondary antibody (1:20, Electron Microscopy Sciences, Hatfield, PA, USA). The samples were counterstained with uranyl acetate and lead citrate before visualization under a Hitachi H-7100 transmission electron microscope.

[^{3}H]-thymidine incorporation

Proliferation of cells was determined by measuring the incorporation of [^{3}H]-thymidine. Cells were seeded in triplicate at a density of 2 x 10^3 cells/well into a 96-well plate and subsequently incubated with or without 5 μM cisplatin for 96 h. After 80 h of incubation, 1 μCi of [^{3}H]-thymidine was added to each well. At the end of the incubation period, the cells were harvested with a semiautomatic cell harvester (Skatron Instruments, Lier, Norway) and transferred onto a Printed Filtermat A (Wallak, Turky, Finland). Incorporated radioactivity was determined using a RackBeta (LKB, Turky, Finland) scintillation counter.

Invasion assay

Serum-induced cell invasion was examined using a 24-well Matrigel invasion chamber (BD Biosciences, Mississauga, ON, Canada) with an 8 μm-pore membrane. A total of 5 x 10^4 cells were incubated within the upper chamber in serum-free medium. The lower chamber contained medium supplemented with 10% fetal bovine serum. After 24 h of incubation, the upper surface of the insert was wiped gently with a cotton swab to remove the non-migrating cells. Cells that had migrated to the lower surface of the membrane were stained with toluidine blue and counted separately by microscopy.

Scratch wound healing assay

Confluent monolayers were obtained by seeding 1 x 10^5 cells onto a 24-well plate the day before the experiment. A scratch was made with a pipet tip in the cell monolayer, followed by washing with PBS to remove cell debris. Immediately after and 24 h after the PBS wash step, the microscopic fields were photographed, and the scratch width was measured using Image J software. For live cell imaging, one day prior to the experiment, 4 x 10^5 cells were seeded onto a 6-well glass-bottom culture plate (MatTek Corporation, Ashland, MA, USA). After the scratch was made, the plate was moved to a PM S1 incubator, and the migration was visualized under a Carl Zeiss LSM780 confocal microscope (Carl Zeiss, Toronto, ON, Canada). Images were captured every 10 min for 2 h. For each cell type, the movements of 30 separate cells were measured. Cell movement was analyzed using the following Image J plugins: manual tracking and chemotaxis tool.

Cell proliferation assay

As a control for cell proliferation during the invasion assay and scratch wound healing test, a total of 2.5 x 10^4 cells were seeded onto a 12-well plate (Fisher Scientific). At the indicated times, the cells were washed with PBS, trypsinized, stained with trypan blue and counted using a hemocytometer.

Production of recombinant proteins

Gal-7 cDNA was cloned into pET-22b(+) using the NdeI and HindIII restriction enzymes. The protein was produced in *E. coli* BL21 (DE3) at 37°C. Isopropyl β-D-1-thiogalactopyranoside (*IPTG*) (1 mM) was added to the bacterial culture at an OD_{600nm} of 0.6–0.7, and the bacteria were further incubated for 4 h. Bacterial pellets were resuspended in lysis buffer (0.7 mg/mL lysozyme, 10 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail), incubated for 1 h at 37°C and centrifuged for 30 min at 15,000 rpm (4°C). The supernatant was then filtered and applied to a lactose-agarose column, and the protein was eluted in 1-mL fractions with a 150-mM lactose solution. Purified fractions were analyzed by

SDS-PAGE. Gal-7 was dialyzed against 20 mM potassium phosphate at pH 7.2 for all subsequent experiments.

Glycan array

A mammalian glycan array (V5.2) was performed by the Consortium for Functional Glycomics (CFG). Briefly, recombinant gal-7^{wt} and gal-7^{R74S} proteins were conjugated to FITC and tested against version 5.2 of the printed array. This array consisted of 609 glycans in replicates of 6. The lists of the glycans and their linkers used in the different versions of the array can be found at http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh.shtml. FITC-conjugated gal-7 was incubated with the sugars, and relative fluorescence units (RFUs) were measured. To eliminate some of the false hits that contained a single very high or low point, the highest and lowest points from each set of six replicates were removed. Consequently, the averages include 4 values rather than 6.

Binding assay

Briefly, a fluorescein isothiocyanate (FITC)/DMSO solution was added to recombinant gal-7 in a 0.1-M $NaHCO_3$ (pH 9.2) solution and then incubated for 2 h at room temperature on a roller. FITC-conjugated gal-7 was then purified using a PD-10 sepharose column (GE Healthcare) and eluted with PBS containing 0.01% (v/v] sodium azide. To measure FITC-gal-7 binding to the cell surface, 2.5 x 10^5 cells were incubated for 30 min with the indicated concentrations of gal-7 and then washed twice with PBS and resuspended in 500 μl PBS. For competition assays, 0.1 M β-lactose was added to cells, which were then incubated with FITC-conjugated gal-7. Samples were analyzed by FACSCalibur (BD Biosciences) and Flowing Software.

In vivo experiments

A mix of 3 independent clones (2 x 10^6 cells) for each transfectant was injected subcutaneously into 6-week-old NOD/SCID mice. Tumor measurements were obtained twice a week. On day 61, the mice were sacrificed, and the primary tumors were harvested and snap-frozen in liquid nitrogen.

Statistical analysis

Statistical significance of the experiments was evaluated using unpaired Student's t-tests. The results were considered statistically significant at $P \leq 0.05$.

Results

Gal-7 expression in human prostate tissues and cancer cell lines

Gal-7 expression in human prostate tissues has not previously been reported. Using immunohistochemistry (IHC), we first investigated the expression pattern of gal-7 in normal prostate tissues. Our results revealed strong nuclear and cytoplasmic expression in the basal cell layers of normal prostate glands, with no staining observed in the luminal epithelial cells (Fig 1A). We then investigated the presence of gal-7 in various types of prostate malignancies (including 32 prostate adenocarcinomas) using commercial tissue microarrays and found negligible gal-7 protein expression in the tumor tissues (Fig 1B and 1C). The low expression in PCa tissues was consistent with expression patterns found during our investigations of gal-7 expression at the mRNA and protein levels in the most common prostate cancer cell line models. Immunoblotting experiments demonstrated that none of these cell lines expressed readily detectable levels of gal-7; however, mRNA was expressed at a low but detectable level in PC3 cells (Fig 1). Taken

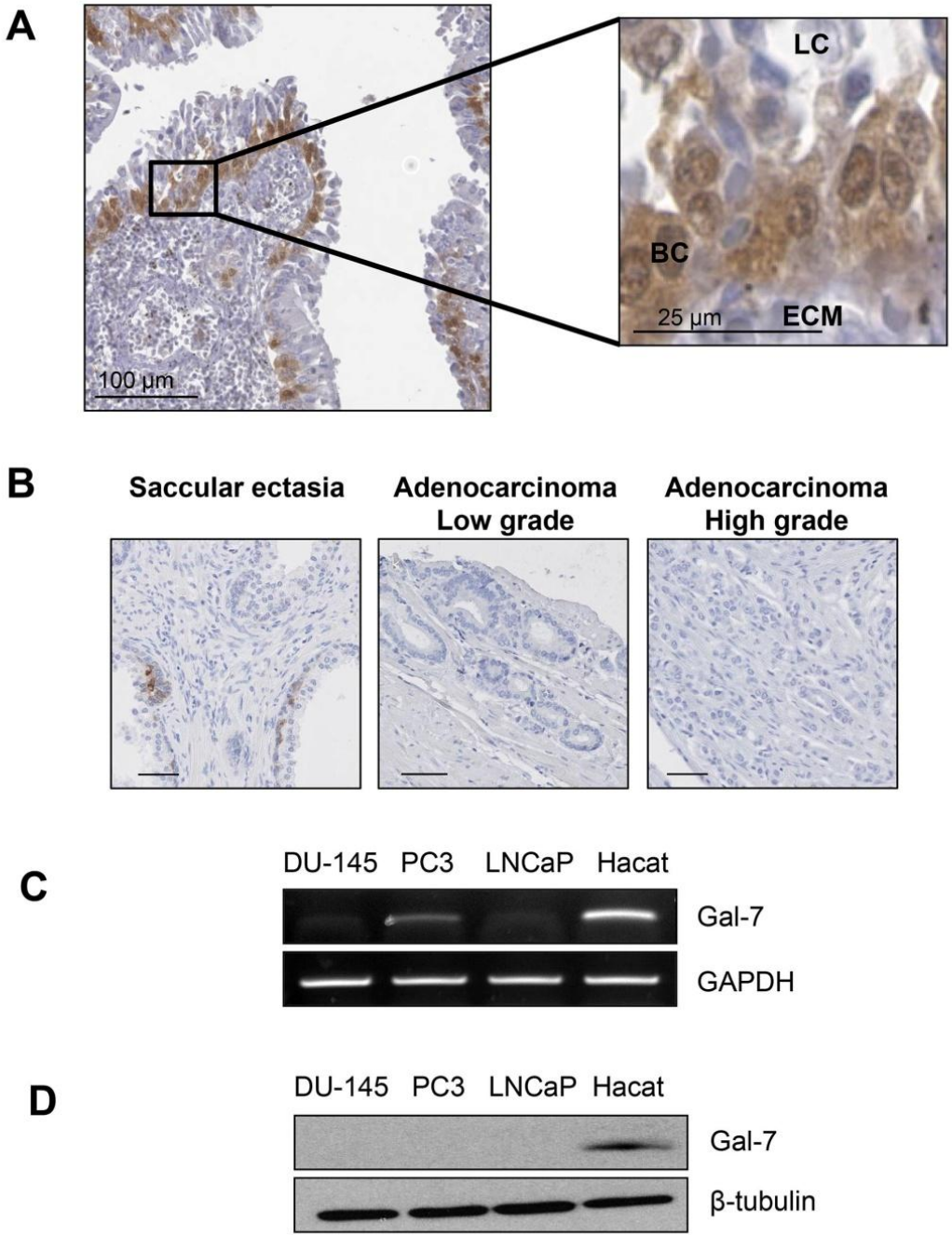

Fig 1. Gal-7 expression in prostate tissues and cancer cell lines. Immunohistochemical (IHC) staining of gal-7 in 3-µm thick sections of formalin-fixed paraffin-embedded (**A**) healthy prostate tissues and (**B**) tissues associated with pathological disorders of the prostate. (**C**) Semi-quantitative RT-PCR and (**D**) western blot analysis of gal-7 expression in DU-145, PC3 and LNCaP human prostate cancer cell lines. The HaCaT keratinocyte cell line was used as a positive control. GAPDH and β-tubulin were used as loading controls. LC: luminal cells, BC: basal cells, and ECM: extracellular matrix.

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together, these results showed that although gal-7 is readily expressed in basal cells within normal prostate tissue, it is completely absent in prostate cancer cells.

Production and characterization of wild-type and CRD-defective gal-7 protein

To investigate the functions of gal-7 in prostate cancer cells and to determine the importance of its CRD, we generated a series of stable transfectants expressing wild-type gal-7 and a mutated form (R74S) of the protein (Fig 2A). This mutation is known to inhibit the ability of gal-7 to bind lactose and to reduce its translocation from the cytosol to the mitochondria and/or the nucleus [17]. Our previous analysis using solution NMR spectroscopy showed that the R74S mutation induced only limited and local changes in gal-7 folding. To further determine the extent to which the CRD of gal-7 is disrupted by the R74S mutation, we compared its binding properties to those of wild-type gal-7 using a CFG glycan array (version 5.2) [31]. The entire list of glycans tested and the results of the binding assays can be found online (http://functionalglycomics.org/). Our results confirmed that the R74S mutation suppressed CRD activity independent of the glycan motifs assessed (Fig 2B, S1 Table). This suppression of CRD activity was confirmed by flow cytometric analysis of the binding of FITC-labeled recombinant gal-7^{wt} and gal-7^{R74S} at the surfaces of DU-145 prostate cancer cells (Fig 2C and 2D). Taken together, these results demonstrate that R74S abolishes the CRD activity of human gal-7.

Characterization of intracellular localization of wild-type gal-7 and gal-7^{R74S}

To investigate the role of gal-7 and its CRD in prostate cancer, DU-145 transfectants expressing either gal-7^{wt} or gal-7^{R74S} were generated. Control transfectants (generated using empty expression vectors) did not express detectable levels of gal-7 (Fig 3A and 3B). Immunoblotting of mitochondrial and nuclear enriched fractions showed detectable expression of gal-7^{wt} in the cytosol, mitochondria and nuclei of DU-145 cells (Fig 3C and 3D). In contrast, there was no detectable expression of gal-7 in the mitochondria of transfectants expressing gal-7^{R74S}. Both proteins were found in the extracellular media of the cells (Fig 3E). Electron microscopy analysis of the DU-145 cells confirmed the expression of gal-7^{wt} in the cytosol, nucleus, and mitochondrial outer membrane, while the expression of gal-7^{R74S} was restricted to the cytosol (S1 Fig). Interestingly, electron microscopic analysis revealed the presence of gal-7^{wt}- and gal-7^{R74S}-rich protrusions at the cytoplasmic membrane (S1 Fig) consistent with previous reports, suggesting that gal-7 associates with the actin cytoskeleton to regulate cell motility [10, 32, 33].

Gal-7 promotes apoptosis in prostate cancer cells

Several studies have reported that ectopic expression of gal-7 renders cervical, gastric and colon cancer cells more sensitive to apoptosis induced by pro-apoptotic drugs [15]. To determine whether this finding is also true in prostate cancer cells, DU-145 transfectants were treated with increasing doses of pro-apoptotic drugs and analyzed for cleavage of Parp-1, which is a commonly used marker of apoptosis [34]. Our results showed that ectopic expression of gal-7 increased the cleavage of Parp-1 induced by etoposide compared to the control cells (Fig 4A). Similar results were obtained when the fragmentation of nuclei in apoptotic cells was visualized with DAPI staining (Fig 4B). The ability of gal-7 to increase the sensitivity of DU-145 cells to apoptosis was also observed using cisplatin as a pro-apoptotic drug (Fig 4C). Interestingly, gal-7^{R74S} was as effective as gal-7^{wt} in increasing the sensitivity of DU-145 to both pro-apoptotic drugs. In both cases, the intracellular localization of gal-7 remained

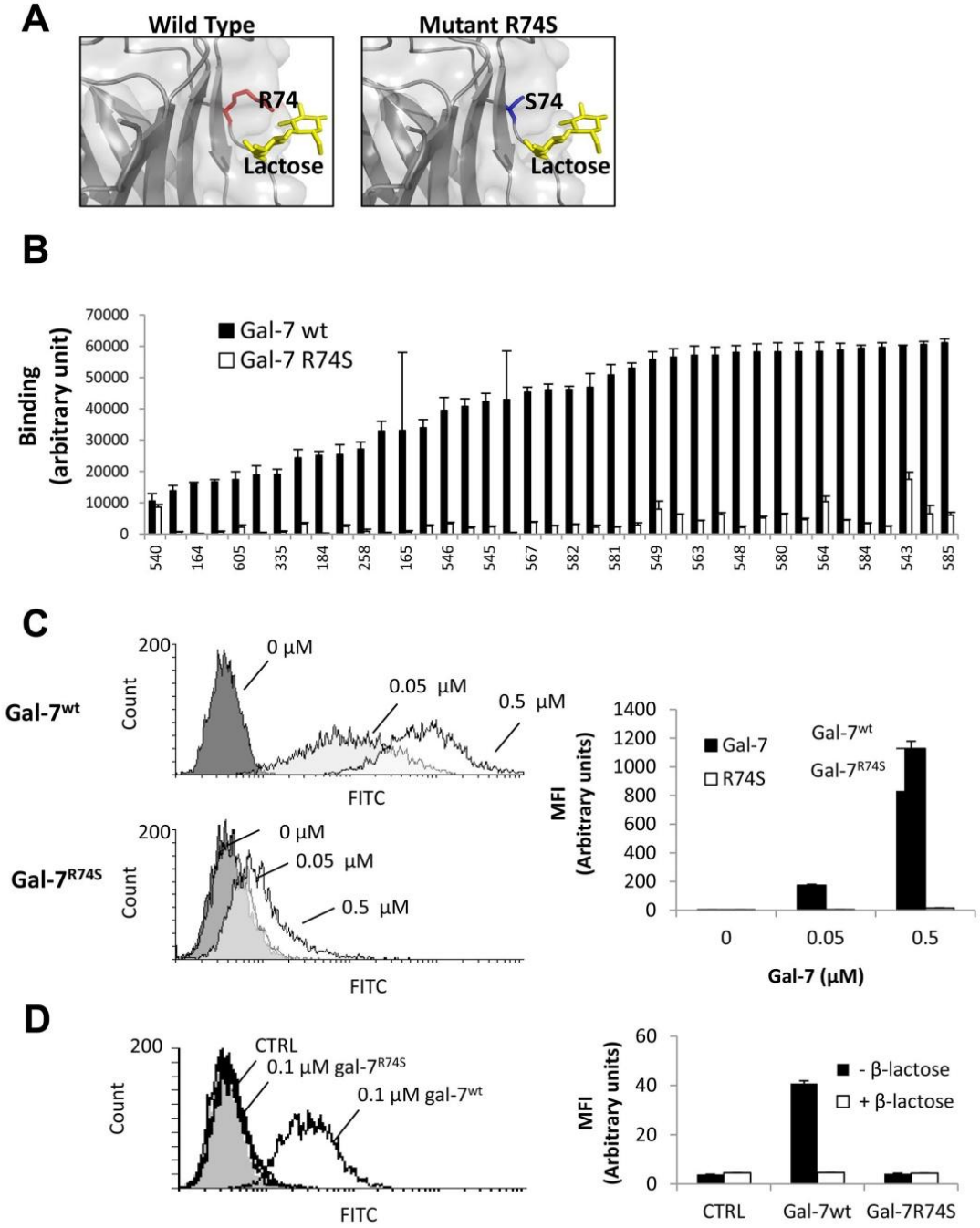

Fig 2. WT and R74S-mutated gal-7 CRD activity. (**A**) 3D view of the gal-7^{wt} and gal-7^{R74S} CRDs in the presence of lactose. (**B**) A glycan array was used to verify the binding of gal-7^{wt} and gal-7^{R74S} to a large variety of sugars. The graph depicts the binding of gal-7^{wt} and gal-7^{R74S} to the sugars. Only RFUs larger than 10,000 are presented. The sugar names are listed in S1 Table. The error bars represent SDs (n = 6). Flow cytometry analysis showing binding of gal-7^{wt} and gal-7^{R74S} to the surfaces of DU-145 cells (**C**) in the absence or (**D**) presence of 0.1 M β-lactose. Binding assays were conducted using the indicated concentrations of FITC-labeled recombinant gal-7. MFI: mean fluorescence intensity. The results represent three independent experiments.

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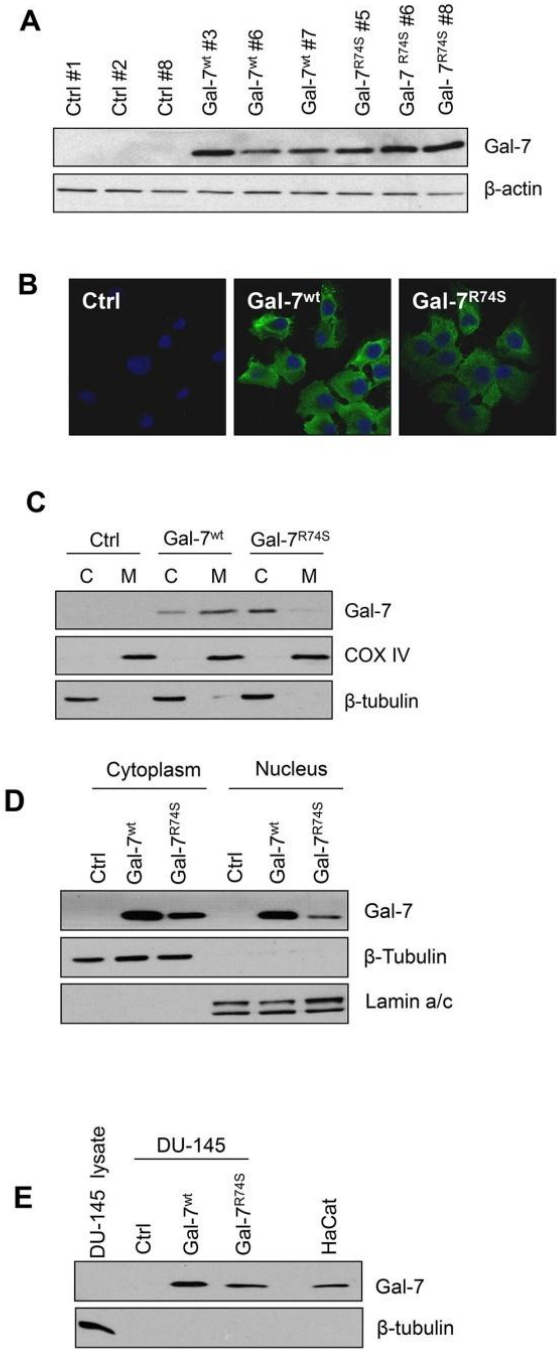


Fig 3. Intracellular localization of wild-type and R74S-mutated gal-7 proteins in DU-145 cells. (**A**) Western blot analysis showing the expression of gal-7 in various stable DU-145 clones transfected with expression vectors encoding wild-type and mutated gal-7. Controls included cells transfected with empty Srα vectors. β-actin was used as a loading control. (**B**) Confocal imaging showing the intracellular distribution of gal-7 in DU-145 transfectants. (**C-D**) Western blot analysis showing gal-7 expression in cytosolic, nuclear and mitochondrial fractions prepared from DU-145 cells. β-tubulin, COX IV and lamin A/C were used as cytosolic,

mitochondrial and nuclear markers, respectively. (**E**) Western blot analysis showing the secretion of gal-7 in the extracellular media of DU-145 cells expressing gal-7^{wt} or gal-7^{R74S}. β-tubulin expression was monitored to exclude the possibility of cell lysis. Intracellular protein extracts from control DU-145 cell and HaCaT cell supernatants were used as positive controls for β-tubulin expression and gal-7 secretion, respectively. All results represent three independent experiments, including a minimum of two independent DU-145 clones.

doi:10.1371/journal.pone.0131307.g003

unchanged during the induction of apoptosis (S2 Fig). Using a (^{3}H]-thymidine incorporation assay, we also measured proliferation of the transfectants in the absence or presence of cisplatin. We found that both gal-7^{wt}- and gal-7^{R74S}-expressing DU-145 cells proliferated at the same rates compared to control cells under normal conditions but proliferated more slowly in presence of cisplatin (Fig 4D), which is consistent with the ability of gal-7 to promote drug-induced apoptosis. Taken together, these results show that gal-7 sensitizes DU-145 cells to pro-apoptotic agents independent of its CRD activity and its intracellular compartmentalization.

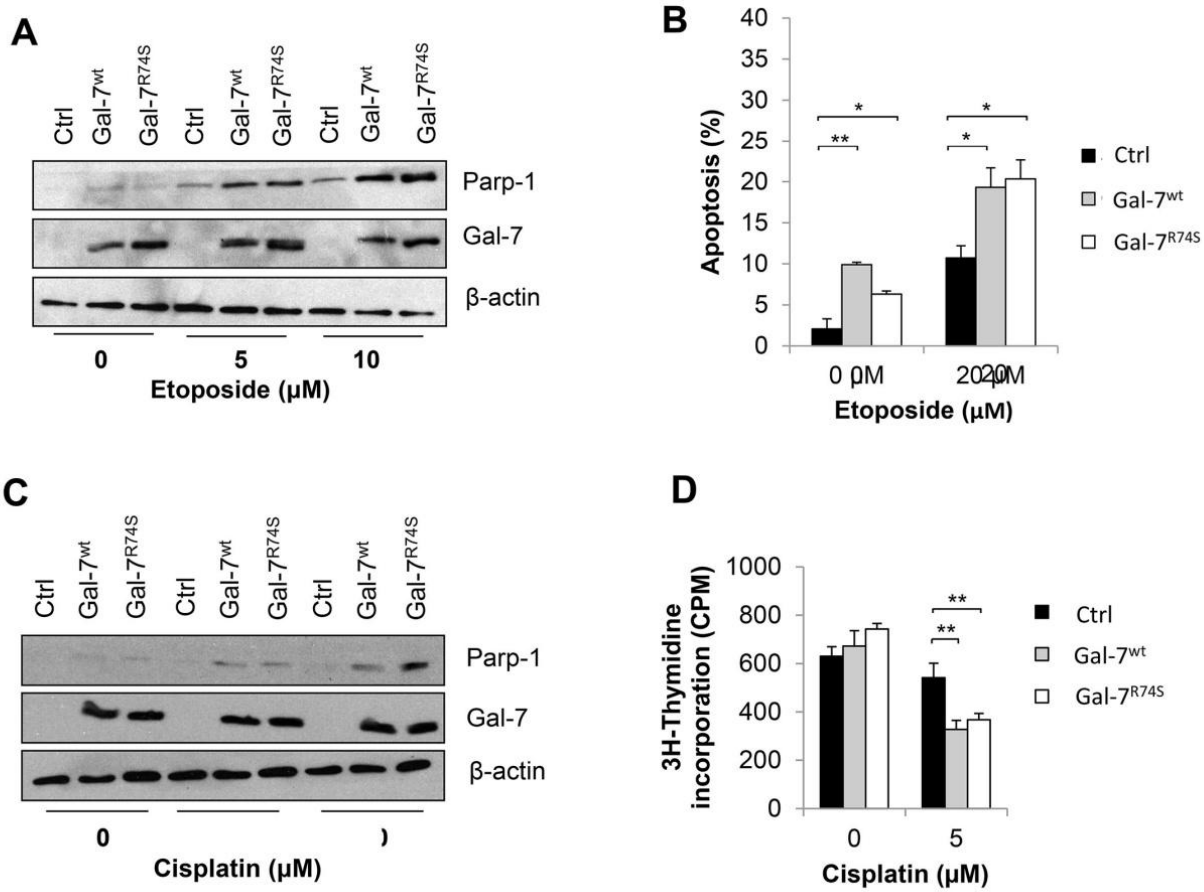


Fig 4. Gal-7 increases the sensitivity of DU-145 cells to apoptosis. (**A**) Cells were incubated for 16 h with the indicated concentrations of etoposide and tested for apoptosis by measuring Parp-1 cleavage by western blot. (**B**) Apoptosis was confirmed by counting the number of cells with a fragmented nucleus visualized by DAPI staining. (**C**) Analysis of Parp-1 cleavage in DU-145 cells treated for 16 h with the indicated concentration of cisplatin. (**D**) Cell proliferation of DU-145 cells treated with or without 5 μM cisplatin measured by (^{3}H]-thymidine incorporation. All results represent three independent experiments, including a minimum of two independent DU-145 clones. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

doi:10.1371/journal.pone.0131307.g004

Gal-7 but not R74S reduces invasive behaviors of DU-145 cells

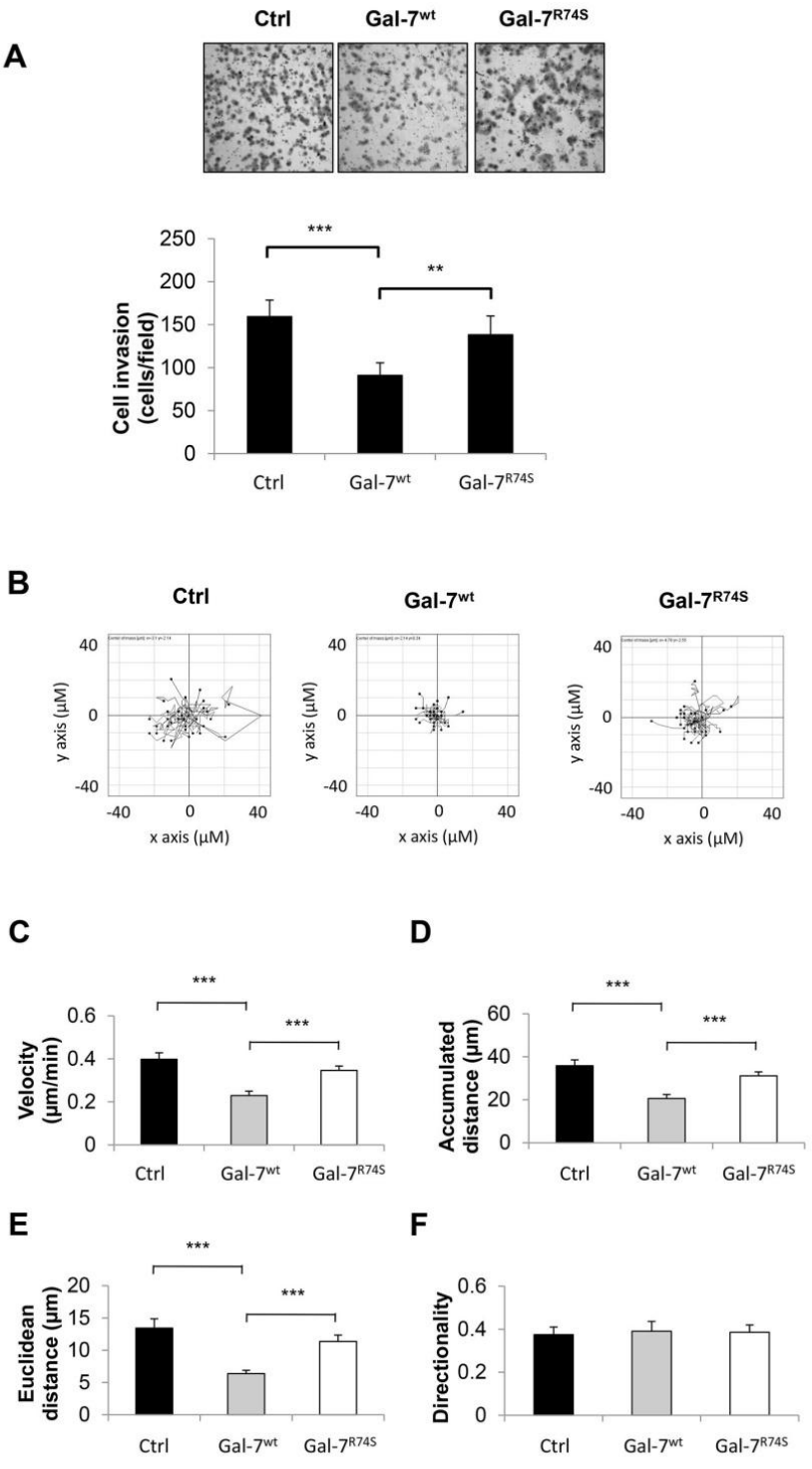
We next investigated whether gal-7 can modulate the invasive behaviors of DU-145 cells using a standard *in vitro* Matrigel invasion assay. We found that ectopic expression of gal-7^{wt} significantly reduced the invasive behaviors of DU-145 cells compared to control cells lacking gal-7 (Fig 5A). A similar difference was not observed for DU-145 cells expressing gal-7^{R74S}. Because cell invasive behaviors might be affected by cell motility, we used live cell imaging to measure cell movements during a scratch wound healing assay (Fig 5B). Our results showed that DU-145 cells expressing gal-7^{wt} significantly reduced cell velocity compared to control cells lacking gal-7 or cells expressing gal-7^{R74S} (Fig 5C). These gal-7^{wt}-expressing cells also had lower accumulated and Euclidean distances of migration (Fig 5D and 5E). The directionality of the DU-145 cells was not affected by expression of the gal-7^{wt} or gal-7^{R74S} proteins (Fig 5F). The use of a standard scratch wound healing assay further confirmed that gal-7^{wt} reduced the cell motility of the DU-145 cells. Again, a similar effect was not observed for the gal-7^{R74S} mutant (S3 Fig). No differences in cell proliferation were observed between the cells expressing gal-7^{wt} or gal-7^{R74S} and the control cells (S4 Fig). The addition of recombinant gal-7^{wt} and gal-7^{R74S} to DU-145 cells had no effect, suggesting that extracellular gal-7 is not involved in reducing invasive behaviors (S5 Fig). Taken together, these results indicate that intracellular gal-7 reduces the invasive behaviors of prostate cancer cells by impairing cell motility in a CRD-dependent manner.

Gal-7 CRD disruption increases tumor growth *in vivo*

We next investigated whether gal-7^{wt} and gal-7^{R74S} impact tumor growth using adult male NOD/SCID mice. Mice were injected subcutaneously with DU-145 transfectants, and tumor size was measured twice a week for 61 days, at which time the tumors were harvested to confirm the expression of gal-7 in the gal-7^{wt}- and gal-7^{R74S}-expressing cells (S6 Fig). Our results showed that the overexpression of gal-7^{wt} led to a modest yet significant ($p \leq 0.05$) reduction in tumor size (Fig 6). Interestingly, the expression of gal-7^{R74S} caused a significant ($p \leq 0.001$) increase in tumor growth compared to both the control and gal-7^{wt}-expressing cells.

Discussion

Our previous results showing that gal-7 is specifically expressed in mammary myoepithelial (basal) cells but not in mammary luminal cells prompted us to investigate whether this molecule is expressed in the basal cells of prostate tissues. Using an anti-gal-7-specific Ab, we found that gal-7 immunostaining in human prostate tissues was consistently strong in the nuclei and cytoplasm of prostate basal cells, with the luminal cells showing no detectable staining. This pattern of expression is thus clearly distinct from those reported for gal-1 and gal-3. Indeed, gal-3 is expressed in luminal cells but not in basal cells, while gal-1 is expressed in the endothelial and stromal fibromuscular cells of the prostate [35]. This distinct expression pattern for gal-7 is also observed in prostate cancer cell lines. Although we found no detectable expression of gal-7 in the prostate cancer cell lines tested, gal-3 has been shown to be readily expressed in both PC-3 and DU-145 cells [36]. Gal-3 expression is also reduced in PCa cells compared with normal prostate cells but is still detectable by IHC in a significant number of samples [36, 37]. Our data, however, clearly showed that gal-3 and gal-7 had distinct properties in PCa cells. For example, in contrast with gal-7, cytoplasmic gal-3 increased Matrigel invasion and cell growth while decreasing apoptosis induction, and nuclear expression had a completely opposite effect [38]. Thus, gal-3 and gal-7 possess completely opposite biological activities in PCa cells. Although future experiments will be needed to confirm these results in other prostatic cell lines (including benign cell lines) and other preclinical PCa models. It is important to note


A
Ctrl
Gal-7wt
Gal-7R74S
Cell invasion (cells/field)
250
200
150
100
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y axis (µM)
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-40
C
Velocity (µm/min)
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D
Accumulated distance (µm)
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E
Euclidean distance (µm)
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F
Directionality

Fig 5. Invasive behavior of DU-145 expressing gal-7^{wt} or gal-7^{R74S}. (**A**) Matrigel invasion assay of DU-145 stable transfectants. Cells were seeded into serum-free medium in the upper chamber, and serum was added to the lower chamber. After 16 h, cells that migrated through the Matrigel and the 8 μm-pore membrane were stained and counted. The results represent the number of cells per microscopic field. (**B**) Plots of 30 cells/sample tracked by live cell imaging during the scratch wound healing test. DU-145 cells were seeded onto a 6-well glass-bottom culture plate. A scratch was made, and images were captured every 10 min for 2 h. Quantifications of the (**C**) velocity, (**D**) accumulated distance, (**E**) Euclidean distance and (**F**) directionality are shown. Error bars represent the SEM. All results represent three independent experiments, including a minimum of two independent DU-145 clones. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

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that we cannot not use the PC3 cells (another classical cell model of human prostate cancer) because these cells express low but significant endogenous galectin-7 (as shown in Fig 1). We cannot use the LNCaP cell model (another commonly used model) because this model in androgen-dependent, in contrast to DU-145. Nevertheless, our findings may have important implications in the development of CRD-specific inhibitors against gal-3 and emphasize the need to develop inhibitors that are highly specific for a given galectin.

Our results showed that gal-7 reduced the invasive behaviors of prostate cancer cells by inhibiting their motility. This phenotype is consistent with the localization of this molecule in lamellipodia and filopodia. In normal cells, gal-7 is also found in motility structures, such as podosomes and primary cilia [32, 33]. Interestingly, a mutation at position 74 completely abolished this activity. In contrast, this mutation did not affect the ability of gal-7 to induce apoptosis, indicating that both functions are mediated by distinct gal-7 sites. One possibility is that gal-7 regulates the stability and/or localization of proteins, such as β-catenin, and that the mutation at position 74 abolishes this interaction. In the cytoplasm, β-catenin is either ubiquitinated for proteasomal degradation or localized at cell-cell contact sites, stabilizing E-cadherin and affecting motility [39]. This interaction between galectins and β-catenin has been reported previously [40–42].

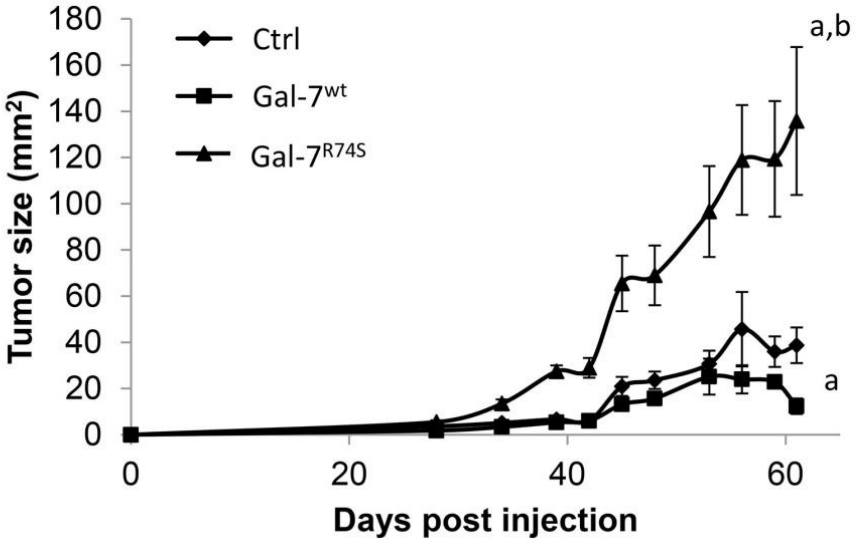

Fig 6. Gal-7^{wt} and gal-7^{R74S} effects on tumor growth. NOD/SCID mice (n = 6) were injected subcutaneously with control DU-145 transfectants or with DU-145 transfectants expressing gal-7^{wt} or gal-7^{R74s}. Tumor growth was monitored twice a week for 61 days. N = 6 mice per group.

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We found that gal-7 overexpression sensitized DU-145 prostate cancer cells to apoptosis induced by cisplatin or etoposide. The dual role of gal-7 in apoptosis has been well documented. Because gal-7 binds to bcl-2, our initial hypothesis was that mitochondrial gal-7 could be responsible for this dual role. However, this assumption is clearly not true because cytoplasmic gal-7^{R74S} displays similar pro-apoptotic functions as the wild-type protein. This similarity has also been reported for the anti-apoptotic functions of gal-7 in breast cancer cells [17]. It is indeed quite clear that although gal-7 is expressed in basal cells in normal prostatic and mammary tissues, it plays a completely different role in prostate and breast cancer. The mechanisms leading to the anti- and pro-tumorigenic functions of cytoplasmic gal-7 remain unknown. One possible mechanism involves modulation of the JNK1 pathway, as suggested by Kuwabara *et al.* [14], who showed that the induction of apoptosis by gal-7 in HeLa cells is correlated with activation of this signaling pathway. It is also possible that gal-7 regulates apoptosis by interacting with bcl-2. Other studies have indeed shown that gal-7 interacts directly with Bcl-2 [16]. Cytoplasmic gal-7 could sequester bcl-2 in the cytoplasm, thereby inhibiting its anti-apoptotic function. Alternatively, given the structural homology among members of the bcl-2 family [43], it is possible that gal-7 binds to other bcl-2 structural homologs, thereby altering the delicate balance between pro- and anti-apoptotic proteins. We are currently investigating these possibilities.

The dual role of galectins in modulating tumor progression has been previously noted but is still unclear. Our data showing that gal-7^{R74S} acts as a tumor suppressor *in vitro* and as a pro-tumorigenic protein *in vivo* suggest that the roles of galectins in cancer likely involve a delicate balance between pro- and anti-tumoral interactions occurring within and outside cancer cells, i.e., in the tumor microenvironment. Because gal-7^{R74S} sensitized DU-145 cells to apoptosis without affecting their invasive behaviors or proliferation, leading to the augmentation of tumor growth *in vivo*, it is clear that alterations in the CRD of gal-7 that shift the balance towards CRD-independent binding partners not only have a profound effect on its intracellular distribution in cancer cells but also drive a phenotypic switch in its role in cancer. *In silico* analysis using a publically available dataset, the cBioPortal for Cancer Genomics, shows that the gene encoding human gal-7 is rarely (less than 1% in prostate adenocarcinoma) mutated in PCa and is not mutated within the CRD-coding region. Similar results were obtained with the COSMIC database (one missense mutation out of 528 cases). This indicates that loss of gal-7 expression in PCa cells is probably due to an epigenetic mechanism and/or to the depletion of basal cells. Our data add, however, a new dimension to the role of galectin CRDs in cancer, emphasizing the use of highly specific inhibitors to target members of the galectin family. The identification of important CRD-independent functions represents a paradigm shift in our understanding of galectin functions. Future investigations will be needed to identify in detail the CRD-independent binding partners involved. The availability of our gal-7^{R74S} model will be useful in this regard.

Supporting Information

S1 Fig. Electron microscopic analysis of gal-7^{wt} and gal-7^{R74S} intracellular localization. Electron microscopic analysis of gal-7 and R74S protein distributions in the cytosol, nuclei, mitochondria and protrusions localized to the cytoplasmic membranes of DU-145 transfectants. Bars represent 100 nm.
(TIFF)

S2 Fig. Intracellular localization of gal-7 during apoptosis. Western blot analysis showing gal-7 expression in cytosolic, (**B**) mitochondrial and (**C**) nuclear fractions prepared from DU-145 cells treated for 16 h with 10 μM etoposide. β-tubulin, COX IV and laminin C were used as

cytosolic, mitochondrial and nuclear markers, respectively. All results represent three independent experiments, including a minimum of two different DU-145 clones.
(TIFF)

S3 Fig. Scratch wound healing assay of DU-145 transfectants. DU-145 transfectants were seeded into a 24-well plate. A scratch was made with a pipet tip, photos were taken at time 0 and at 24 h, and the width of the scratch was measured. The results represent the distances of the migration of the cells, and the bars represent the SDs. The results represent three independent experiments. $^{***}P \leq 0.001$.
(TIFF)

S4 Fig. Gal-7^{wt} and gal-7^{R74S} effects on cell proliferation. As a control for cell proliferation, cells were seeded into a 24-well plate and counted at the indicated time using trypan blue staining. The results represent three independent experiments.
(TIFF)

S5 Fig. Extracellular gal-7 effect on cell motility. (**A**) Plots of 30 cells/sample tracked by live cell imaging during a scratch wound healing assay. DU-145 cells were seeded onto a 6-well glass-bottom culture plate and treated with the indicated concentration of gal-7^{wt}. A scratch was made, and images were captured every 10 min for 2 h. Quantifications of the (**B**) velocity, (**C**) accumulated distance, (**D**) Euclidean distance and (**E**) directionality are shown. The error bars represent the SEMs. All results represent three independent experiments, including a minimum of two independent DU-145 clones.
(TIFF)

S6 Fig. Gal-7 mRNA expression in DU-145 tumors. NOD/SCID mice (n = 6) were injected subcutaneously with DU-145 transfectants expressing the empty vector or gal-7^{wt}/gal-7^{R74s}. On day 61, the mice were sacrificed, and the primary tumors were harvested and snap-frozen in liquid nitrogen. Tumor mRNA was extracted and gal-7 expression was measured by RT-PCR. N = 6 mice per group.
(TIFF)

S1 Table. Glycan array. The names of the different sugars used in the glycan array are listed. Only those sugars for which gal-7^{wt} had an RFU of larger than 10,000 are presented.
(TIFF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ML JS LG YSP. Performed the experiments: ML MV BGL AAG. Analyzed the data: ML MV BGL AAG JS LG YSP. Contributed reagents/materials/analysis tools: JS LG YSP. Wrote the paper: ML YSP.

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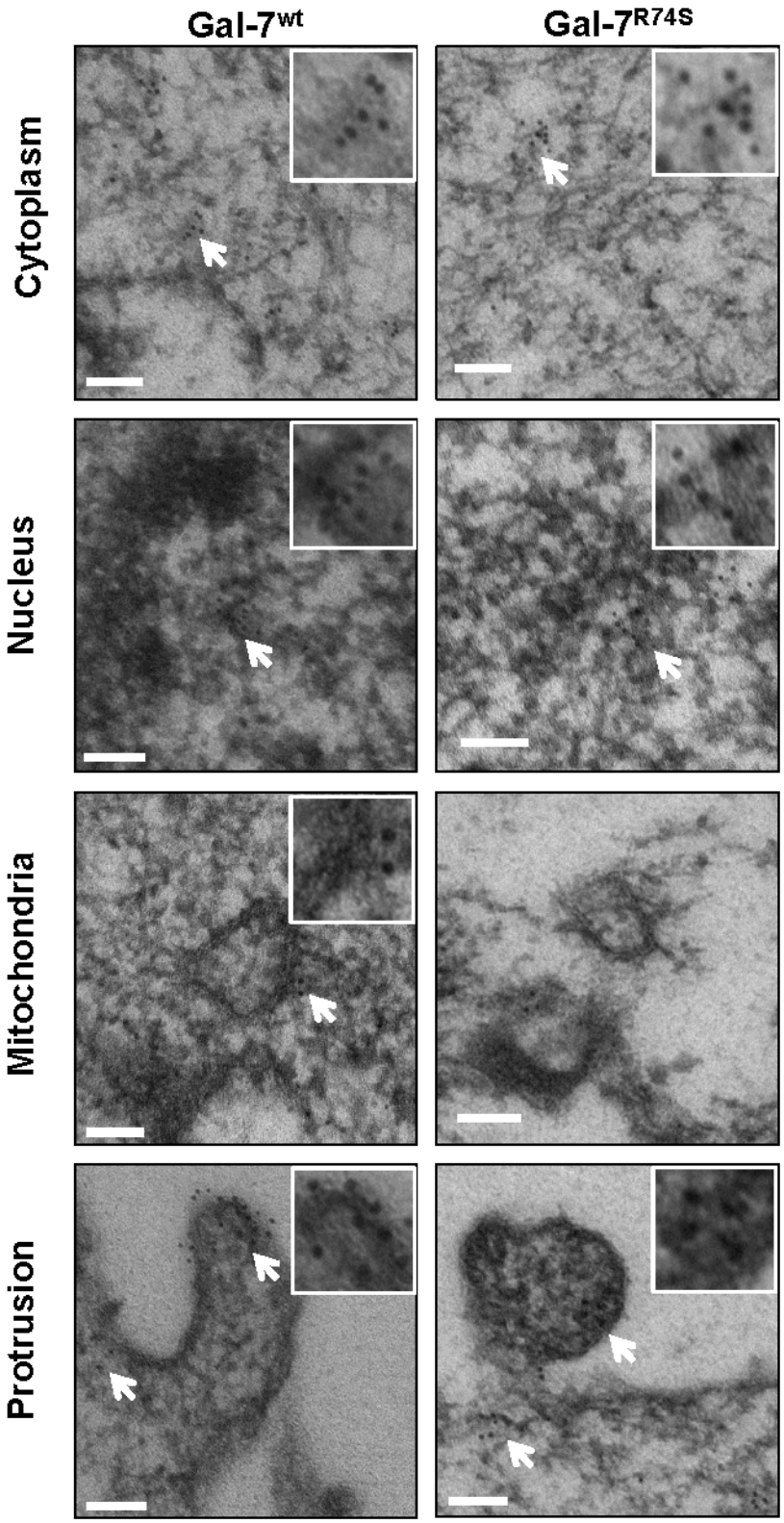

S1 Fig. Electron microscopic analysis of gal-7^{wt} and gal-7^{R74S} intracellular localization. Electron microscopic analysis of gal-7 and R74S protein distributions in the cytosol, nuclei, mitochondria and protrusions localized to the cytoplasmic membranes of DU-145 transfectants. Bars represent 100 nm.

S1 Table. Glycan Array. Structure of the different sugars used in the glycan array assay. Only sugars for which gal-7^{wt} had a RFU larger than 10 000 are presented.

Chart Number	Glycan sructure
540	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
607	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
164	Galb1-4GlcNAcb1-3Galb1-4Glcb-Sp0
73	Fuca1-2Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
605	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
337	GlcNAca1-4Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
335	GalNAca1-3(Fuca1-2)Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
575	GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24
184	GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
74	Fuca1-2Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
258	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
162	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
165	Galb1-4GlcNAcb1-3Galb1-4Glcb-Sp8
606	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
546	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
574	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24
545	GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp25
333	GalNAcb1-3Gala1-4Galb1-4GlcNAcb1-3Galb1-4Glcb-Sp0
567	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp25
541	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp24
582	GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAb1-2)Mana1-6(GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24
547	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp24
581	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAb1-2)Mana1-6(Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24
544	GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
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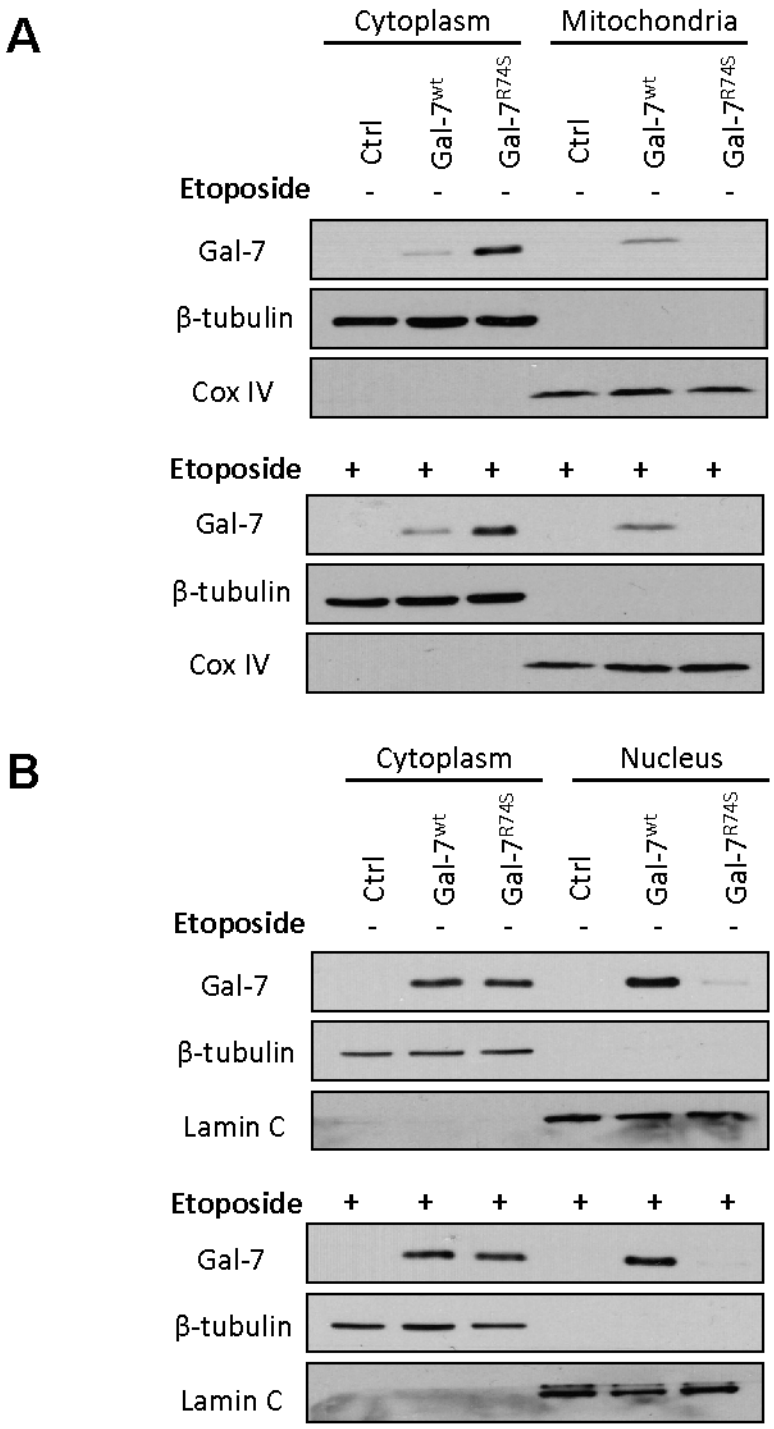
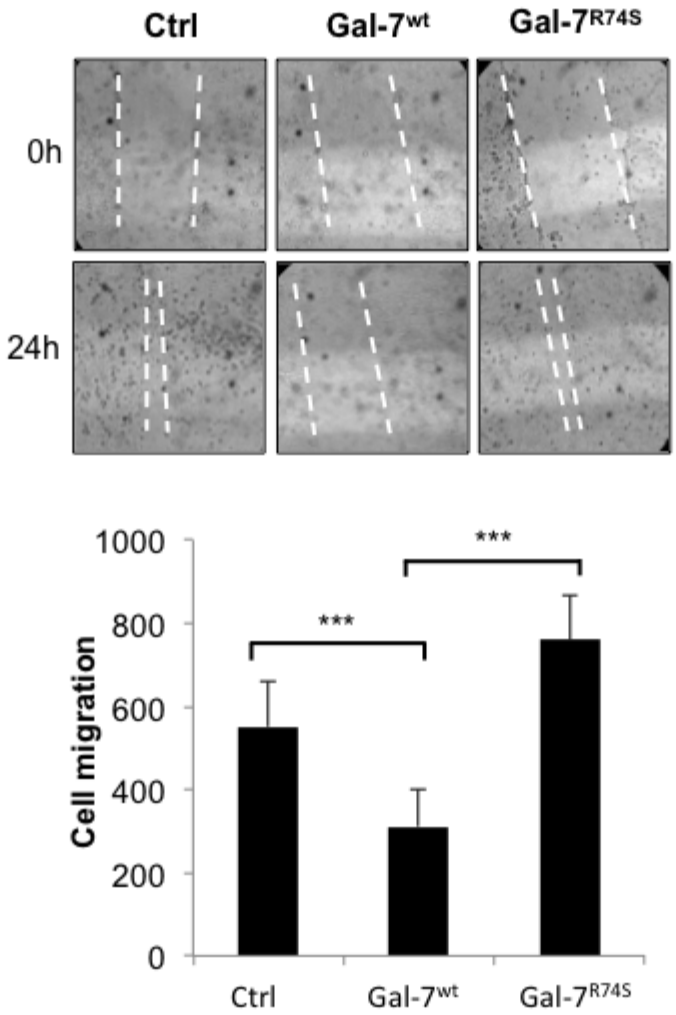
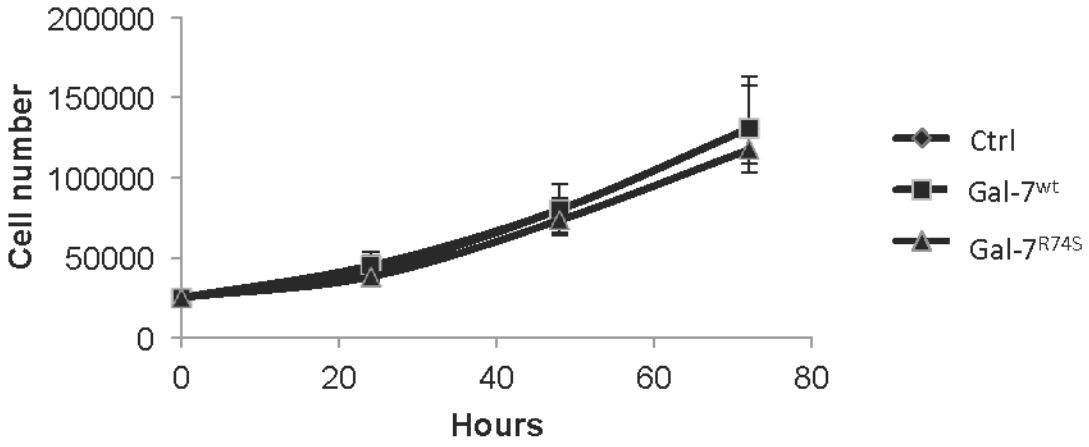


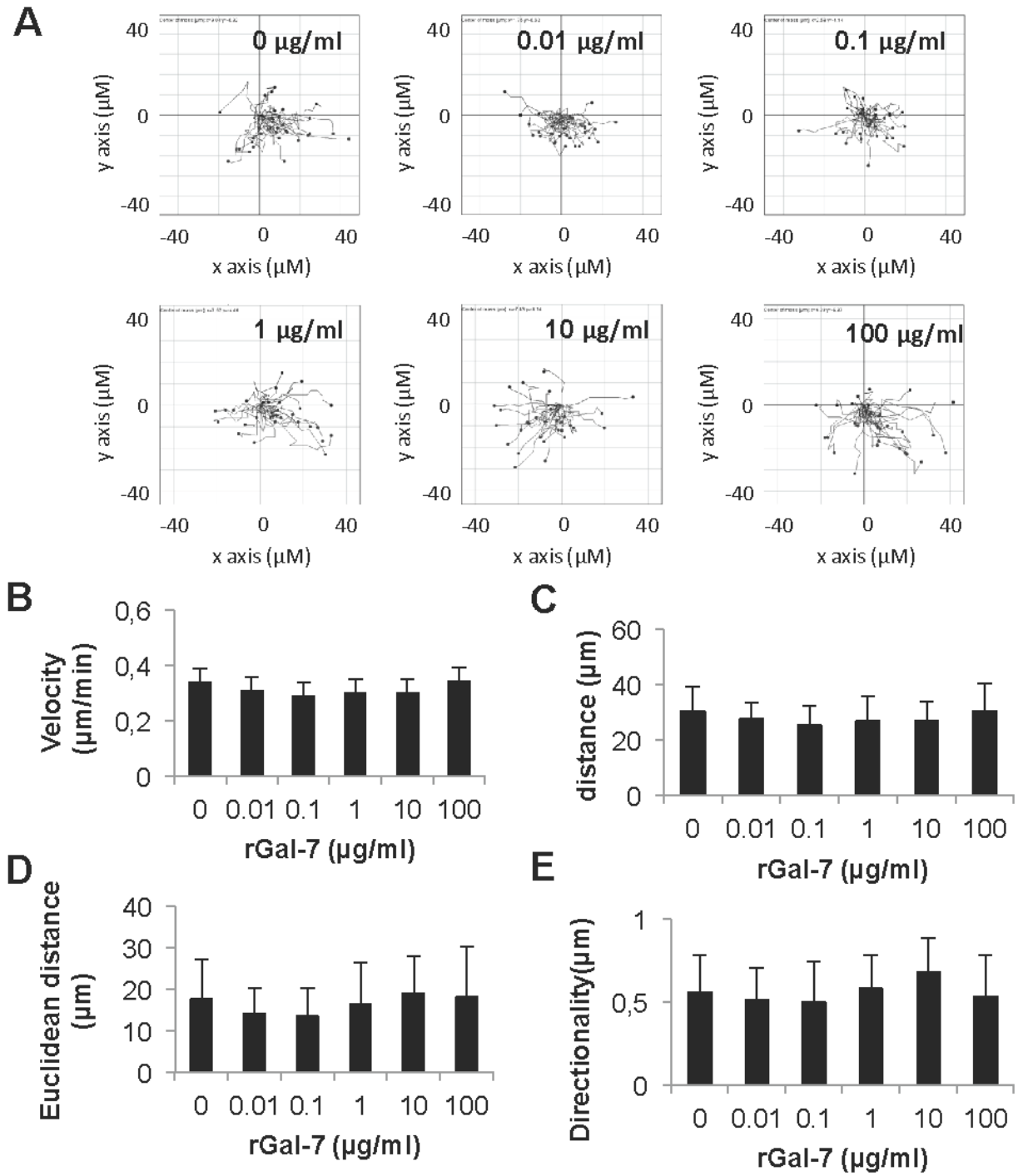
S2 Fig. Intracellular localization of gal-7 during apoptosis. Western blot analysis showing gal-7 expression in cytosolic, (**B**) mitochondrial and (**C**) nuclear fractions prepared from DU-145 cells treated for 16 h with 10 µM etoposide. β-tubulin, COX IV and laminin C were used as cytosolic, mitochondrial and nuclear markers, respectively. All results represent three independent experiments, including a minimum of two different DU-145 clones.



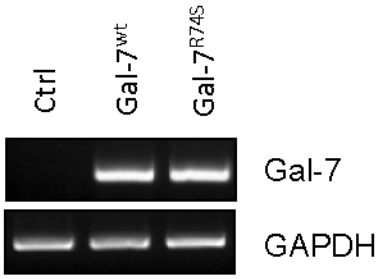
S3 Fig. Scratch wound healing assay of DU-145 transfectants. DU-145 transfectants were seeded into a 24-well plate. A scratch was made with a pipet tip, photos were taken at time 0 and at 24 h, and the width of the scratch was measured. The results represent the distances of the migration of the cells, and the bars represent the SDs. The results represent three independent experiments. ***P ≤ 0.001.



S4 Fig. Gal-7^{wt} and gal-7^{R74S} effects on cell proliferation. As a control for cell proliferation, cells were seeded into a 24-well plate and counted at the indicated time using trypan blue staining. The results represent three independent experiments.



S5 Fig. Extracellular gal-7 effect on cell motility. (**A**) Plots of 30 cells/sample tracked by live cell imaging during a scratch wound healing assay. DU-145 cells were seeded onto a 6-well glass-bottom culture plate and treated with the indicated concentration of gal-7^{wt}. A scratch was made, and images were captured every 10 min for 2 h. Quantifications of the (**B**) velocity, (**C**) accumulated distance, (**D**) Euclidean distance and (**E**) directionality are shown. The error bars represent the SEMs. All results represent three independent experiments, including a minimum of two independent DU-145 clones.



S6 Fig. Gal-7 mRNA expression in DU-145 tumors. NOD/SCID mice (n=6) were injected subcutaneously with DU-145 transfectants expressing the empty vector or gal-7^{wt}/ gal-7^{R74s}. On day 61, the mice were sacrificed, and the primary tumors were harvested and snap-frozen in liquid nitrogen. Tumor mRNA was extracted, and gal-7 expression was measured by RT-PCR. N=6 mice per group.

CHAPITRE 3

EXPRESSION ET FONCTIONS DE LA GALECTINE-7 DANS LE CANCER DE L'OVAIRE

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RÉSUMÉ

Il y a un besoin urgent de développer de nouvelles stratégies pour le diagnostic et le traitement du cancer de l'ovaire. Dans la présente étude, nous avons vérifié l'expression et la fonction de la galectine-7 (gal-7) dans les cellules du cancer épithélial de l'ovaire (EOC). Nous avons analysé par immunohistochimie l'expression de gal-7 dans des micromatrices tissulaires. Les résultats démontrent que gal-7 est absente de l'ovaire normal, mais qu'il est possible de détecter la présence cytoplasmique de la protéine dans tous les sous-types histologiques d'EOC. Son expression est plus fréquente dans les tumeurs de haut grade et dans les échantillons de métastases. L'expression de gal-7 corrèle significativement avec la survie des patientes atteintes d'adénocarcinomes séreux. De plus, en utilisant des lignées cellulaires d'EOC, nous avons découvert que l'expression de gal-7 est induite par des formes mutés de p53. Au niveau des mécanismes, des tests d'invasion au travers de matrigel et l'imagerie en temps réel indiquent que gal-7 induit le potentiel invasif des cellules cancéreuses de l'ovaire par l'induction de MMP-9 et par l'augmentation de la motilité. Les cellules EOC sécrètent également gal-7. La protéine gal-7 recombinante tue les cellules T Jurkat ainsi que les cellules T humaines du sang périphérique, suggérant que la gal-7 a aussi des propriétés immunosuppressives. En résumé, notre étude valide l'importance clinique de la surexpression de la gal-7 dans le cancer ovarien et suggère qu'elle pourrait être ciblée afin d'améliorer le pronostic des patientes atteintes de cette maladie.

CONTRIBUTION DES AUTEURS

Yves St-Pierre et moi avons décidé du plan expérimental. J'ai réalisé l'ensemble des expériences de cet article et participé à chacune d'entre elles. Andrée-Anne Grosset et Louis Gaboury ont participé au marquage immunohistochimique des micromatrices tissulaires et à leur évaluation. Maria Vladoiu a participé à la production de galectine-7 recombinante. Yves St-Pierre et moi avons écrit l'article et tous les auteurs ont révisé et approuvé le manuscrit final.

Expression and functions of galectin-7 in ovarian cancer

Marilyne Labrie[1], Maria Claudia Vladoiu[1], Andrée-Anne Grosset[1], Louis Gaboury[2] and Yves St-Pierre[1]

[1] INRS-Institut Armand-Frappier, Laval, Québec, Canada

[2] Institute for Research in Immunology and Cancer, Downtown Station, Montréal, Québec, Canada

Correspondence to: *Yves St-Pierre,* ***email:*** *yves.st-pierre@iaf.inrs.ca*

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ABSTRACT

There is a critical need to develop effective new strategies for diagnosis and treatment of ovarian cancer. In the present work, we investigated the expression of galectin-7 (gal-7) in epithelial ovarian cancer (EOC) cells and studied its functional relevance. Immunohistochemical analysis of gal-7 expression in tissue microarrays showed that while gal-7 was not detected in normal ovarian tissues, positive cytoplasmic staining of gal-7 was detected in epithelial cells in all EOC histological subtypes but was more frequent in high grade tumors and metastatic samples. Gal-7 expression correlated with a significant difference in the overall survival of patients with ovarian serous cystadenocarcinoma. Furthermore, using human EOC cell lines, we found that gal-7 expression was induced by mutant p53. Mechanistically, Matrigel invasion assays and live cell imaging showed that gal-7 increased the invasive behavior of ovarian cancer cells by inducing MMP-9 and increasing cell motility. EOC cells can also secrete gal-7. Recombinant human gal-7 kills Jurkat T cells and human peripheral T cells, suggesting that gal-7 also has immunosuppressive properties. Taken together, our study validates the clinical significance of gal-7 overexpression in ovarian cancer and provides a rationale for targeting gal-7 to improve the outcome of patients with this disease.

INTRODUCTION

Epithelial ovarian cancer (EOC) accounts for approximately 85–90% of total ovarian cancers and is the leading cause of gynecologic malignancies in North America and worldwide [1]. Despite improvements in various treatments, the 5-year survival of patients with advanced stage disease remains less than 40%. Largely, this is due to the fact that EOC tends to be diagnosed at advanced stages, the lack of effective treatments for patients and incomplete understanding of the molecular underpinnings of the disease progression. Therefore, it is essential to identify potential biomarkers and novel therapeutic targets that are involved in ovarian cancer initiation, progression and outcome to help improve clinical outcomes.

Members of the galectin family are recognized for their ability to bind β-galactosides via their carbohydrate recognition domain (CRD). They play an important role in several physiological processes including cell migration, programmed cell death and regulation of the immune response (as reviewed in [2]). They are also involved in a number of pathological conditions including cancer. In fact, galectins have been shown to induce systemic and local immunosuppression by killing immune cells. They can also bind to the surface of tumor cells to form cell surface lattices that stabilize expression of key growth factor receptors. In doing this, galectins increase cell growth, motility, and the overall invasive properties of cancer cells [3, 4]. However, so far most of the attention has been focused on galectin-1 and galectin-3, and we still know very little about how gal-7 expression affects cancer progression and its distinctive role. Unlike other galectins, gal-7 is strictly expressed in epithelial cells. In fact, this galectin is generally considered to be a marker of epithelial differentiation. It is present in normal epithelial cells of various tissues and is rarely expressed in any other cell types including hematopoietic cells, muscle cells, neurons,

Table I: Expression of gal-7 in ovarian tumors.

	Gal-7 expression		Total
	Positive (n=44)	Negative (n=68)	(n=112)
Normal	0 (0 %)	7 (100 %)	7
Adjacent	0 (0 %)	17 (100 %)	17
Benign serous	3 (50 %)	3 (50 %)	6
Benign mucinous	1 (8.33 %)	11 (91.77 %)	12
Bordeline serous	5 (83.33 %)	1 (16.67 %)	6
Bordeline mucinous	1 (100 %)	0 (0 %)	1
Serous	14 (43.73 %)	18 (56.25 %)	32
Mucinous	5 (71.43 %)	2 (28.57 %)	7
Endometrioid	8 (61.54 %)	5 (38.46 %)	13
Transitional cell	4 (80 %)	1 (20 %)	5
Clear cell	3 (75 %)	1 (25 %)	4
Granular	0 (0 %)	2 (100 %)	2

fibroblasts, adipocytes, or endothelial cells. While gal-7 expression in normal epithelial cells has been relatively well documented, we are just beginning to understand its role in cancers of epithelial origin. For example, in breast cancer we found that gal-7 was exclusively expressed in aggressive subtypes including HER2-positive and basal-like breast cancer [5]. We also found that gal-7 expression in cell lines that express low or undetectable levels of gal-7 resulted in increased metastasis to the lung and bone and larger osteolytic lesions [5]. This is in contrast to earlier reports showing that gal-7 is absent in some types of malignant cells and may sensitize cells to apoptosis induced by chemotherapeutic agents [6-9]. Thus, while prior studies cannot predict whether any changes in gal-7 expression will result in resistance to apoptosis in epithelial cancers, differential expression patterns in epithelial cancers may represent a helpful biomarker. Differential expression of galectins may facilitate differential diagnoses if it is correlated with stage/grade, especially in cases where a morphological diagnosis is controversial. Accordingly, because galectins are implicated in a wide range of diseases, intensive efforts have been employed to identify highly selective and potent galectin inhibitors. In the present work, we investigated expression of gal-7 in EOC cells and studied its functional relevance.

RESULTS

Gal-7 expression is increased in ovarian cancer tumors

To study the role of gal-7 in ovarian cancer, we first conducted an *in silico* analysis of *gal-7* gene expression in EOC cells using publically available datasets from the Gene Expression Omnibus (GEO) repository of the National Center for Biotechnology Information (NCBI). Interestingly, a microarray dataset (GDS1375) obtained from profiling a murine model of ovarian cancer indicated that while *gal-7* mRNA was rarely detectable at early and intermediate stages of transformation, it was expressed at significantly higher levels at later stages of transformation ($P<0.001$) (Fig. 1A) [10]. These data prompted us to assess gal-7 expression in human ovarian tissues by immunohistochemistry (IHC) using TMAs constructed from samples obtained from a cohort of 112 patients with different EOC histological subtypes and containing core samples from matched normal adjacent tissues (n=17) and normal (n=7) ovarian tissues. While we found no detectable expression of gal-7 in normal ovarian tissues, including the single layer of ovarian surface epithelium, gal-7 positive staining was detected in epithelial cells in all EOC histological subtypes (Table I and Fig. 1B-C). Positive staining for gal-7 was seen primarily in the cytoplasm of tumor cells (Fig. 1B). No correlation was found between expression of gal-7, the age of the patients or the stage of the disease (Supplementary Table S1).

However, we found that gal-7 was present more frequently in metastatic samples compared to non-metastatic samples (**P**<0.05) (Fig. 1D). Histopathological scoring further revealed that gal-7 expression was significantly higher in high grade, borderline and metastatic tumors when compared to benign tumors. Low grade tumors also had significantly lower scores than metastatic samples (Fig. 1C and E). Analysis of the public RNAseq datasets obtained from the cBio Cancer Genomics Portal (http://cbioportal.org) also showed a significant correlation (P<0.03) between overexpression of *gal-7* mRNA and a lower overall survival of patients with ovarian serous cystadenocarcinoma (Fig. 2A and Supplementary Table S2).

Gal-7 expression in ovarian cancer cells is induced by mutant forms of p53

High expression of gal-7 in cancer cells is somewhat paradoxical because gal-7 has generally been considered to be a pro-apoptotic protein under the control of p53 [11]. However, in breast cancer cells gal-7 can be induced by mutant p53 to promote cancer progression. These data may explain why gal-7 is often constitutively expressed

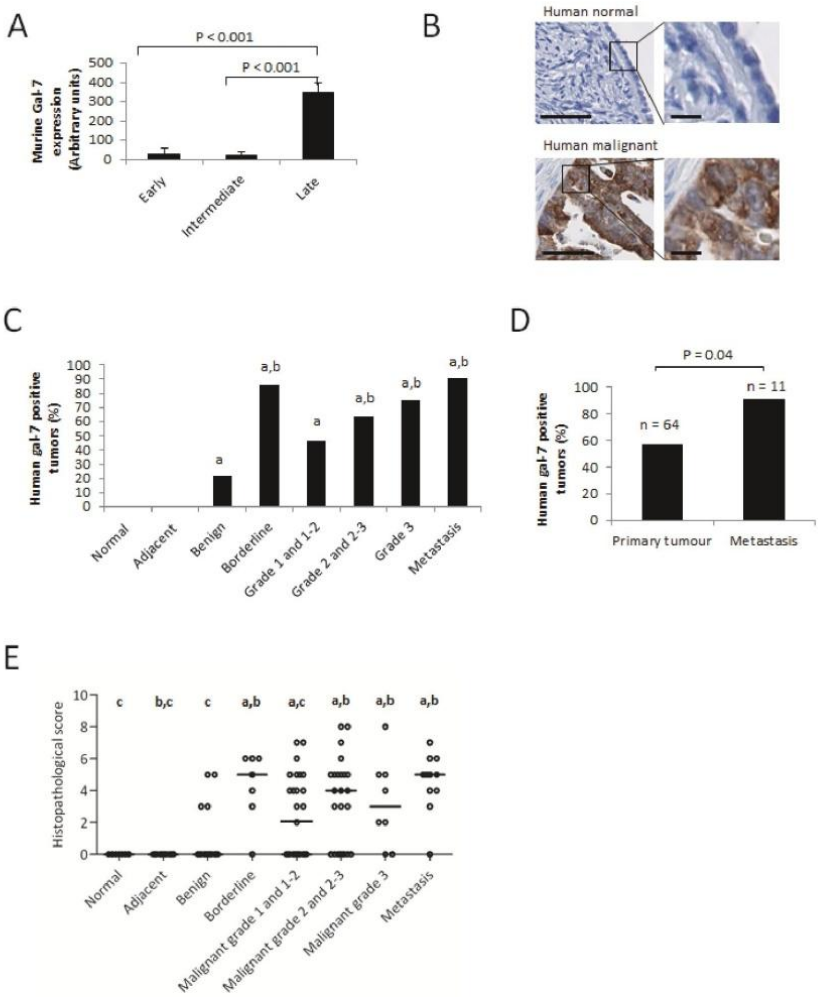


Figure 1: Expression of gal-7 in ovarian tissues. (A) mRNA expression of *gal-7* in EOC at different stages of transformation. The data were obtained from a publicly accessible gene profiling of an *in vitro* mouse model of EOC progression [10]. (B) Representative IHC staining of gal-7 expression in normal or malignant human ovarian tissue samples. Bars represent 50 µm (left panel) and 10 µm (right panel) (C) Percentage of gal-7 positive samples in different subtypes of human ovarian cancer. (D) Comparative analysis between the percentages of human non-metastatic primary tumors and metastatic samples expressing gal-7. (E) Differences in IHC scorings of gal-7 expression among different human ovarian cancer subtypes. Error bars represent SEM. a: $p \leq 0.05$ compared to normal tissue samples; b: $p \leq 0.05$ compared to benign tumors samples; c: $p \leq 0.05$ compared to metastatic samples.

in cancer cells harboring mutant forms of p53 [12]. To determine whether a similar scenario exists in ovarian cancer, we tested whether mutant forms of p53 that are commonly expressed in ovarian cancer could regulate gal-7 expression. According to the International Agency for Research on Cancer (IARC) database, mutations at codons His175, Met243 and Arg273 in the *p53* gene are the most frequently occurring mutations in ovarian cancer (Supplementary figure S1) [13]. Interestingly, OVCAR-3 cells, which harbor a $p53^{R248Q}$ mutation, readily express gal-7 (Fig. 2B). In contrast, gal-7 was not detected in ovarian cancer cells harboring a wild-type p53 (A2780 and COV434 cells) or cells with a $p53^{null}$ genotype (e.g., SK-OV-3) [13] Transfection of SK-OV-3 cells with expression vectors encoding p53 with mutations at codons 175, 273, and 248 all induced *de novo* expression of gal-7 at both the mRNA and protein level compared to cells transfected with a control (empty) vector (Fig. 2 C-D). The use of siRNA specific for p53 further support the idea that gal-7 is regulated by p53 mutants (Fig. 2E).

Where do we find gal-7 in ovarian cancer cells?

Our IHC staining in ovarian cancer tissues revealed that gal-7 was found mostly in the cytoplasm of cancerous cells. However, we found gal-7 in the supernatants of both OVCAR-3, and SK-OV-3 cells transfected with an expression vector encoding human gal-7 (Fig. 3A). The presence of gal-7 in the supernatants was confirmed by ELISA (with a range between 100 pcg/ml and 2.5 ng/ml). Confocal microscopy of permeabilized and non-permeabilized OVCAR-3 cells confirmed that endogenous gal-7 was also bound to the cell surface and in the

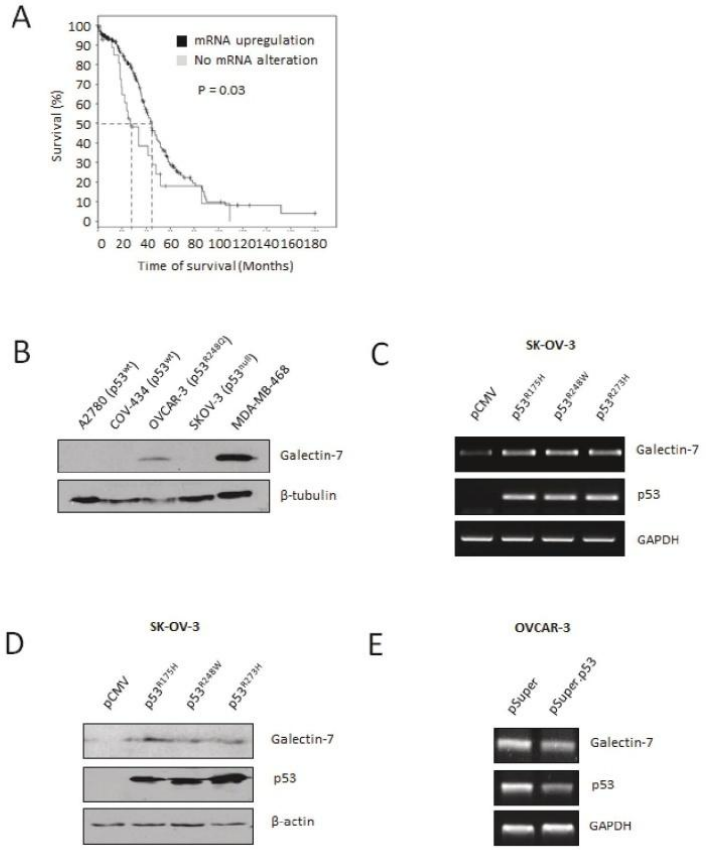


Figure 2: Gal-7 expression in EOC cells is associated to poor overall survival and is controlled by mutant forms of p53. (A) *In silico* analysis showing overall survival of ovarian serous cystadenocarcinoma cancer patients according to the mRNA expression level of *gal-7*. Data were obtained from the cBio portal RNAseq datasets [40]. (B) Western blot analysis of *gal-7* expression in commonly used human ovarian cancer cell lines. The human MDA-MB-468 basal-like breast cancer cells, which readily express gal-7, were used as a positive control [12]. β-tubulin was used as an internal loading control. (C and D) *De novo* expression of gal-7 as measured by RT-PCR and western blot respectively in SK-OV-3 transfected with expression vectors encoding mutant forms of human *p53*. An empty pCMV vector was used as a control for transfection. β-actin and GAPDH were used as internal loading controls. (E) RT-PCR analysis of *gal-7* mRNA expression in OVCAR-3 cells following suppression of endogenous p53 mutant. GAPDH was used as an internal loading control. Results are representative of three independent experiments.

cytoplasm (Fig. 3B). Similar data were obtained using SK-OV-3 cells transfected with an expression vector encoding gal-7 (Fig. 3C). Addition of β-lactose to the culture media decreased binding of gal-7 to SK-OV-3 cells, indicating that the binding was CRD-dependent. The ability of gal-7 to bind cell surface receptors of A2780 and SK-OV-3 cells was further confirmed by measuring the binding of FITC-labeled recombinant gal-7 by flow cytometry (Fig. 3 D-E). This binding was inhibited by the addition of β-lactose (Supplementary figure S2). Taken together, these results indicate that gal-7 exhibits dual localization in ovarian cancer cells, being found in both the extracellular compartment (cell surface and extracellular medium) as well as the intracellular compartment.

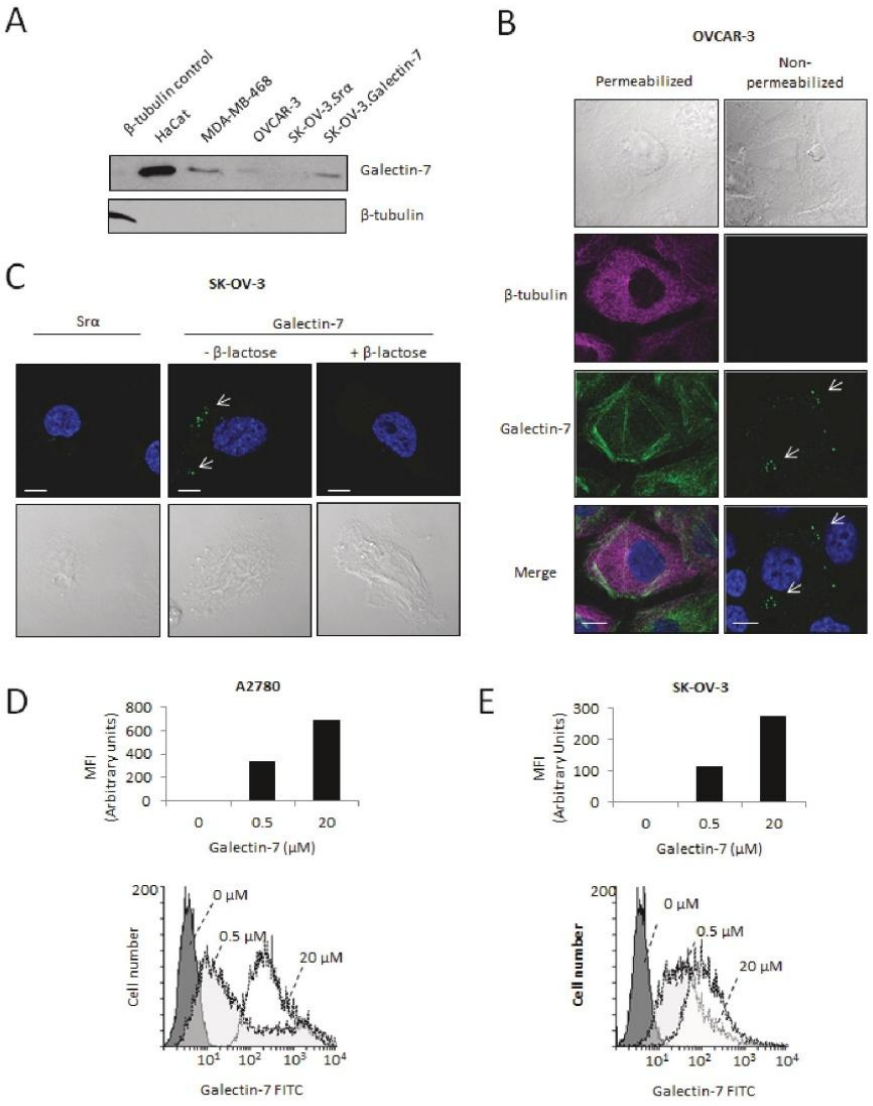


Figure 3: Release of gal-7 by ovarian cancer cell lines. (A) Western blot analysis of cell culture supernatants collected from ovarian cancer cells. The HaCat keratinocyte cell line and the MDA-MB-468 cells were used as positive controls. (B) Confocal microscopy imaging of gal-7 (green) in ovcar-3 cells. Gal-7 was detected by immunofluorescence in permeabilized or non-permeabilized cells to detect the intracellular and extracellular protein, respectively. Staining of β-tubulin (magenta) was used as a control to monitor permeabilization. (C) Binding of extracellular gal-7 to SK-OV-3 cells transfected with the empty Srα (control) vector or the same vector encoding the *gal-7* gene. Cells were incubated with or without 0.1M β-lactose to inhibit the gal-7 binding to glycosylated receptors. Nuclei were stained with DAPI (blue). Bar represents 10 μm. (D) Flow cytometry analysis showing binding of gal-7 to the surface of A2780 and (E) SK-OV-3 cells. Binding assays were conducted using the indicated concentrations of FITC-labeled recombinant gal-7. MFI; mean fluorescent intensities. Results are representative of three independent experiments.

Gal-7 increases the invasive behavior of ovarian cancer cells

Release of galectins in the tumor microenvironment has been shown to play a central role in cancer progression, most notably by increasing the invasive properties of cancer cells through lectin-dependent interactions (As Reviewed in [14]). To test whether this was the case for gal-7 in ovarian cancer cells, A2780 cells were treated with increasing concentrations of recombinant human gal-7 and tested for their invasive properties using a standard Matrigel invasion assay. We found that addition of gal-7 to A2780 cells induced a dose-dependent and significant increase in their invasive behavior ($P<0.001$) (Fig. 4A). Interestingly, this increase correlated with higher expression of MMP-9 (Fig. 4B). The use of GM6001, a broad range MMP inhibitor, significantly reduced the invasive behavior that was observed after treatment with recombinant gal-7 ($P<0.005$) (Fig. 4C). Inhibition of endogenous gal-7 expression by siRNA also induced a significant reduction in their invasive properties ($P<0.001$) (Fig. 4D-E). However, suppression of gal-7 did not correlate with reduced levels of MMP-9 (Fig. 4F), suggesting that gal-7 may also function independently of MMPs, or that other MMPs are involved.

Because gal-7 has been associated with the motility of mouse keratinocytes [15], we next investigated the effect of gal-7 on cell motility, using live cell video microscopy. We found that repression of gal-7 significantly reduced the velocity ($P<0.001$) and the accumulated migration distance ($P<0.001$) of OVCAR-3 cells without affecting the directionality or the Euclidean distance of migration (Fig. 4G). Interestingly, addition of recombinant gal-7 to A2780 cells had no effect on cellular motility, suggesting that intracellular rather than extracellular gal-7 is involved (Supplementary fig. S3). This possibility is real because gal-7 has previously been shown to co-localize with actin-rich structures (podosomes) implicated in cell motility [15]. Indeed, we found that gal-7 co-localized with cortical actin (Fig. 4H). Taken together, these results indicate that both intracellular and extracellular gal-7 increase the invasive behavior of ovarian cancer cells.

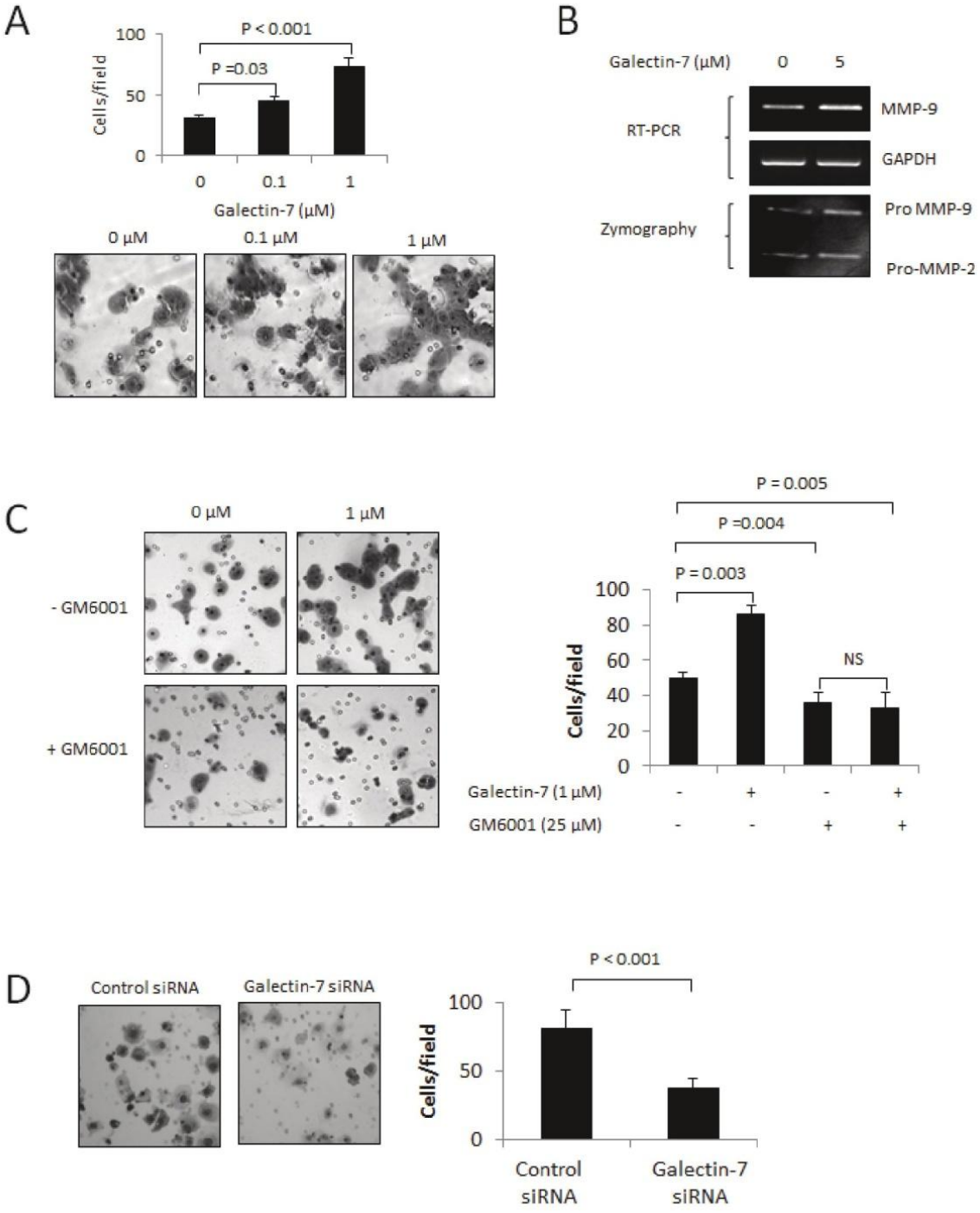
Extracellular gal-7 induces apoptosis of lymphocytes and monocytes

Galectins are well known for their ability to induce local immunosuppression within the tumor microenvironment. While this is well-known for galectin-1 and galectin-3, a recent report by Yamaguchi *et al.* suggests that this may well be the case for gal-7 [16]. We tested this possibility using Jurkat T cells, which do not express gal-7 mRNA or protein (Fig. 5A-B). Using FITC-labeled recombinant gal-7, we found that gal-7 bound to the surface of Jurkat T cells in a dose-dependent manner (Fig. 5C). This binding was inhibited by the addition of β-lactose and unlabeled recombinant gal-7, indicating that binding of gal-7 to Jurkat cells was dependent on its specific interaction with cell surface glycans (Fig. 5D-E). Flow cytometric analysis also revealed that binding of gal-7 to Jurkat T cells induced a significant increase in annexin V staining (Fig. 6A), suggesting that gal-7 triggers apoptosis of T cells. This hypothesis was supported by western blot analysis of PARP-1 cleavage (Fig. 6B). The effect of gal-7 on apoptosis was dependent on its lectin activity, as tested in experiments using lactose as an inhibiting sugar (Fig. 6C). Similar results were obtained using freshly isolated human PBMCs. Multiparametric analysis by flow cytometry showed that CD14-positive monocytes and CD4- or CD8-positive T cells all showed increased annexin V staining following incubation with recombinant gal-7 (Fig. 6D-H). Interestingly, although we found that gal-7 binds to the surface of ovarian cancer cells, it did not induce detectable apoptosis (Supplementary figure S4).

DISCUSSION

There is a critical need to develop effective new strategies for the treatment of patients with EOC. In the present work, we examined the clinical significance of gal-7 overexpression in ovarian cancer and investigated its potential as a therapeutic target for the treatment of ovarian cancer. More specifically, we found that while gal-7 is not detected in normal ovarian tissues, its expression is induced in benign, borderline and malignant tumors. In fact, analysis of histopathological scores indicated that its expression is significantly augmented in high grade, borderline and metastatic tumors compared to benign tumors. Moreover, overexpression of *gal-7* mRNA correlated with a poor overall survival of patients with serous cystadenocarcinomas, which account for the vast majority of EOC. We found that mutant forms of p53 most likely induce gal-7 expression in ovarian cancer cells, because *de novo* expression of the most common mutant forms of p53 induced gal-7, while suppression of p53 by siRNA reduces gal-7 expression. Finally, our *in vitro* data support the idea that gal-7 expression has the potential to impact ovarian cancer progression by increasing the invasive properties of ovarian cancer cells and by killing immune cells. Taken together, our study validates the clinical significance of gal-7 overexpression in ovarian cancer and provides a rationale for targeting gal-7 to improve the outcome of patients with this disease.

Mutations in the *p53* gene are among the most common genetic alterations found in cancer. In ovarian cancer, these mutations are observed in more than 90% of the cases and are more frequently observed in later stage tumors compared to early stage tumors [17-20]. In many cases, the presence of specific mutations is associated with a gain of function, often by inducing expression of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P < 0.001
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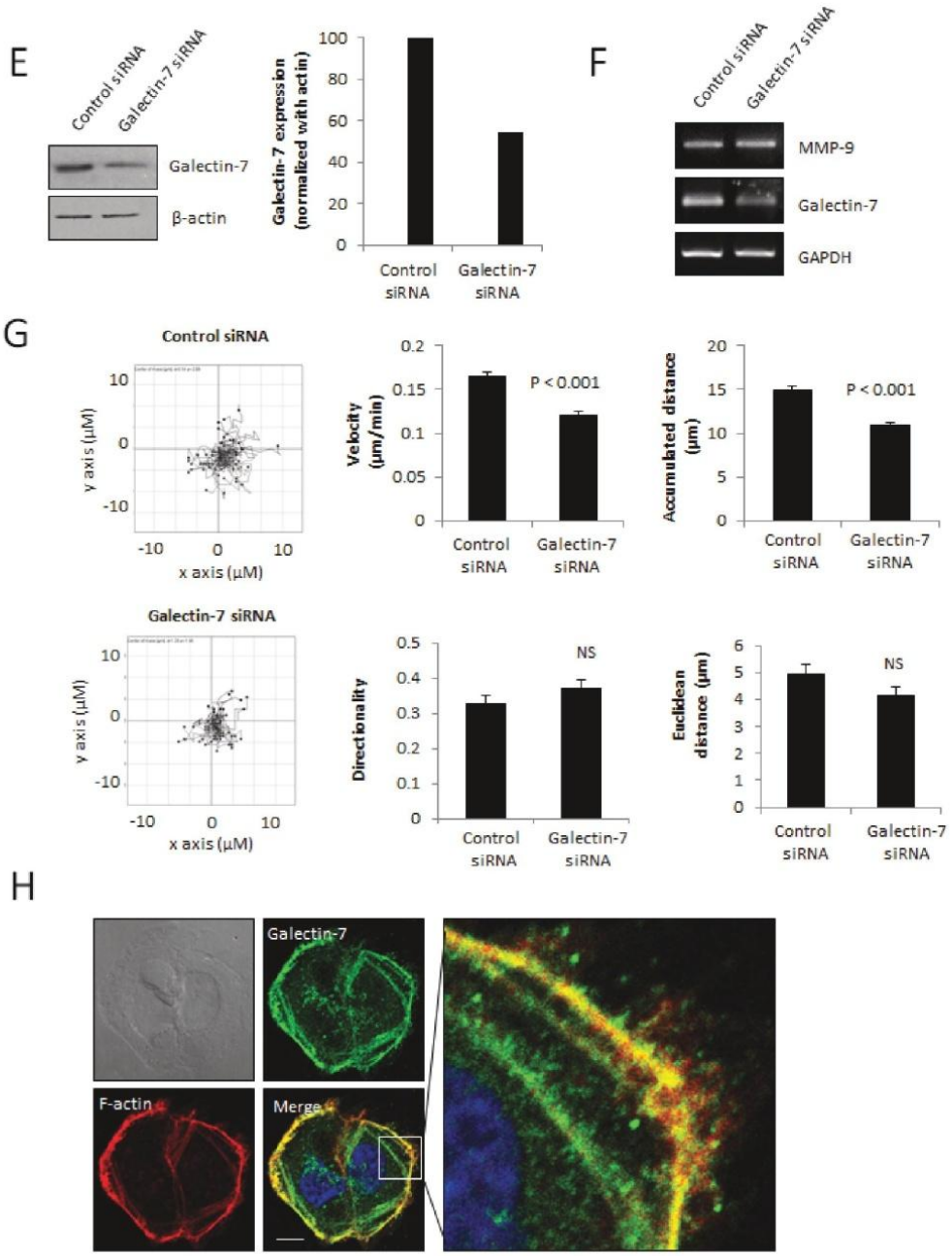


Figure 4: Gal-7 increases the invasive behavior of ovarian cancer cells. (A) Migration of A2780 cells through Matrigel in absence and presence of increasing concentrations of recombinant gal-7. (B) RT-PCR and zymography showing expression of MMP-9 by A2780 cells with or without 5 μM recombinant gal-7. (C) Effect of GM6001 on gal-7-induced Matrigel invasion of A2780 cells. (D) Migration of OVCAR-3 cells through Matrigel following suppression of gal-7. (E) Western blot analysis showing specific gal-7 suppression by the siRNA. (F) Effect of gal-7 suppression on *MMP-9* mRNA expression and (G) *in vitro* cell motility. Velocity, accumulated distance, Euclidean distance and directionality were measured on individual OVCAR-3 cells (n = 60) tracked by live cell imaging using a scratch wound healing test. Images were captured every 10 min for 2 h. Error bars represent SEM. (H) Intracellular distribution of gal-7 inside OVCAR-3 cells. Gal-7 (green) and F-actin (red) were detected by immunofluorescence and visualized by confocal imagery. Nuclei were stained with DAPI (blue). Bar represents 10 μm. Results are representative of three independent experiments. Error bars represent SD except for (G) which are SEM.

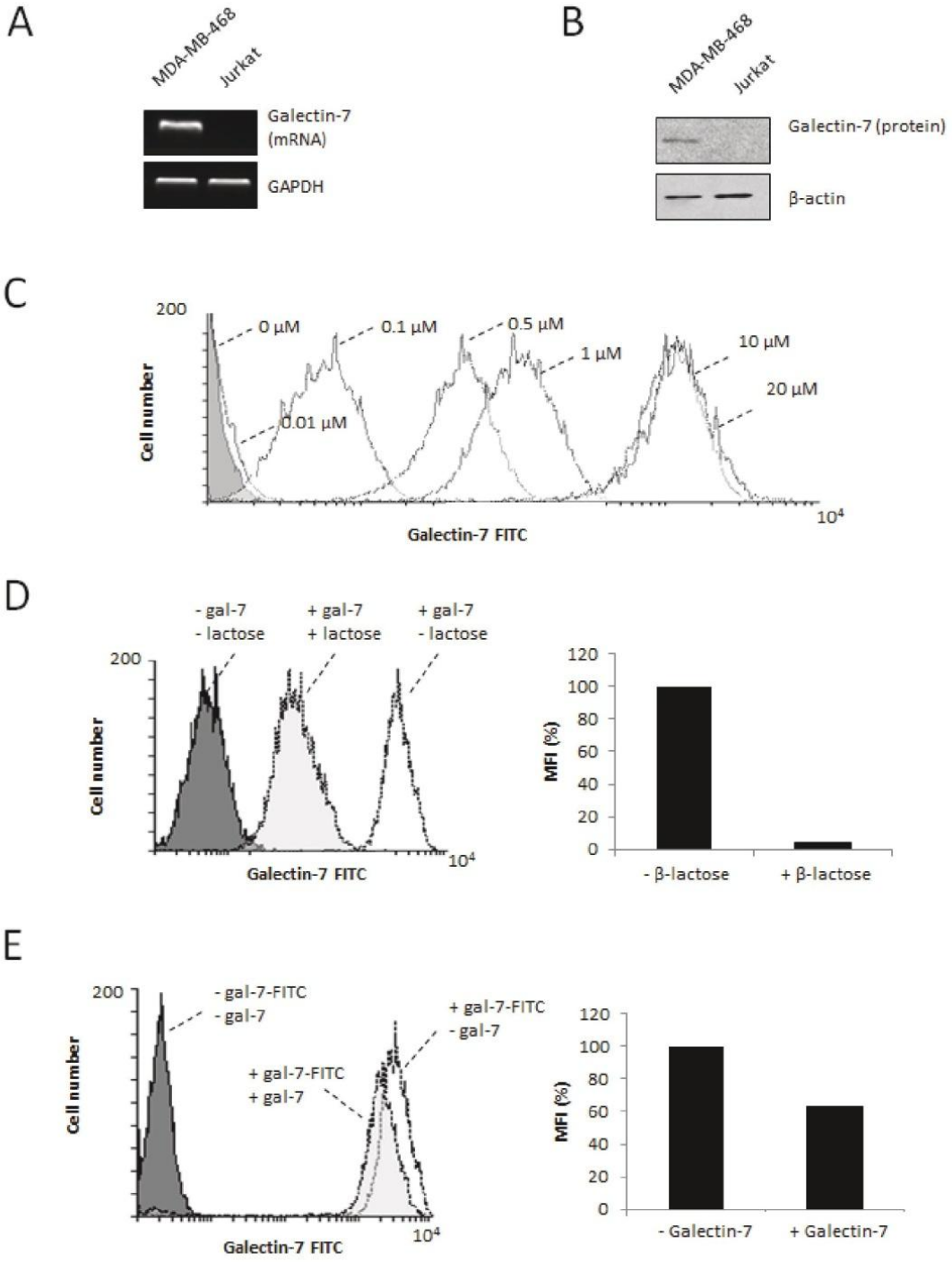
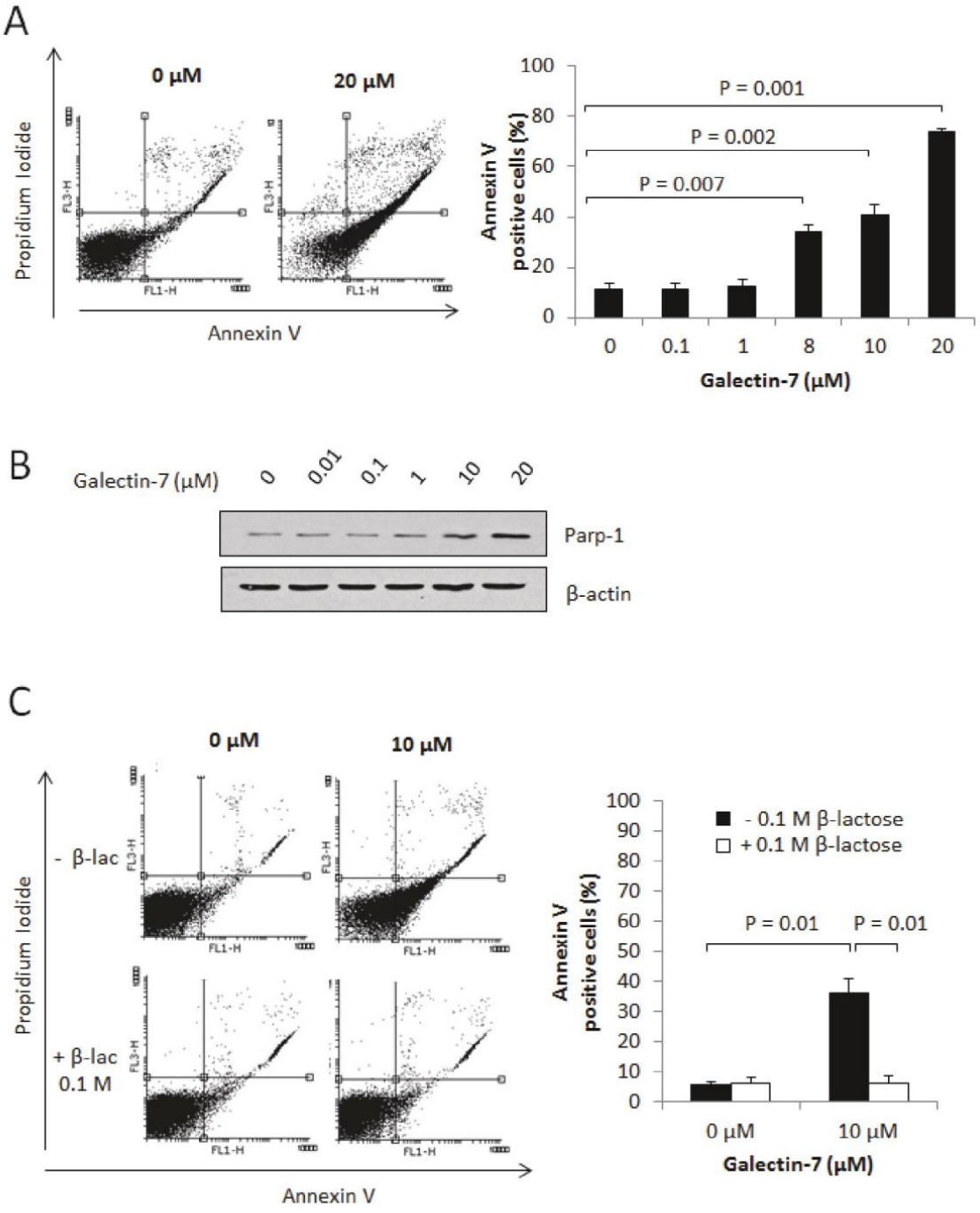


Figure 5: Binding of recombinant gal-7 to Jurkat cells. RT-PCR (A) and western blot (B) analysis of gal-7 expression in Jurkat cells. (C) Binding of gal-7 to the surface of Jurkat T cells. Increasing concentrations of recombinant FITC-labeled gal-7 were added to Jurkat T cells. Binding was measured by flow cytometry after 30 min of incubation. In some experiments, binding was measured in presence or absence of (D) 0.1 M β-lactose or (E) 20 μM of non-conjugated (cold) recombinant gal-7. Results are representative of three independent experiments.


A
0 µM
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P = 0.001
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Galectin-7 (µM)
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Galectin-7 (µM)
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Parp-1
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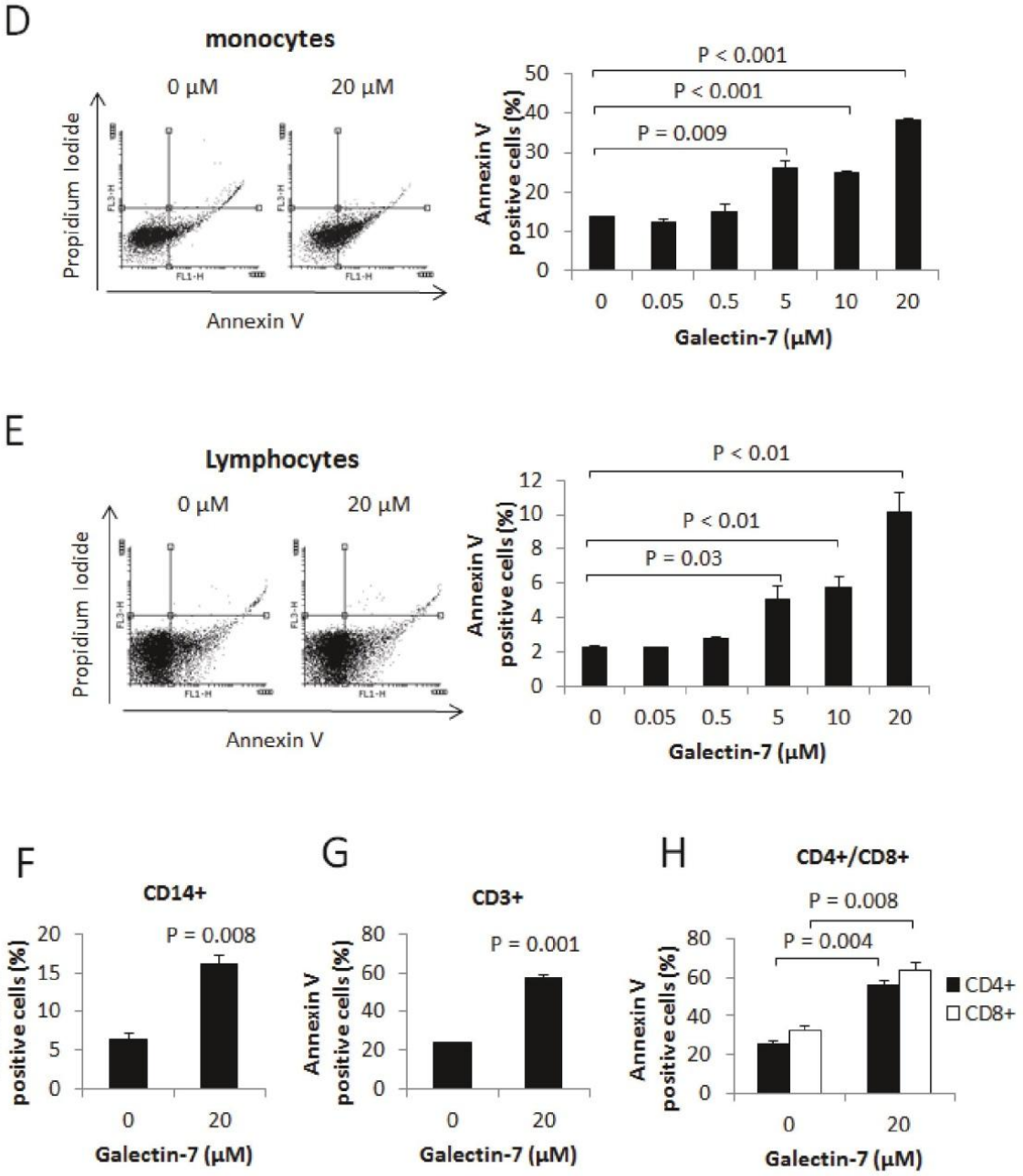

Figure 6: Gal-7 induces apoptosis of T cells. Jurkat cells were treated for 4 h with increasing concentrations of recombinant gal-7. Apoptosis was monitored by measuring (A) Annexin V staining and (B) Parp-1 cleavage. (C) Effect of β-lactose on gal-7 induced apoptosis. (D-E) Freshly isolated PBMCs were collected from healthy individuals and treated for 4 h with increasing concentration of recombinant gal-7. Apoptosis was measured on gated populations of monocytes and lymphocytes and on cell populations expressing cell surface receptors (F-H). Cells in the lower right quadrant are representative of annexin-positive, early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells. The percentage of cell death was obtained by adding the percentage of cells found in the lower and the upper right quadrants. Results are representative of three independent experiments. Error bars represent SD.

genes involved in various aspects of cancer biology [21]. However, the mechanism by which specific mutant forms of p53 can induce gal-7 in ovarian cancer cells is currently unknown. Our findings suggest that gal-7 might in fact be part of a gain-of-function pathway that is frequently observed in many types of cancer [21]. This possibility is supported by our data showing that gal-7 may promote cancer progression by increasing the invasive properties of ovarian cancer cells. While we found that recombinant gal-7 had no effect on cell motility, we found that repression of gal-7 decreased the velocity and accumulated distance of migration of OVCAR-3 cells. These data suggest that ovarian cancer cell motility was modulated by intracellular gal-7, which co-localized with the actin cytoskeleton within cortical actin. This finding is reminiscent of a previous report by Gendronneau *et al.* [15], who showed that keratinocytes isolated from gal-7 KO mice had reduced motility when compared to normal keratinocytes. They had also found that gal-7 was localized in the podosomes of these cells, indicating that gal-7 is involved in organization of the actin cytoskeleton. Such a role has been well documented for galectin-1, which binds to the actin cytoskeleton and participates in actin polymerization-depolymerization by modulating the activity of small GTPase [22, 23]. Alternatively, release of gal-7 in the tumor microenvironment by EOC cells may favor establishment of a local immunosuppressive response by killing immune cells. This possibility has been reported in the case of galectin-1, which is also overexpressed in EOC [24]. Galectin-1 is indeed released by SK-OV-3, but not OVCAR-3 cells [25]. Galectin-3 is also expressed in EOC, although it is mainly found inside the cells and does not vary among benign, borderline and malignant mucinous and serous tumors [26]. In fact, it is becoming clear that many cell types do express more than one intracellular galectin, suggesting existence of functional redundancy. For example, recombinant galectin-1, -3, -8 and -9 have all been shown to induce apoptosis in Jurkat T cells [16, 27-30]. However, in contrast to galectin-3, we found that killing of Jurkat T cells by gal-7 is independent of CD45 (Supplementary Fig. S5). This suggests that expression of galectins by EOC may induce apoptosis of specific infiltrating immune cell populations depending on their fine carbohydrate specificities [8, 31]. Such redundancy is often necessary to increase the maintenance of important gene functions and to limit losses following mutations/deletions of specific genes (functional compensation). Future *in vivo* investigations are required to determine how the "galectinome" of EOC carves development of local immune suppression and whether development of CRD-specific galectin inhibitors is an attractive avenue for the treatment of EOC.

Although relatively high concentrations of soluble gal-7 were required to trigger apoptosis or cell invasion *in vitro*, the concentrations we used for our *in vitro* studies, however, were comparable to concentrations commonly used to study galectin-apoptosis in T cells *in vitro*. Such range of concentrations are not unexpected since *in vitro* studies are usually carried out using recombinant galectins that display suboptimal biological activity [32]. It is also important to note that extracellular galectins bind to a relatively large number of cell surface glycoprotein receptors [33], which is known to concentrate galectins by forming spatial gradients that allow them to exert their biological activity following binding to their respective receptors localized on responding cells [34, 35]. In fact, total galectin concentrations in tissues can reach up to 70-80 mg/kg tissue or approximately 1-2 μM [36]. Thus, miroenvironmental concentrations of gal-7 in ovarian cancer could be quite high, and may explain, at least in part, why galectins are now considered as key mediators of development of distant metastasis through induction of local and systemic immunosuppression [37-39]. It will be interesting to determine whether gal-7 is found in ascites of patients with ovarian cancer and whether levels of gal-7 correlate with disease progression, remission and/or response to treatment.

It is becoming clear that gal-7 plays a central role in cancer of epithelial origin. There are now numerous reports showing that gal-7 can modulate the progression of many types of carcinoma. This has been well established in breast cancer cells. In fact, there is increasing evidence that targeting galectins in cancer may help to reduce immunosuppression during metastatic progression of the disease [38]. In EOC, our findings add to a recent report by Kim *et al.* who showed that gal-7 was associated with a lower survival rate of Korean patients with EOC [26]. While the authors reported that gal-7 expression in tissue samples was primarily detected in the nuclei and occasionally in the cytoplasm, our IHC, confocal microscopy and western blot analysis suggest that extracellular gal-7 is released outside the cells and may have a significant impact on tumor progression by inducing immunosuppression and increasing the invasive behavior of tumor cells. Thus, these functions may explain Kim's findings and ours showing that gal-7 is associated with metastasis and poor overall survival. Taken together, these studies provide proof-of-principle that targeting gal-7 may represent a valuable strategy to overcome cancer-associated immunosuppression and dissemination of metastasis in EOC, restrain tumor growth and prevent metastatic disease.

MATERIAL AND METHODS

Cell lines and reagents

The COV-434, HACAT and MDA-MB-468 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM). The A2780, Jurkat and OVCAR-3

cells were maintained in RPMI 1640 medium, and the SK-OV-3 cell line was maintained in McCoy's 5A medium. The culture medium were supplemented with 10% [v/v] fetal bovine serum, 2 mmol/L l-glutamine, 10 mmol/L HEPES buffer, and 1 mmol/L sodium pyruvate. All cell culture products were purchased from Life Technologies (Burlington, ON, Canada).

Vectors and transfections

The vectors encoding mutant p53 (R175H, plasmid 16436; R273H, plasmid 16439; R248W, plasmid 16437) were obtained from Addgene (Cambridge, MA, USA). pSuper and pSuper-p53 siRNA vectors were kindly provided by Dr. Reuven Agami (The Netherlands Cancer Institute, Amsterdam, Netherlands). The empty pCMV vector was produced by excising the p53 cDNA from the $p53^{R175H}$ vector using the EcoRI restriction enzyme. Control siRNA and gal-7 siRNA were purchased from Santa Cruz (Dallas, Texas, USA). cDNA encoding the human *gal-7* gene has been previously described [39]. For transfection, the cells were plated at equal densities 24 h prior to transfection and then transfected with the indicated vectors or siRNAs using the Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol.

Immunohistochemistry

Ovarian tissue microarrays (TMA) were purchased from Lifespan Biosciences (Seattle, WA, USA) and US Biomax (Rockville, MD, USA). Immunostaining reactions for gal-7 were performed using the Discovery XT automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA). Deparaffinized sections were incubated in cell conditioning 1 (pH 8.0) buffer for antigen retrieval and then stained for 60 min with the anti-human gal-7 polyclonal antibody (R&D Systems, Minneapolis, MN) using a 1:150 dilution. The slides were counterstained with hematoxylin and scanned at a high resolution using the Nanozoomer Digital Pathology (Hamamatsu, Bridgewater, NJ). The percentage of staining was scored from 0 to 4 according to the percentage of positive cells displaying gal-7 expression within a sample ($0 = 0\%$; $1 \leq 25\%$; $2 \leq 50\%$; $3 \leq 75\%$; $4 \leq 100\%$). The intensity of staining was also scored from 0 to 4, with a score of 0 representing no staining and a score of 4 representing the strongest staining observed. Histological scores were calculated by adding both scores.

Immunofluorescence

Cells were fixed in paraformaldehyde 3% [w/v] for 15 min, permeabilized in PBS/Triton X-100 0.1% [v/v] for 5 min and blocked 30 min at 4°C in 1% [w/v] PBS/BSA. Goat anti-human gal-7 (dilution 1:100), rabbit anti-β-tubulin (dilution 1:100, New England Biolabs), rabbit anti-goat Alexa Fluor 488 (dilution 1:500, Life Technologies) and donkey anti-rabbit Alexa Fluor 647 (dilution 1:500, Life Technologies) antibodies were used. Filamentous actin was stained with Alexa Fluor 594 conjugated-phalloidin (dilution 1:500, Life Technologies). All antisera were diluted in PBA and all washing steps were performed with PBS. Nuclei were stained with ProLong Gold Antifade Reagent with DAPI (Life Technologies). Cells were visualized with a LSM 780 laser-scanning microscope (Zeiss, Jena, Germany).

RNA isolation and RT-PCR

Total cellular RNA was isolated from cells using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 µg of cellular RNA in a total reaction volume of 20 µL using the reverse transcriptase Omniscript (QIAGEN, Mississauga, ON, Canada). After reverse transcription, the human *gal-7* (gene ID 3963, forward primer: 5'- TCC CAA TGC CAG CAG GTT CCA TGT -3' and reverse primer: 5'- GAA GCC GTC GTC TGA CGC GAT GAT-3'), *TP53* (gene ID 3963, forward primer: 5'- CCAGCCAAAGAAGAAACCAC-3' and reverse primer 5'- TATGGCGGGAGGTAGACTGA-3'), *MMP-9* (gene ID 4318, forward primer 5'- caacatcacctattggatcc-3' and reverse primer 5' cgggtgtagagtctctcgct-3') and *GAPDH* (gene ID 2597, forward primer: 5'- CGG AGT CAA CGG ATT TGG TCG TAT-3' and reverse primer: 5'- CAG AAG TGG TGG TAC CTC TTC CGA -3') cDNAs were amplified using the following conditions: 94ºC for 3 min, followed by 30 to 35 cycles of the following: 94°C for 40 sec, 60°C for 40 sec, and 72°C for 40 sec, followed by a final extension step at 72°C for 10 min. PCR was performed in a thermal cycler (MJ Research, Watertown, MA). The amplified products were analyzed by electrophoresis using 1.5% [w/v] agarose gels, SYBR Safe (Life Technologies) DNA gel staining and UV illumination.

Western blotting and ELISA analysis

For whole cell extracts, cells were homogenized and resuspended in RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitors (Roche, Laval, QC, Canada). For extracellular media, cells were cultured to confluence in 12-wells plates in 600 µl of media for 48 h. Media was centrifuged at 3,000 rpm for 10 min to remove cell debris. Equal amounts of whole-cells extract (20 to 60 µg) or extracellular media (40 µl) were separated on SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad Laboratories,

Mississauga, ON, Canada). The membranes were first blocked with 5% milk [w/v] in TBS/0.5% Tween 20 [v/v] for 60 min at room temperature and subsequently blotted overnight in a solution of TBS containing 3% BSA [w/v] and 0.5% Tween 20 [v/v]. The following antibodies were used: a goat anti-gal-7 polyclonal antibody (1:1000), a rabbit anti-Poly-(ADP-ribose) polymerase (PARP)-1 (p25) polyclonal antibody (1:5000; Epitomics, Burlingame, CA, USA), a rabbit anti-p53 (1:1000; Santa Cruz), a mouse anti-β-actin (1:20000; Sigma-Aldrich, St. Louis, MO, USA) and a rabbit anti-β-tubulin (1:1000) polyclonal antibodies. Secondary antibodies consisted of horseradish peroxidase-conjugated donkey anti-rabbit (GE Healthcare, Buckinghamshire, England), donkey anti-goat (R&D Systems) or sheep anti-mouse (GE Healthcare) IgG. Detection was performed using the enhanced chemiluminescence method (GE Healthcare). For extracellular media, an ELISA was performed according to the manufacturer's recommendations (Ray Biotech, Norcross, GA, USA).

Gelatin zymography

A confluent monolayer of cells was seeded on a 24-well plate in 300 µl serum-free RPMI medium. The supernatant was harvested after 24 hr and centrifuged at 3,000 rpm to remove cell debris. To increase the protein concentration, 200 µl of media was concentrated by centrifugation for 1 h at 60ºC in a SpeedVac system. A total of 40 µl was then electrophoresed under non-reducing conditions using 7.5% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Merck, Darmstadt, Germany). To remove SDS after the electrophoresis, the gel was washed twice for 1 h in a solution containing 50 mM Tris, 5 nM CaCl2, 0.2% [v/v] NaN_3 and 2.5% [v/v] Triton X-100. After an overnight incubation in the same buffer containing only 1% [v/v] Triton X-100, the gels were stained with Coomassie brilliant blue and destained in 45% methanol 15 % acetic acid [v/v]. Gelatinolytic activity was identified as a clear band on a blue background. The lowest band (72 kDa) corresponds to MMP-2, the highest band (92 kDa) to MMP-9.

Production of recombinant gal-7

Gal-7 cDNA was cloned into pET-22b(+) using NdeI and HindIII restriction enzymes. The protein was produced in *E. coli* BL21 cells (DE3) at 37°C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) was added to the bacteria culture at an OD_{600nm} = 0.6-0.7 and the bacteria were further incubated for 4 h. Bacterial pellets were resuspended in lysis buffer (0.7 mg/mL lysozyme, 10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail), incubated for 1 h at 37°C and centrifuged for 30 min at 15,000 x *g* (4°C). The supernatant was then filtered and applied to a lactose-agarose column and the protein was eluted in 1 mL fractions with 150 mM lactose solution. Purified fractions were analyzed by SDS-PAGE. Gal-7 was dialyzed against 20 mM potassium phosphate at pH 7.2 for all subsequent experiments.

FITC conjugation and gal-7 binding assay

Briefly, 10 µl of a 2 mg/ml fluorescein isothiocyanate (FITC)/DMSO solution was added to 300 µl of 1.7 µg/µl recombinant gal-7 in a 0.1 M $NaHCO_3$ pH 9.2 solution and incubated for 2 h at room temperature on a roller. FITC-conjugated gal-7 was then purified using a PD-10 Sepharose column (GE healthcare) and eluted with PBA containing 0.01% [v/v] sodium azide. To measure FITC-gal-7 binding to the cell surface, 2.5 x 10^5 cells were incubated for 30 min with the indicated concentrations of gal-7 and then washed twice with PBA and resuspended in 500 µl PBA. For the competition assays, 0.1 M β-lactose or 20 µM non-conjugated gal-7 were added to the cells at 4 °C for 30 min prior to the addition of FITC-conjugated gal-7. The samples were analyzed using a FACSCalibur (BD Biosciences) and the Flowing Software.

Annexin V/PI staining

Apoptosis was measured by flow cytometry using Annexin V conjugated with Alexa Fluor 488 (Life Technologies) and propidium iodide. Briefly, 2.5 x 10^5 cells were incubated with different concentrations of recombinant gal-7 at 37°C for 4 h. The cells were washed once in PBS and once in binding buffer (0.01 M HEPES, 0.14 M NaCl, 2.5 mM $CaCl_2$, pH 7.4). Subsequently, the cells were stained for 15 min with Annexin V (2.5 µl Annexin V in 50 µl binding buffer) in the dark at room temperature. A total of 400 µl of binding buffer containing 0.25 µg/ml propidium iodide was added to the cells before analysis by flow cytometry.

PBMCs isolation and characterization

Monocytes and lymphocytes were isolated from 30 ml heparinized blood. The blood was diluted 1:1 with ice-cold 2% (v/v) FBS-PBS and layered onto a Lymphoprep density gradient solution (Stemcell Technologies, Vancouver, BC, Canada) before centrifugation at room temperature for 20 min at 800 x *g*. The PBMC interphase was washed twice in RPMI 1640 and resuspended in RPMI-1640 containing 10% (v/v) FBS. Immunophenotyping of monocytes and T cell subpopulations were performed by membrane immunofluorescence staining of cell surface markers using the following antibodies: mouse anti-human

phycoerythrin (PE)-conjugated CD14, mouse anti-human allophycocyanin (APC)-conjugated CD3, mouse anti-human CD4 PE-conjugated, mouse anti-human APC-conjugated CD8. Each analysis was carried out with at least 10,000 cells. All antibodies were purchased from BD Biosciences.

Invasion assay

Serum-induced cell invasion was examined using a 24-well Matrigel invasion chamber with 8-µm pores membrane. A total of 2 x 10^5 cells were incubated in the upper chamber in serum-free medium. The lower chamber contained 10% [v/v] FBS. After 24 h, the upper surface of the insert was wiped gently with a cotton swab to remove non-migrating cells. Cells that had migrated to the lower surface of the membrane were stained with 1% borax/1% toluidine blue [w/v] and counted separately under a microscope. To inhibit endogenous extracellular proteases, the same volume of GM 6001 negative control solution (Santa Cruz) or GM 6001 (Santa Cruz) was directly added into the upper chamber and incubated with cells for the whole duration of the experiment.

Live cell imaging

One day prior to the experiment, a confluent monolayer of cells was seeded into a 6-well glass bottom culture plate (MatTek Corporation, Ashland, MA, USA). A scratch with a pipette tip was made within the cell monolayer, followed by a wash with PBS to remove cell debris. The plate was moved to an incubator PM S1 and migration was visualized with a LSM 780 laser scanning microscope. Images were captured every 10 min for the indicated time. For each condition, the movement of 30 to 60 different cells was recorded. Cell movement was analyzed using the Image J plugins manual tracking and chemotaxis tool.

Statistical analysis

Statistical significance of the experiments was evaluated using the unpaired Student's t-test or the Fisher's exact test. Results were considered statistically significant at $P \leq 0.05$.

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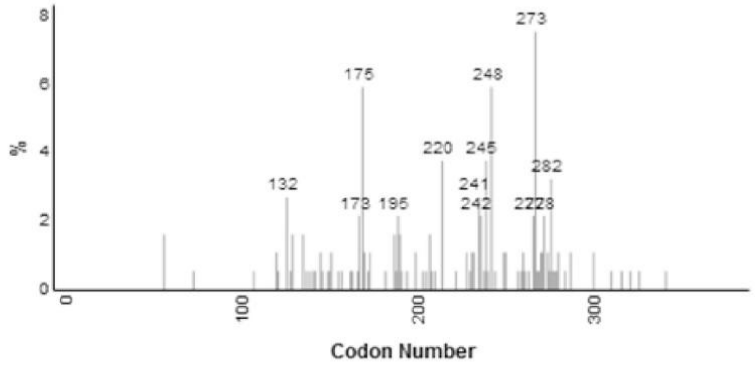
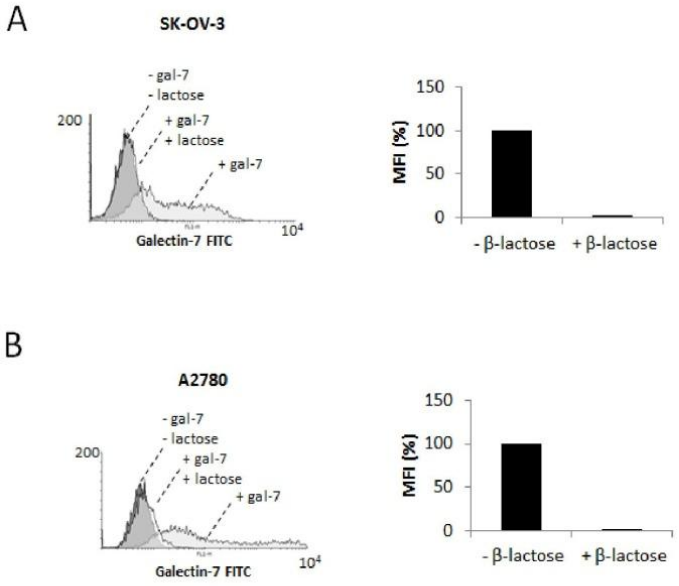
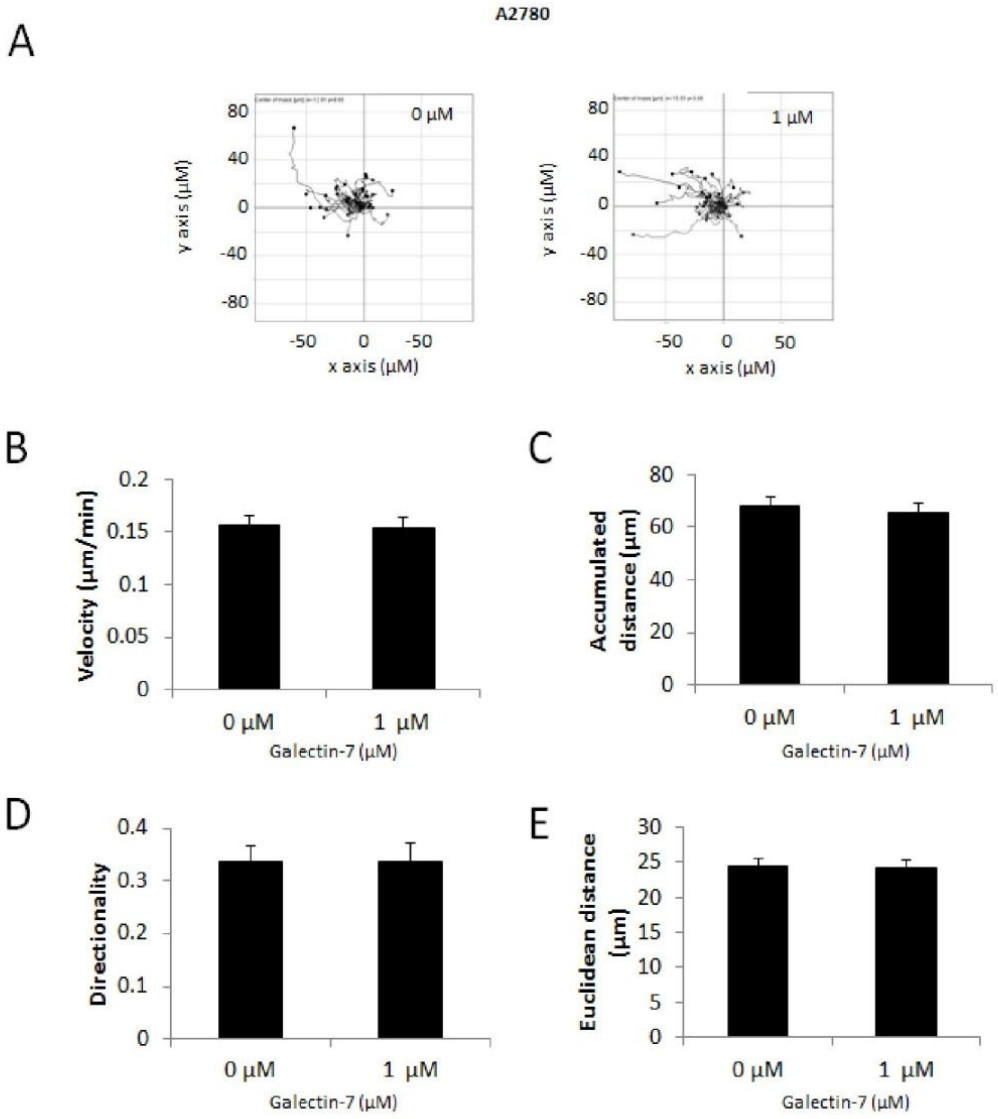
Supplementary Material



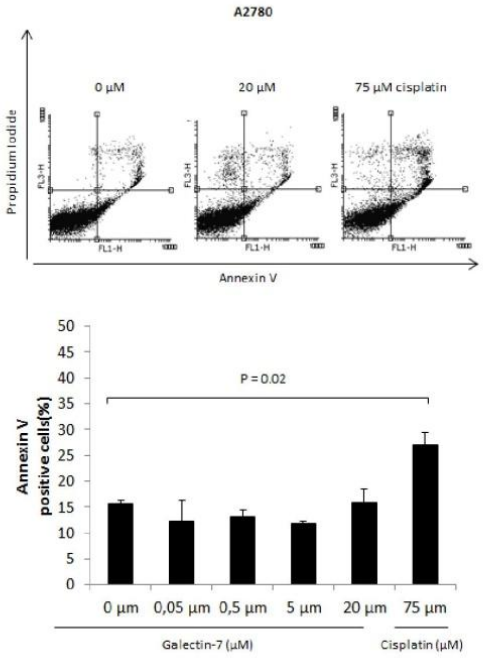
Supplementary figure S1: Must common p53 mutations in ovarian cancer cells. Frequency and codon distribution of p53 mutation among ovarian cancer cells, according to IARC database (n=186) [14].



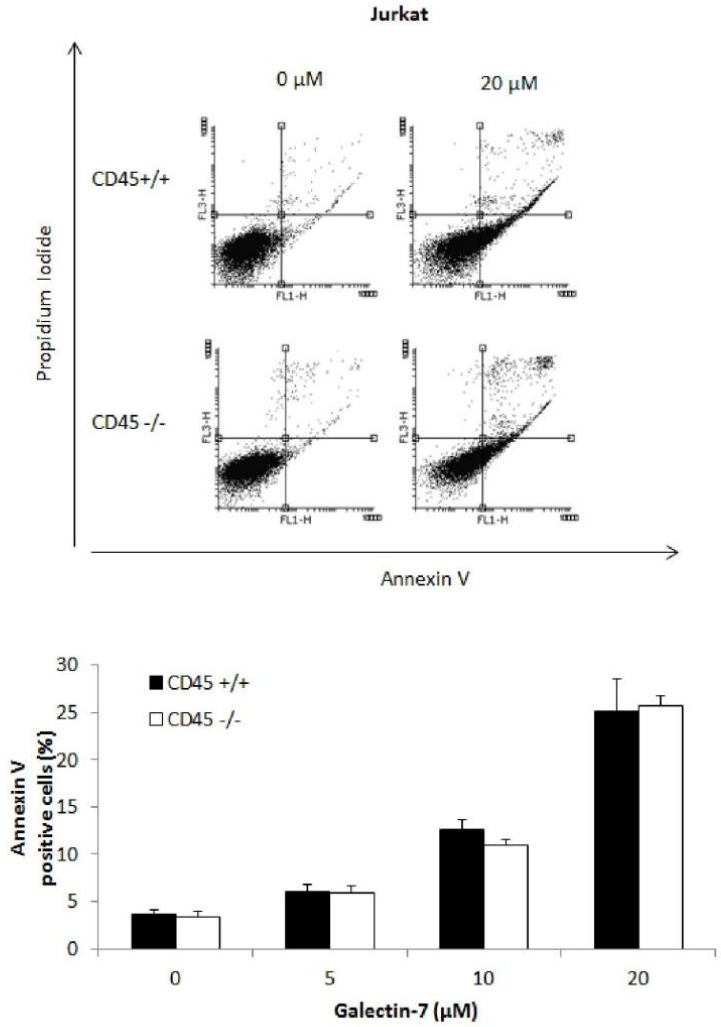
Supplementary figure S2: Binding of recombinant gal-7 on SK-OV-3 and A2780 cells. 0.5 µM recombinant FITC-labeled gal-7 was added to SK-OV-3 and A2780 cells in the presence or absence of 0.1 M β-lactose. Binding was measured by flow cytometry. Results are representative of three independent experiments.



Supplementary figure S3: Recombinant galectin-7 does not modulate the motility of A2780 cells. A2780 cells were seeded into a 6-well glass bottom culture plate. Cells were treated with or without 1 µM recombinant galectin-7, 2 h prior to the experiment. A scratch was made and images were captured every 15 min for 7.5 h. **(A)** Plots represent 30 cells/sample, tracked by live cell imaging during scratch wound healing test. Histograms represent the **(B)** velocity, **(C)** accumulated distance, **(D)** Euclidean distance and **(E)** directionality of the cells. Error bars represent SEM. Results are representative of three independent experiments.



Supplementary figure S4: Extracellular galectin-7 does not induce apoptosis of A2780 cells. A2780 cells were treated for 4 h with increasing concentration of recombinant galectin-7. As a positive control of apoptosis, cells were also treated with 75 µM cisplatin. The induction of apoptosis was determined by flow cytometric analysis of Annexin V-alexa 488 and PI-staining. Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells. Results are representative of three independent experiments. Error bars represent SD.



Supplementary figure S5: Extracellular galectin-7 induces apoptosis of Jurkat cells, independently of CD45 receptor. $CD45^{+/+}$ and $CD45^{-/-}$ Jurkat cells were treated for 4 h with or without 20 µM recombinant galectin-7. The induction of apoptosis was determined by flow cytometric analysis of Annexin V-alexa 488 and PI-staining. Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells. Results are representative of three independent experiments. Error bars represent SD.

Supplementary Table S1: Patients characteristics according to galectin-7 expression.

Outcome	All cases (n=64)	Galectin-7 expression		P value
		Positive	Negative	
Median age, years (range)	48 (22-75)	50 (33-75)	45 (22-68)	0.15
Stage				0.99
I	43	25	18	
II	10	5	5	
III	11	6	5	
Grade				0.12
1 and 1-2	31	13	18	
2 and 2-3	25	16	9	
3	8	6	2	

Supplementary Table S2: Overall survival of ovarian serous cystadenocarcinoma cancer patients according to the mRNA expression level of gal-7.

	Total number of patients	Number of deceased patients	Median of survival (Months)
Overexpression	28	21	26.94
Unaltered expression	230	126	44.06

[a]Data were obtained from the cBio portal RNAseq datasets [10].

CHAPITRE 4

UNE NOUVELLE SIGNATURE PROTÉIQUE AUGMENTE LA VALEUR PRÉDICTIVE DE CA125 DANS LE CANCER DE L'OVAIRE

Article en préparation

RÉSUMÉ

Le cancer de l'ovaire est la 5^{e} cause de mortalité dans le monde occidental. Malgré l'amélioration de différents traitements, le taux de survie sur cinq ans des patientes qui ont une maladie de stade avancé est de moins de 40 %. L'hétérogénéité de la réponse aux traitements complique le processus de prise de décision clinique et reflète la complexité génétique qui mène à l'hétérogénéité intra-tumorale. Il est clair qu'il y a un besoin urgent de développer des biomarqueurs cliniques fiables afin d'identifier les patientes à différents niveaux de risque concernant leur pronostic. Dans cette étude, nous avons étudié l'expression des galectines dans le cancer ovarien et nous avons examiné leur potentiel en tant que biomarqueurs. En utilisant des micromatrices tissulaires, construites à partir de tumeurs primaires de 209 patientes atteintes de cancer de l'ovaire séreux de haut grade, nous avons identifié une nouvelle signature de protéines qui prend en considération l'expression des galectines, à la fois dans le stroma péritumoral et les cellules cancéreuses. Notre étude démontre une augmentation de l'expression de la galectine-1 stromale et des galectines-8 et -9 épithéliales dans les tumeurs primaires associées à une faible réponse à la chimiothérapie. Cette signature permet également d'augmenter la valeur prédictive de CA125 pour la survie et la récidive sur cinq ans, ainsi que la récidive post-chimiothérapie. Des analyses univariées et multivariées ont révélées que les galectines-8 et -9 sont toutes deux des facteurs indépendants pour la prédiction de la résistance à la chimiothérapie et la survie, respectivement. Pour les patientes CA125 négatives, la galectine-9 épithéliale est associée à un taux de survie sur cinq ans plus bas alors que la galectine-1 stromale et la galectine-8 épithéliale étaient toutes deux associées à un plus haut taux de récidive. Ensemble, ces résultats démontrent qu'il serait pertinent de poursuivre ces études afin de déterminer si les galectines sont de bons biomarqueurs et cibles thérapeutiques pour le cancer de l'ovaire. D'un point de vue fondamental, notre projet contribue à mieux comprendre l'hétérogénéité de cette maladie.

CONTRIBUTION DES AUTEURS

Yves St-Pierre, Anne-Marie Mes-Masson et moi avons décidé du plan expérimental. J'ai réalisé l'ensemble des expériences de cet article et participé à chacune d'entre elles. J'ai également effectué les différentes analyses statistiques. Laudine Communal a participé à la validation des anticorps ainsi qu'au marquage et à l'évaluation du marquage en immunofluorescence des micromatrices tissulaires. Yves St-Pierre et moi avons écrit l'article et tous les auteurs ont révisé et approuvé le manuscrit final.

A new protein signature that potentiates CA125 predictive value in ovarian cancer

Marilyne Labrie[1], Laudine Communal[2], Anne-Marie Mes-Masson[2] and Yves St-Pierre[1]

[1] INRS-Institut Armand-Frappier, Laval, Quebec, Canada.

[2] CR-CHUM, Montreal, Quebec, Canada.

Corresponding Author: Yves St-Pierre, INRS-Institut Armand-Frappier, 531 Blv des Prairies, Laval, Quebec H7V 1B7, Canada. Phone: (450) 686-5354; Fax: (450) 686-5501; E-mail: yves.st-pierre@iaf.inrs.ca

Keywords: galectins, ovarian cancer, biomarker, CA125

ABSTRACT

Ovarian cancer (OC) is the 5th leading cause of cancer-related deaths in the Western world. Despite improvements in various treatments, the 5-years survival of patients with advanced stage disease remains less than 40%. The non-uniformity of response to therapy complicates the clinical decision-making process and reflects the genetic complexity that gives rise to intra-tumor heterogeneity. Clearly, there is an urgent need to develop reliable clinical biomarkers to identify patients at different levels of risk for specific outcomes. Here, we have investigated the expression of galectins in OC and examined their potential as biomarkers. Using tissue microarrays constructed from primary tumors collected from 209 patients with high grade serous carcinoma, we have identified a new protein signature that takes into account the expression of galectins in both cancer and stromal cells. Our study showed that increased protein expression of stromal gal-1 and epithelial gal-8/9 in primary tumors was associated with a poor response to treatment in patients with high grade serous carcinoma (HGSC). This signature also increased the predictive value of CA125 on five-year disease-free survival, post-chemotherapy treatment and five-year overall survival. Univariate and multivariate analyses revealed that gal-8 and gal-9 were both independent predictors of chemoresistance and overall survival, respectively. In CA125-negative patients, epithelial gal-9 was associated with a lower 5 year OS while stromal gal-1 and epithelial gal-8 were both associated with a lower 5 year DFS. Overall, these data provide impetus for further studies to determine the potential of galectins as biomarkers and actionable targets for the treatment of OC. From a fundamental point of view, our project contributes to better define the heterogeneity of the disease.

INTRODUCTION

The poor survival rate of patients with epithelial ovarian cancer (EOC) is largely due to the high-grade serous carcinoma (HGSC) histological subtype. Approximately 20% of patients with HGSC do not respond to chemotherapy, and for those who initially respond, emergence of drug resistance during subsequent cycles of chemotherapy is a common problem. Such non-uniformity to response to therapy complicates the clinical decision-making process. It is thus critical to develop reliable and easily measurable biomarkers that provide a statistical probability to respond to therapy [213]. At present, the most commonly clinically validated biomarker for monitoring disease progression and

assessing response and relapse to treatment is the carbohydrate antigen 125 (CA125). Unfortunately, serum CA125 lacks specificity and sensitivity, as a single marker, for early EOC detection and prognosis [214]. Moreover, the rate of false negatives is relatively high, especially in the early stages of ovarian cancer [215, 216]. Thus, although CA125 may be useful for monitoring disease progression and response to treatment in some patients with EOC, it is difficult to interpret the meaning of an abnormally high level of serum CA125 without additional information.

Given the influence of the immune stroma on cancer progression, the development of panels of immune biomarkers with sensitivity and specificity of detection is a promising avenue to yield robust predictive tools for prognosis and monitoring response to therapy in ovarian cancer. Immune cells and cancer cells closely interact at every steps of disease progression. Such cross-talk has a profound impact on disease progression as it can both inhibit and enhance tumor growth. Because of their known ability to induce local immunosuppression and to confer cancer cells with resistance to apoptosis, members of the galectin family are emerging as a new class of actionable immune biomarkers in cancer [217, 218]. Such Galectin-specific signatures has been shown to serve as reliable protein biomarkers to monitor disease progression in the case of prostate cancer [144, 219]. In the present work, using tissue microarrays (TMAs) constructed from tumors collected from patients with HGSC, we report the identification of a new protein signature that takes into account the expression of galectins in both stromal and epithelial cancer cells. We further show that such signature can be potentially used as reliable predictors of overall survival, disease recurrence and chemoresistance in patients with HGSC, most notably in patients who are CA125 negative.

RESULTS

Galectin expression in normal ovaries, fallopian tubes, and ovarian cancer subtypes

We initially investigated galectin-1, -3, -4, -7, -8, and -9 expression patterns by IHF using a TMA containing multiple ovarian cancer subtypes and normal ovarian tissues and a TMA constructed from normal fallopian tissue samples. This allowed us to establish the galectin signature in normal tissues (Fig. 1) and various ovarian cancer subtypes (Supplementary Fig. 1). Overall, we found that every histological subtypes of ovarian cancer has a distinctive galectin signature. For example, Gal-1 was mainly found

in serous subtype while gal-4 was associated with mucinous carcinomas. Gal-3, -7, -8 and -9 were found in all histological subtypes.

Galectins expression in HGSC

Our subsequent studies were conducted on TMAs constructed with HGSC samples. A particular attention was paid to galectin expression in stromal versus epithelial cancer cells since we and others had previously found that galectins can be expressed by either stromal and/or cancer cells [220-222]. Examples of such stromal vs. epithelial signatures are shown in Fig. 2. Overall, our results showed that 45% to 65% of HGSC display epithelial expression of either gal-1, -3, -7, -8 or -9 (Fig.2c). Stromal gal-1 and -9 were found in 67% of tumors and stromal gal-3 and gal-8 were expressed in 42% and 31% of tumors, respectively. Stromal gal-7 was observed in only 8% of the tumors (Fig.2c). Interestingly, 83% of gal-9 positive tumors displayed cytosolic/perinuclear puncta (Fig.2a and Supplementary Fig.2), consistent with the association of gal-9 with endosomes [22]. Confocal imagery analysis of gal-9 IF staining in three ovarian cancer cell lines and the use of a different anti-human galectin-9 antibody was used to confirm the presence of those puncta (Supplementary Fig.2). IF staining of gal-9, LC3B and COV IV in TOV-1369TR cells demonstrated that gal-9 perinuclear puncta are not associated with autophagosomes nor mitochondria.

Predictive values of galectins in HGSC patients

The presence of galectins in the epithelial or the stromal compartments of HGSC was associated with the clinical data of the patients (Table I and Supplementary Table III). We found that epithelial gal-3, -7 and -9, as well as stromal gal-3 and -7 had no significant association to any clinical data. Epithelial gal-1 was significantly associated with a higher CA125 value and there was a tendency towards the association between epithelial gal-1 expression and a higher FIGO stage and the recurrence of the disease. Epithelial gal-8 was more frequent in patients with the recurrence of the disease, although it did not reach a significant level of $P \leq 0.05$. Stromal gal-1 was significantly associated with the recurrence of the disease and it was more frequently expressed in samples with higher CA125 values. There was also a tendency towards the association between stromal gal-8 and gal-9 with a lower rate of death and higher CA125 values,

respectively. In the case of gal-9, perinuclear puncta were associated with a higher rate of death, although it did not reach a significant level of $P \leq 0.05$. Taken together, those results indicate that epithelial gal-1 and gal-8, stromal gal-1 and gal-9 as well as gal-9 perinuclear puncta are associated with a bad prognosis. On the other hand, stromal gal-8 might be associated with a good prognosis. Kaplan-Meier curves were used to correlate the expression of galectins to 5-years OS, 5-years DFS and chemoresistance. We found a significant association between stromal gal-1 staining and 5-years DFS and between epithelial gal-8 and chemoresistance (Fig. 3a). In a number of cases, we found an association between positive staining of a given galectins and 5-years OS, 5-years DFS or chemoresistance that fell just short of the traditional definition of statistical significance (Supplementary Fig. 3).

Galectin signatures: an added value to CA125 in patients with HGSC

We have next determined whether the galectin signature could increase the predictive value of CA125, the commonly used biomarker for ovarian cancer. As expected, our data confirmed the predictive value of CA125 in predicting 5-years OS, 5-years DFS or chemoresistance (Fig. 3b). Our results showed that expression of gal-9 in cancer cells increases the predictive value of CA125 (Fig. 3c), although the p value ($p = 0.180$) fell just short of the traditional definition of statistical significance. In CA125-negative patients, epithelial gal-9 was associated with a lower 5 year OS ($p = 0.041$), while stromal gal-1 was associated with a lower 5 year DFS ($p = 0.005$) (Fig. 3c).

DISCUSSION

The treatment of ovarian cancer is severely complicated by high frequency of disease recurrence and the development of resistance to treatment. This problem is exacerbated by the absence of reliable biomarkers that can identify with high accuracy patients that would benefit. At present, the most common biomarkers is CA125, which unfortunately lacks the sensitivity and specificity necessary to identify patients that would benefit of more aggressive treatments and to follow up treatment response and early detection of recurrence. In search for new biomarkers for ovarian cancer, we have identified a new galectin signature that takes into account the expression of multiple galectins in both cancer and tumor-associated stromal cells. More specifically, we found

that galectin signatures in both cancer and stromal cells can be used: 1) to predict five-year disease-free survival, post-chemotherapy treatment and five-year overall survival in HGSC patients; and 2) to potentiate the predictive value of CA125. Taken together, galectin signatures may be very useful to help clinicians to identify patients with a high probability of resistance to chemotherapy, especially in patients who are CA125-negative.

Immune cells and cancer cells closely interact at every steps of disease progression. Such cross-talk has a profound impact on disease progression as it can both inhibit and enhance tumor growth. Not surprisingly, there is increasing interest at examining the potential of immune and stromal components as predictive biomarkers. Such strategy has exploited to stratify breast cancer patients and to develop powerful predictors of cancer prognosis, recurrence risk, and metastasis [223-226]. Because of their known ability to induce local immunosuppression and to confer cancer cells with resistance to apoptosis, members of the galectin family are emerging as a new class of actionable biomarkers in cancer. Although most studies have focused on gal-1 and gal-3 as predictive biomarkers, recent studies have shown that multiple galectins are expressed at abnormally high levels in cancer. In breast cancer, analysis of the stromal signature showed that gal-1, -3, -9-positive stroma were preferentially found in triple-negative and HER2 subtypes [222]. Such expression of gal-1, -3, and -9 signature has also been reported in the stroma and cancer cells in the case of squamous cervical cancer [221]. Such signature is somewhat similar to that we found in HGSC. However, although gal-1 correlated with a poor prognosis in all cases, the prognostic value of gal-9 differs significantly in squamous cervical cancer and HGSC. Indeed, in squamous cervical cancer, expression of gal-9 in cancer cells was associated with a better survival [221]. Stromal gal-9 is also often associated with a better survival [227]. This was clearly not the case for HGSC. Such dual role for a galectin in cancer cells has been well documented for gal-3, gal-7 and gal-8 and has been attributed to distinct subcellular localization [65, 222, 228]. These observations emphasize the importance of using immunohistochemical subcellular localization analyses to accurately measure the predictive potential of galectins and other proteins that are known to traffic from one cell type to the other. It will be interesting to determine whether the galectin signature found in tissue samples of HGSC patients can be detected in plasma. This is a real possibility since galectins are small soluble molecular weight proteins that are readily present in the plasma of healthy individuals and are abnormally high in patients with cancer [229-231].

This might well be the case for ovarian cancer since a recent study reported a significant difference in serum gal-1 between EOC patients with non-metastatic and those with metastatic disease [201]. Such « liquid biopsies » are not only less invasive and less costly, they represent a better way to assess or bypass spatial heterogeneity of tissue biopsies. Measure of validated biomarkers in plasma, for example, allows for a closer follow up of patients conditions and response to treatment in daily practice.

In summary, our study brings support to the idea that because of their critical role in inducing local immunosuppression in cancer, galectins have the potential as prognostic tools for the follow up of patients with ovarian cancer. We believe that such signature could be used to distinguish between benign and malignant disease and to monitor response to therapy in women with ovarian cancer. Future work with independent cohort of patients will be necessary to confirm the predictive value of this signature and to determine whether this signature can be incorporated in multiple biomarkers panels for risk stratification of patients with ovarian cancer.

MATERIALS AND METHODS

Ethics statement

A written informed consent was obtained from all subjects providing tissue specimens. This study was conducted in accordance with guidelines and approval of the institutional ethical review boards of the CHUM ethics committee.

Tumor and normal tissue specimen

Tumor and normal tissue specimens were harvested from patients treated at Centre Hospitalier de l'Université de Montréal (CHUM) from 1992 to 2012. All samples were evaluated by a gynecologic-oncologist pathologist who assigned the disease stage, tumor grade and histopathological subtypes according to the criteria established by the International Federation of Gynecology and Obstetrics. Only tumors from patients that did not received preoperative chemotherapeutic treatment were included in this study. The clinical data on disease-free interval were defined according to computed tomographic (CT) imaging, alone or combined with blood CA125 levels. Formalin-fixed paraffin-embedded material from each primary tumor and normal tissue samples was

used to construct tissue microarrays (TMA). For immunohistofluorescence (IHF), three different TMAs were used: the multi-subtype TMA, with 63 samples of various EOC subtypes (Table I) and 8 normal ovarian tissue samples, the HGSC TMA with 209 samples of HGSC (Table II) in duplicate and a TMA with a total of 14 normal fallopian tubes samples.

IHF

Immunostaining reactions were performed using the BenchMark XT automated stainer (Ventana Medical System Inc., Tucson, AZ, USA). Deparaffinized sections were incubated in cell conditioning 1 (pH 8.0) (Ventana Medical System Inc) buffer for antigen retrieval and then stained for 60 min with the mouse anti-gal-1 (1:4000, Proteintech Group (3G10D2), Chicago, IL, USA), rabbit anti-gal-3 (1:2000, Abcam (EP2775Y), Cambridge, MA, USA), goat anti-gal-4 (1:300, Santa Cruz (T20), CA, USA), goat anti-gal-7 (1:100, R&D Systems (AF1339), Minneapolis, MN, USA), rabbit anti-gal-8 (1:50, Abcam (Ab183637)), rabbit anti-gal-9 (1:100, Abcam (Ab69630)) and a mix of mouse anti-cytokeratin-7 (1:200, Thermo Scientific, OV-TL 12/30), anti-cytokeratin-18 (1:200 Santa Cruz, DC-10) and anti-cytokeratin-19 (1:200 Thermo Scientific, A53-B/A2.26). After washing three times in PBS, sections were than incubated for 45 min in secondary antibodies consisting of a goat anti-mouse Alexa Fluor 750 (1:500, life technologies, Burlington, ON, Canada), goat anti-mouse Cy5 (1:500 life technologies), donkey anti-goat-biotin (1:300, Jackson ImmunoResearch, West Grove, PA, US), streptavidin Alexa Fluor 488 (1:500, Jackson ImmunoResearch), donkey anti-goat Alexa Fluor 488 (1:500, life technologies), goat anti-rabbit Cy5 (1:500, life technologies) and goat anti-rabbit Alexa Fluor 488 (1:500, life technologies). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The slides were scanned with a 20X 0,75NA objective with a resolution of 0,3225 µm (VS110, Olympus, Center Valley, PA, USA) and the intensity of the staining was quantified as MFI, using VisiomorphTM software (Visiopharm, Denmark). For the stroma, the percentage of staining was scored from 0 to 3 according to the percentage of positive cells displaying the protein expression within a sample (0 = 0 %; 1 ≤ 30 %; 2 ≤ 60 %; 3 ≥ 61 %). The intensity of staining was also scored from 0 to 3, with a score of 0 representing no staining and a score of 3 representing the strongest staining observed. Histological scores were calculated by multiplying both scores. The

validation of antibodies specificity was assessed by western blot and IHF using lysates of ovarian cancer cell lines and TMA of those respective cell lines.

EOC cell lines

The HGS ovarian cancer cell line OV-4453 was established from the ascites of a patient whereas TOV-1369TR and TOV-1369 (2) were established from a HGS ovarian tumor of a patient before (TOV-1369TR) and after (TOV-1369(2)) chemotherapy treatment. All cell lines were maintained in ovarian surface epithelium (OSE) medium (Wisent, QC, Canada) supplemented with 10% [v/v] fetal bovine serum.

IF

Cells were fixed in 3 % (w/v) paraformaldehyde for 15 min, permeabilized in 0.1 % (v\v) PBS/Triton X-100 for 5 min and blocked overnight at 4 ºC in 1 % (w/v) PBA. Rabbit anti-human gal-9 (1:100, Abcam), goat anti-human gal-9 (1:50, R&D Systems), rabbit anti-human LC3B (1:200, Sigma) and rabbit anti-human Cox IV (1:500, New England Biolabs, Ipswich, MA) primary antibodies were used. Secondary antibodies were a donkey anti-rabbit Alexa Fluor 647 (1:500, life technologies) and a donkey anti-goat Alexa Fluor 488 (1:500, life technologies). All antisera were diluted in 1% (w/v) PBA, and all washing steps were performed with PBS. Nuclei were stained with ProLong Gold Antifade Reagent with DAPI (Life Technologies). Cells were visualized under a Carl Zeiss LSM780 confocal microscope, and digitized images were generated using Carl Zeiss ZEN software (Zeiss, Jena, Germany).

Statistical Analysis

All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA) where $P \leq 0.05$ were considered significant. Roc curves were used to determine the optimal threshold for positive and negative values of galectin expression. Protein expression of galectins were correlated to one another using Pearson's correlation test (two-tailed) and correlated to the patients' characteristics using Fisher exact test, T test and Kruskal Wallis Test. Overall survival (OS) and disease free survival (DFS) were calculated as the time elapse between the day of the diagnosis and the death of the

patient or the clinically proven recurrence, respectively. Taxol/Carboplatin resistance was considered as a recurrence of the disease in the 2 years following the last day of chemotherapy treatment. Survival curves (OS, DSF and Taxol/Carboplatin resistance) were plotted by the Kaplan-Meier estimator and compared using the log rank test. Univariate and multivariate Cox proportional hazard models were used to determine the hazard ratio for each marker.

FINANCIAL SUPPORT

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FIGURE LEGENDS

Figure 1: Galectins expression in normal ovarian and fallopian tube tissues. IHF staining of gal-1, -3, -4, -7 -8 and -9 in normal (A) ovarian and (B) fallopian tube tissues. Cytokeratin staining was used to identify epithelial cells. Bar represent 50 µm.

Figure 2: Galectins expression and tissular distribution in HGSC. (A) The expression of gal-1, -3, -7, -8 and -9 was detected by IHF in HGSC (A) cancer cells and (B) stroma. Cytokeratin staining was used to identify epithelial cancer cells. Cytokeratin negative tissue was considered as stroma. Bar represent 20 µm. (C) Percentage of positive tumors expressing galectins in the cancer cells and the stroma of 209 HGSC samples.

Figure 3: Galectins predictive value of OS, DFS and chemoresistance. (A) Kaplan-Meier curves of 5-years OS, 5-years DFS and chemoresistance according to stromal gal-1, epithelial gal-8 and gal-9 puncta in HGSC samples. Blue bar: galectin negative tumors, red bar: galectin positive tumors. (B) Kaplan-Meier curves of 5-years OS and 5-years DFS according to the presence of circulating CA125. Blue bar: CA125 negative samples, red bar: CA125 positive samples. (C) Kaplan-Meier curves of 5-years OS according to gal-9 puncta presence and 5-years DFS according to stromal gal-1

presence in the tumors of CA125 positive or negative patients. Blue bar: galectin negative tumors, red bar: galectin positive tumors.

Supplementary figure 1: Galectins expression in different histological subtypes of ovarian cancer. (A) Positive IHF staining of gal-1, -3, -4, -7 -8 and -9 in a single mucinous tumor. Cytokeratin staining was used to identify epithelial cancer cells. Bar represent 50 µm. Percentage of positive tumors displaying expression of gal-1, -3, -4, -7, -8 and -9 in (B) clear cell, (C) endometrioid, (D) mucinous and (E) serous ovarian tumors.

Supplementary figure 2: Gal-9 puncta in ovarian cancer cells. (A) IHF staining of gal-9 puncta in HGSC. Cytokeratin staining was used to identify epithelial cancer cells. Bar represent 20 µm. (B) IF staining of gal-9 (red) was detected by confocal imagery in Tov-1369TR, Tov-1369(2) and OV-4453 ovarian cancer cell lines. Nuclei were stained with DAPI (blue). (C) IF staining of gal-9 (red) was detected by confocal imagery in Tov-1369TR with a goat anti-gal-9 antibody (R&D). (D) IF staining of gal-9 (red), LC3B (green, left panel) and COX IV (green, right panel) in TOV-1369TR cells. LC3B and Cox IV antibodies were used to stain autophagosome and mitochondria, respectively. Nuclei were stained with DAPI (blue). Bars represent 10 µm.

Supplementary figure 3: Galectins predictive value of OS, DFS and chemoresistance. Kaplan-Meier curves of (A) 5-years OS, (B) 5-years DFS and (C) chemoresistance according to epithelial or stromal presence of gal-1, -3, -7, -8 and -9. Blue bar: galectin negative tumors, red bar: galectin positive tumors.

Table I: Correlation between the patients characteristics and galectins expression.

	Gal-1 (stroma)		Gal-8 (epith)		Gal-9P (epith)	
	Low	High	Low	High	Low	High
Age[1]						
Mean	61	61	63	61	61	62
Figo Stage[2]						
Low (I-II)	13 (7)	19 (10)	17 (9)	17 (9)	16 (8)	18 (9)
High (III-IV)	49 (26)	111 (58)	67 (34)	98 (49)	72 (38)	84 (44)
CA125[3]	P = 0.125					
Median	453.4	793	530.32	618	548	769.5
Residual disease[2]						
No	10 (6)	26 (16)	18 (11)	20 (12)	20 (13)	17 (11)
Yes	42 (27)	80 (51)	58 (35)	70 (42)	53 (34)	67 (43)
5-yrs recurrence[2]	P = 0.008		P = 0.065			
No	18 (10)	17 (6)	21 (11)	16 (8)	18 (10)	17 (9)
Yes	41 (22)	112 (60)	62 (32)	96 (49)	70 (38)	81 (44)
5-yrs death[2]					P = 0.109	
No	31 (16)	54 (28)	36 (18)	53 (27)	45 (23)	40 (21)
Yes	31 (16)	76 (40)	48 (24)	62 (31)	43 (23)	62 (33)

Results are expressed as n (%). [1]T test, [2]Fisher's exact test, [3]Kruskal-Wallis test

Table II: Univariate and multivariate Cox analysis.

	Univariate analysis				Multivariate analysis			
		95 % confidence interval				95 % confidence interval		
Variables	Hazard ratio	Lower	Upper	P-Value	Hazard ratio	Lower	Upper	P-Value
5-years OS								
Age at diagnosis	1.019	1	1.039	0.046	1.027	0.999	1.057	0.059
Figo				0.006				0.239
I	1				1			
II	0.739	0.198	2.753		0.178	0.030	1.059	
III	2.508	0.921	6.832		0.713	0.206	2.474	
IV	3.423	1.157	10.126		0.622	0.155	2.497	
Residual Disease				≤ 0.001				≤ 0.001
No residual disease	1				1			
≤ 1 cm	1.629	0.794	3.343		1.103	0.477	2.552	
>1 cm ≤ 2 cm	2.608	1.240	5.486		1.471	0.599	3.610	
> 2 cm	4.414	2.274	8.568		4.088	1.749	9.551	
CA125	1.862	1.193	2.905	0.006	2.107	1.178	3.769	0.012
Gal-9P (epith)	1.537	1.041	2.271	0.031	2.309	1.378	3.868	≤ 0.001
5-years DFS								
Age at diagnosis	1.005	0.989	1.021	0.541				
Figo				≤ 0.001				0.386
I	1				1			
II	1.632	0.575	4.634		1.137	0.336	3.854	
III	4.408	1.797	10.809		1.781	0.595	5.334	
IV	6.839	2.586	18.085		2.253	0.669	7.591	
Residual Disease				≤ 0.001				≤ 0.001
No residual disease	1				1			
≤ 1 cm	1.418	0.850	2.366		1.023	0.577	1.813	
>1 cm ≤ 2 cm	2.446	1.407	4.252		1.637	0.863	3.107	
> 2 cm	3.515	2.149	5.749		2.701	1.486	4.909	
CA125	1.770	1.249	2.509	≤ 0.001	1.459	0.949	2.243	0.085
Gal-1 (stroma)	1.534	1.071	2.199	0.020	1.416	0.931	2.152	0.104
Chemoresistance								
Age at diagnosis	0.995	0.972	1.017	0.638				
Figo								
I	1			≤ 0.001	1			0.003
II	4.457	0.548	36.233		1.352	0.145	12.624	
III	15.125	2.094	109.238		3.818	0.466	31.274	
IV	34.439	4.410	268.959		20.124	1.912	211.861	
Residual Disease				≤ 0.001				0.048
No residual disease	1				1			
≤ 1 cm	1.821	0.853	3.889		0.967	0.429	2.180	
>1 cm ≤ 2 cm	3.577	1.652	7.743		1.270	0.525	3.075	
> 2 cm	4.255	2.014	8.987		2.827	1.143	6.991	
CA125	2.701	1.588	4.593	≤ 0.001	2.756	1.366	5.558	0.005
Gal-8 (epith)	1.757	1.078	2.866	0.024	2.379	1.247	4.539	0.009

Figure 1

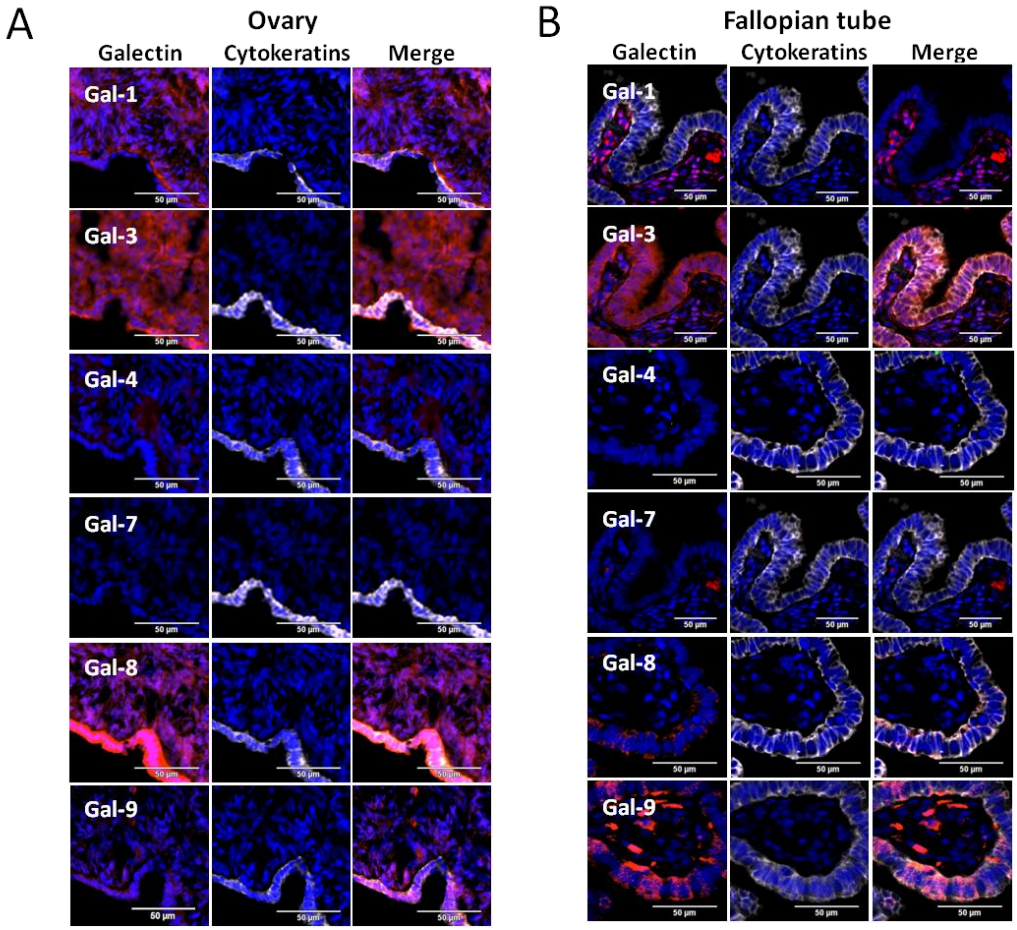

A
Ovary
Galectin
Cytokeratins
Merge
Gal-1
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9
B
Fallopian tube
Galectin
Cytokeratins
Merge
Gal-1
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9

Figure 2

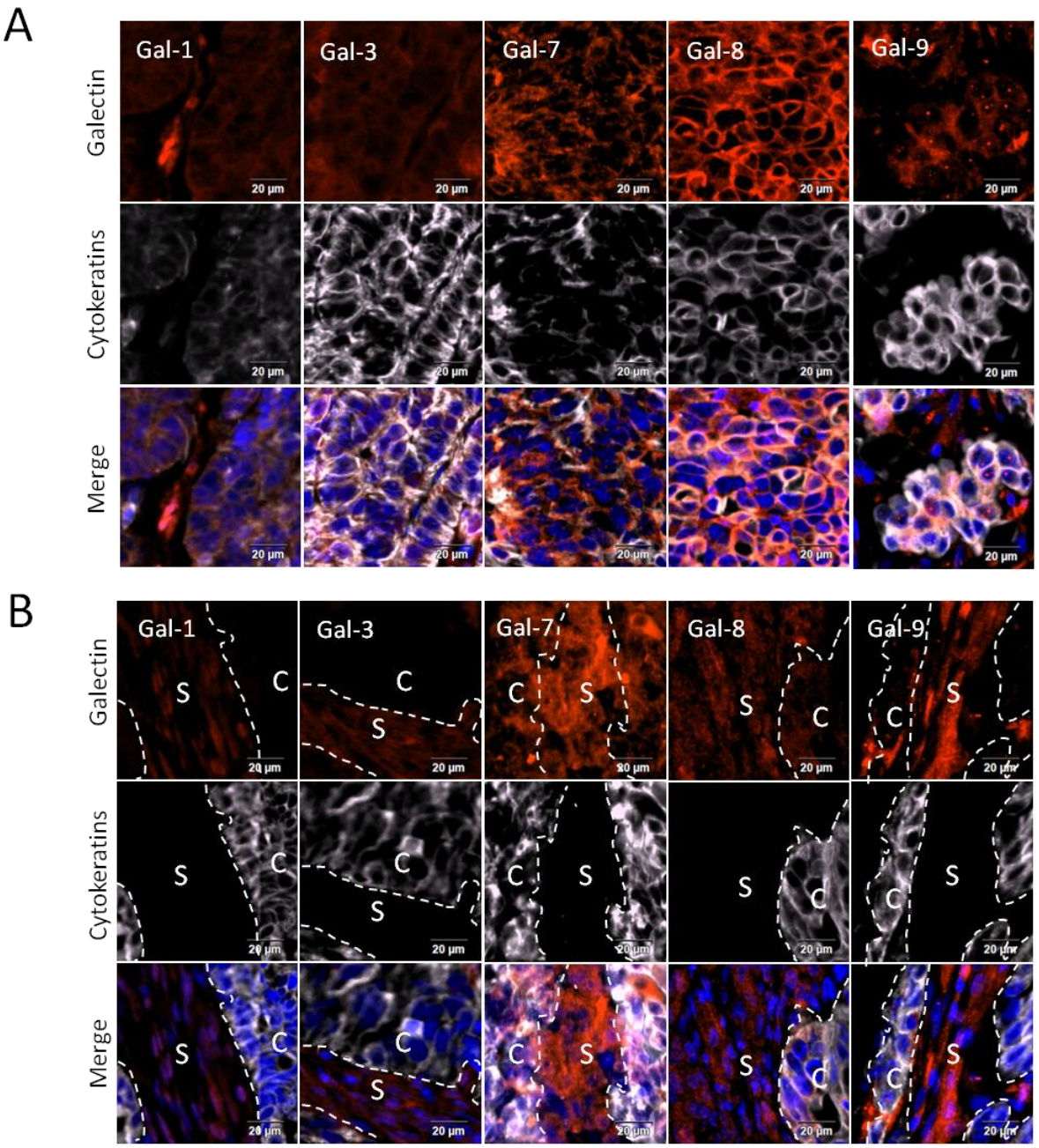

A
Galectin
Cytokeratins
Merge
Gal-1
Gal-3
Gal-7
Gal-8
Gal-9
20 µm
B
Galectin
Cytokeratins
Merge
Gal-1
Gal-3
Gal-7
Gal-8
Gal-9
S
C
20 µm
C
Positive tumors (%)
80
60
40
20
0
Gal-1
Gal-3
Gal-7
Gal-8
Gal-9
Epithelial
Stromal

Figure 3

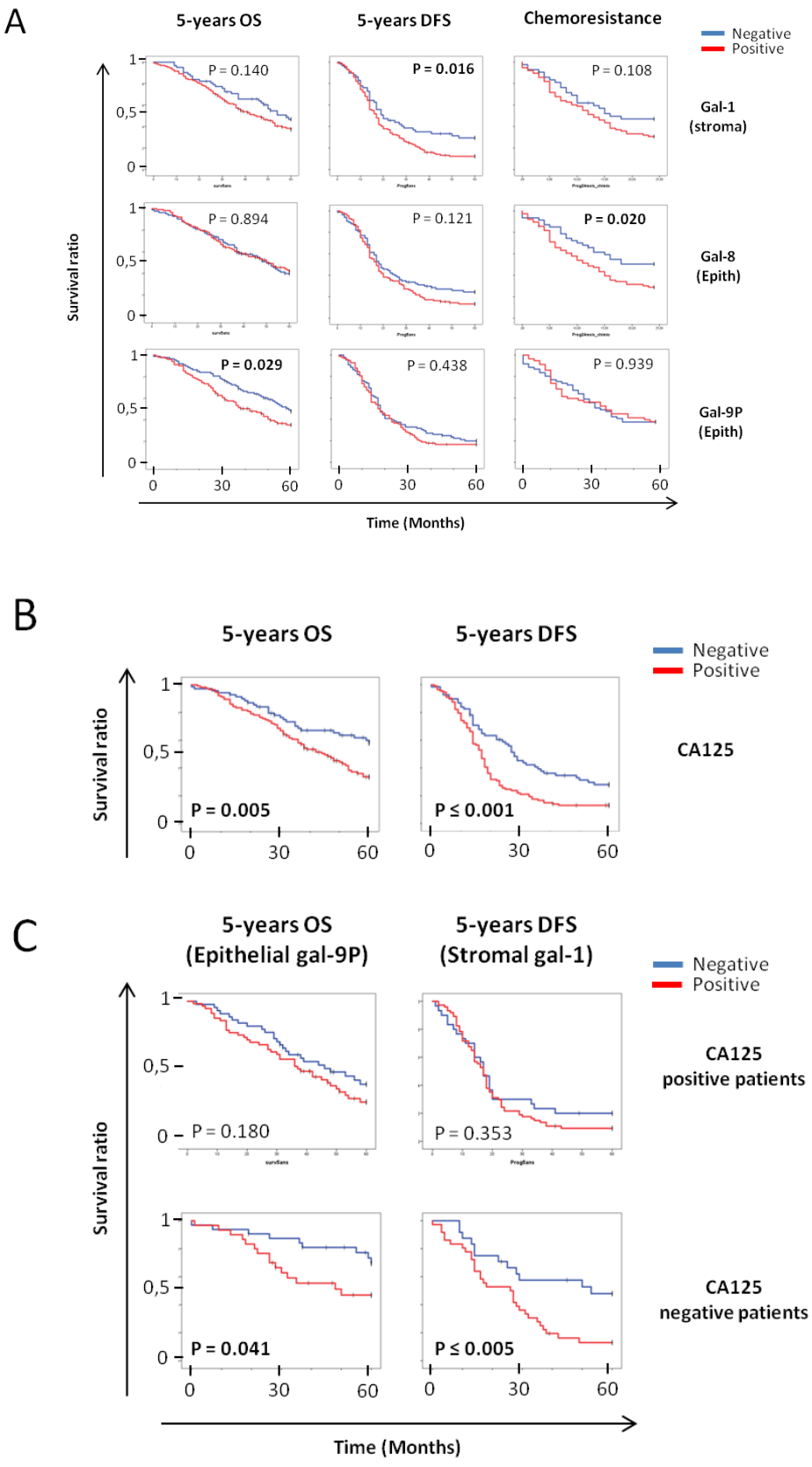

A
5-years OS
5-years DFS
Chemoresistance
Negative
Positive
P = 0.140
P = 0.016
P = 0.108
Gal-1 (stroma)
P = 0.894
P = 0.121
P = 0.020
Gal-8 (Epith)
P = 0.029
P = 0.438
P = 0.939
Gal-9P (Epith)
Survival ratio
1
0,5
0
0
30
60
Time (Months)
B
5-years OS
5-years DFS
Negative
Positive
CA125
Survival ratio
P = 0.005
P ≤ 0.001
C
5-years OS (Epithelial gal-9P)
5-years DFS (Stromal gal-1)
Negative
Positive
CA125 positive patients
P = 0.180
P = 0.353
CA125 negative patients
P = 0.041
P ≤ 0.005
Survival ratio
Time (Months)

Tableau S1

Supplementary table I: Patients characteristics (multi-subtype tissue microarray (n=63))

	Clear cell (n=12)	Endometrioid (n=21)	Mucinous (n=14)	Serous (n=16)
Age range (mean)	47-73 (57)	43-80 (59)	28-82 (54)	33-79 (57)
Grade				
Borderline	0	0	12	3
I (Low)	0	11	1	2
II	4	5	1	5
III (High)	8	5	0	6
Stade				
I	6	13	11	2
II	2	1	1	3
III	4	6	2	10
IV	0	1	0	1

Tableau S2

Supplementary table II: Patients characteristics (HGSC tissue microarray (n=209))

Age range (median)	36-89 (64.5)
Figo Stage	
I	13
II	23
III	148
IV	25
CA-125 range (median)	5-13763 (667)
Residual disease	
No residual disease	40
≤ 1 cm	51
>1 cm ≤ 2 cm	30
> 2 cm	50
Unknown	38
Chemotherapy	
Taxol/carboplatin	147
Other	60
Unknown	2
Reccurence	
Yes	172
No	37
Mortality	
Yes	140
No	69

Tableau S3

Supplementary Table III: Correlation between the patients characteristics and galectins expression in cancer cells and stroma of HGSC

	Gal-1		Gal-3		Gal-7		Gal-8		Gal-9	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
Cancer cells										
Age										
Mean	61	62	64	59	59	63	63	61	62	61
Figo Stage	**P = 0.056**									
Low (I-II)	29 (14)	13 (6)	13 (7)	20 (11)	13 (7)	20 (11)	17 (9)	17 (9)	16 (8)	18 (9)
High (III-IV)	84 (41)	**77 (38)**	58 (31)	98 (52)	71 (38)	85 (45)	67 (34)	98 (49)	82 (43)	74 (39)
CA-125	**P = 0.016**									
Median	453.36	**809.5**	587.9	697.44	765.5	559.5	530.32	618	553	697.44
Residual disease										
No	21 (13)	15 (9)	12 (8)	25 (16)	17 (11)	20 (13)	18 (11)	20 (12)	21 (13)	16 (10)
Yes	63 (40)	59 (37)	50 (32)	69 (44)	51 (33)	68 (44)	58 (35)	70 (42)	60 (38)	60 (38)
Recurrence	**P = 0.092**						**P = 0.065**			
No	23 (12)	12 (6)	15 (8)	20 (11)	18 (10)	17 (9)	21 (11)	16 (8)	21 (11)	14 (8)
Yes	75 (40)	**78 (41)**	56 (30)	94 (51)	63 (34)	87 (47)	62 (32)	**96 (49)**	76 (41)	75 (40)
Death										
No	49 (28)	36 (21)	30 (16)	56 (30)	41 (22)	45 (24)	36 (18)	53 (27)	47 (25)	38 (20)
Yes	53 (31)	34 (20)	41 (22)	62 (33)	43 (23)	60 (32)	48 (24)	62 (31)	51 (27)	54 (28)
Stroma										
Age										
Mean	61	61	60	62	61	1	61	61	61	62
Figo Stage										
Low (I-II)	13 (7)	19 (10)	16 (9)	16 (9)	31 (17)	1 (1)	22 (12)	11 (6)	10 (5)	23 (13)
High (III-IV)	49 (26)	111 (58)	92 (49)	63 (34)	141 (75)	14 (7)	109 (58)	47 (25)	51 (28)	100 (54)
CA-125	**P = 0.125**								**P = 0.074**	
Median	453.4	793	548.5	828.5	589.5	769.5	618	587.9	510	**767.5**
Residual desease										
No	10 (6)	26 (16)	20 (13)	17 (11)	35 (23)	2 (1)	23 (15)	15 (9)	13 (9)	23 (15)
Yes	42 (27)	80 (51)	72 (47)	45 (29)	107 (69)	10 (6)	85 (54)	35 (22)	34 (23)	81(54)
Recurrence	**P = 0.008**									
No	18 (10)	17 (6)	19 (10)	16 (9)	31 (17)	4 (2)	24 (13)	11 (6)	11 (6)	23 (13)
Yes	41 (22)	**112 (60)**	86 (47)	62 (34)	137 (75)	11 (6)	103 (56)	47 (25)	46 (26)	100 (56)
Death							**P = 0.027**			
No	31 (16)	54 (28)	46 (25)	38 (20)	77 (41)	7 (4)	53 (28)	**78 (41)**	24 (13)	58 (32)
Yes	31 (16)	76 (40)	62 (33)	41 (22)	95 (51)	8 (4)	34 (18)	24 (13)	37 (20)	65 (35)

Figure S1

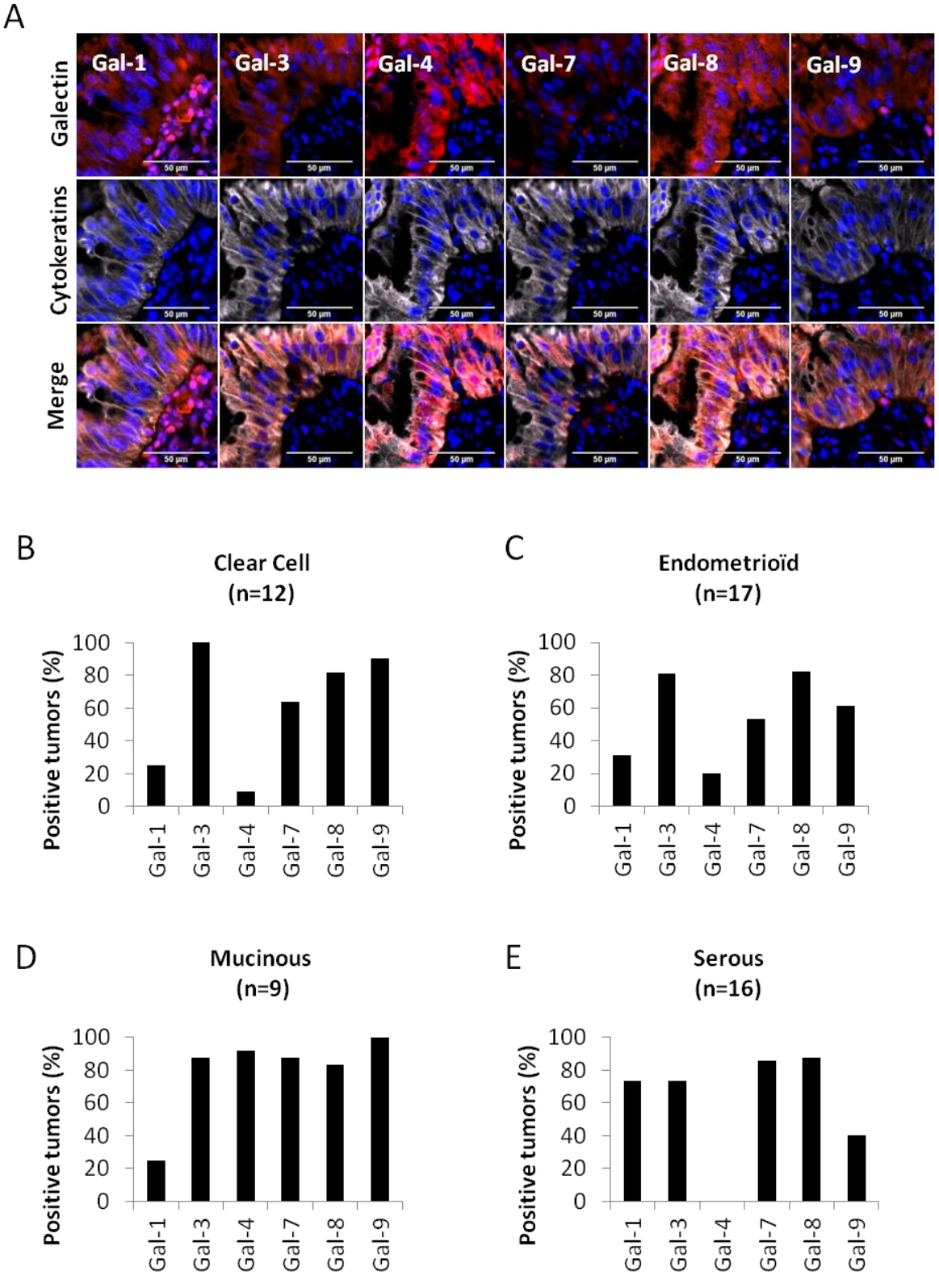

A
Galectin
Cytokeratins
Merge
Gal-1
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9
50 µm
B
Clear Cell
(n=12)
C
Endometrioïd
(n=17)
D
Mucinous
(n=9)
E
Serous
(n=16)
Positive tumors (%)
100
80
60
40
20
0
Gal-1
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9

Figure S2

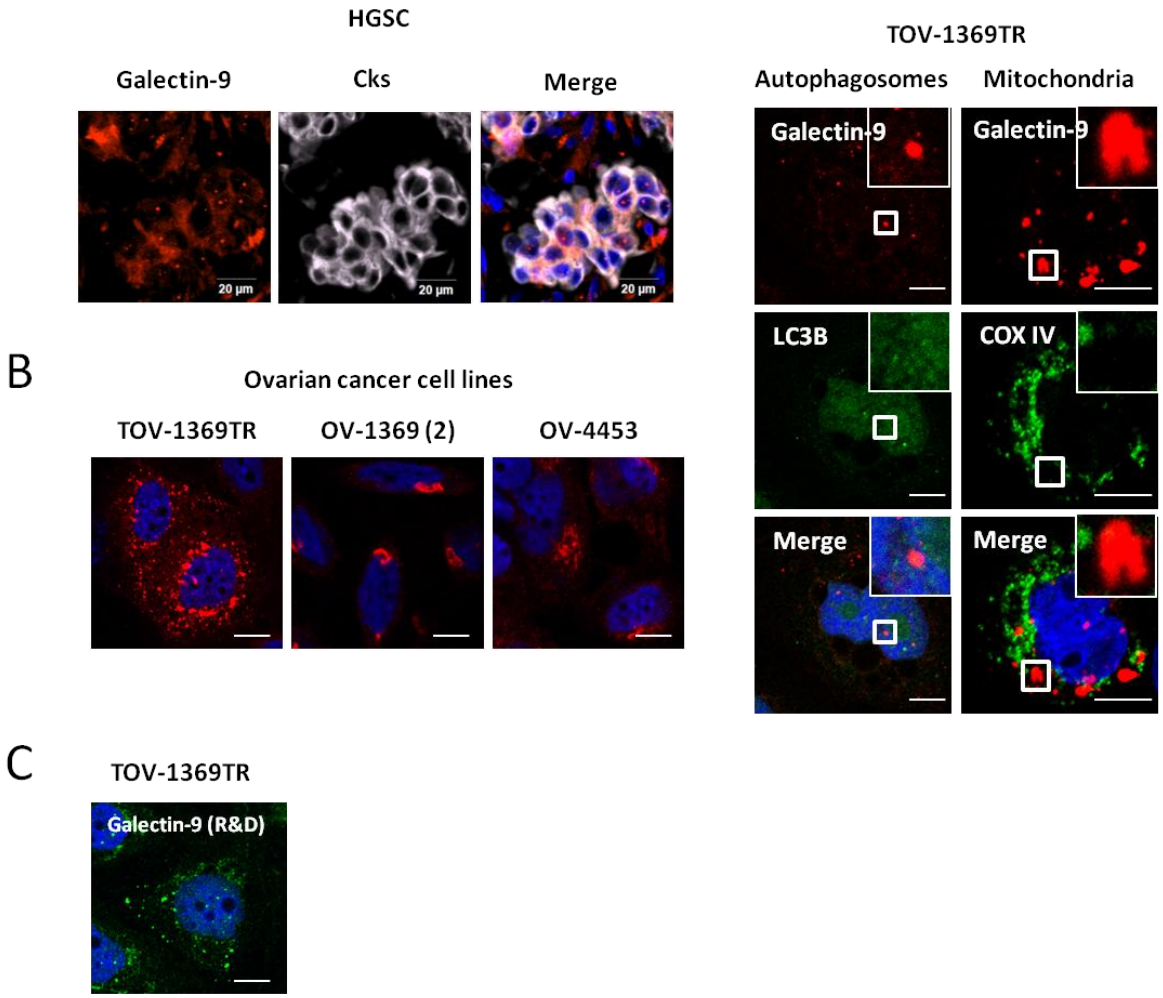

HGSC
Galectin-9
Cks
Merge
20 µm
20 µm
20 µm
TOV-1369TR
Autophagosomes
Mitochondria
Galectin-9
Galectin-9
LC3B
COX IV
Merge
Merge
B
Ovarian cancer cell lines
TOV-1369TR
OV-1369 (2)
OV-4453
C
TOV-1369TR
Galectin-9 (R&D)

Figure S3

A

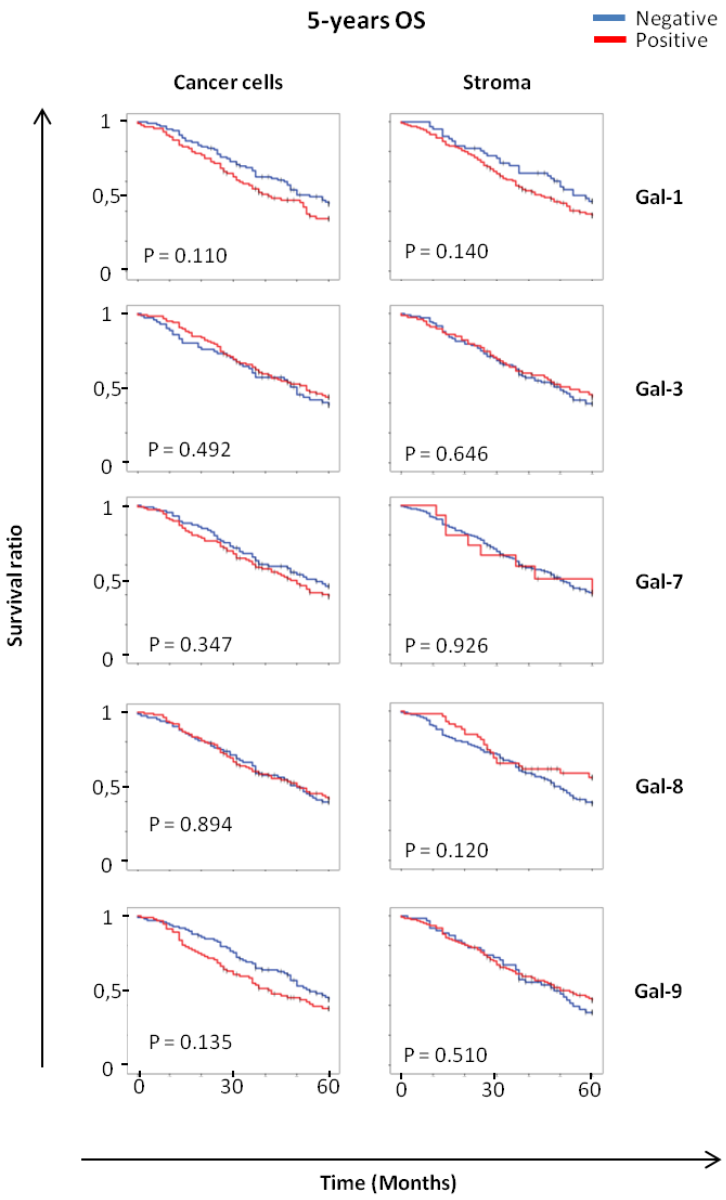
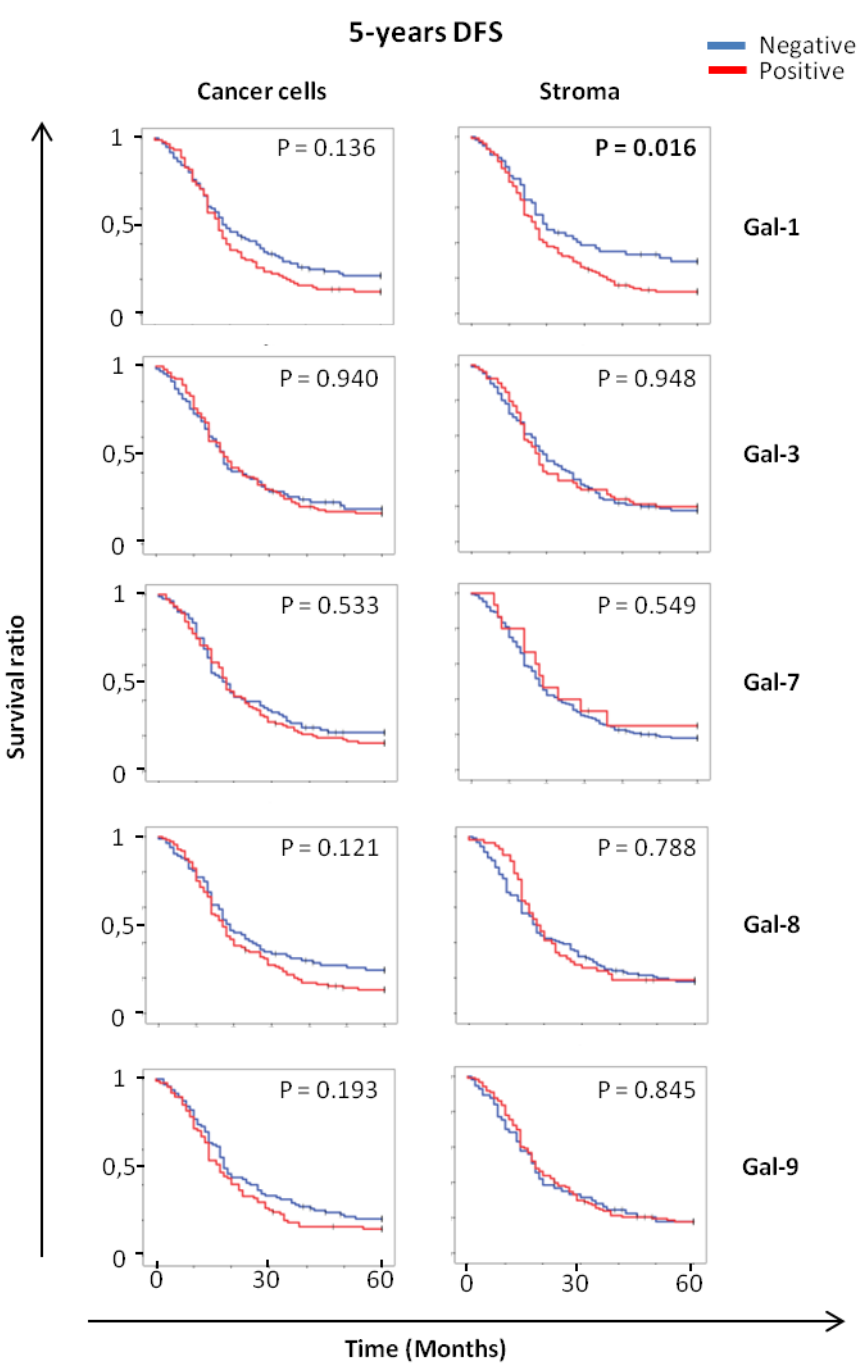

5-years OS
Negative
Positive
Cancer cells
Stroma
Survival ratio
Gal-1
Gal-3
Gal-7
Gal-8
Gal-9
P = 0.110
P = 0.140
P = 0.492
P = 0.646
P = 0.347
P = 0.926
P = 0.894
P = 0.120
P = 0.135
P = 0.510
1
0,5
0
0
30
60
Time (Months)

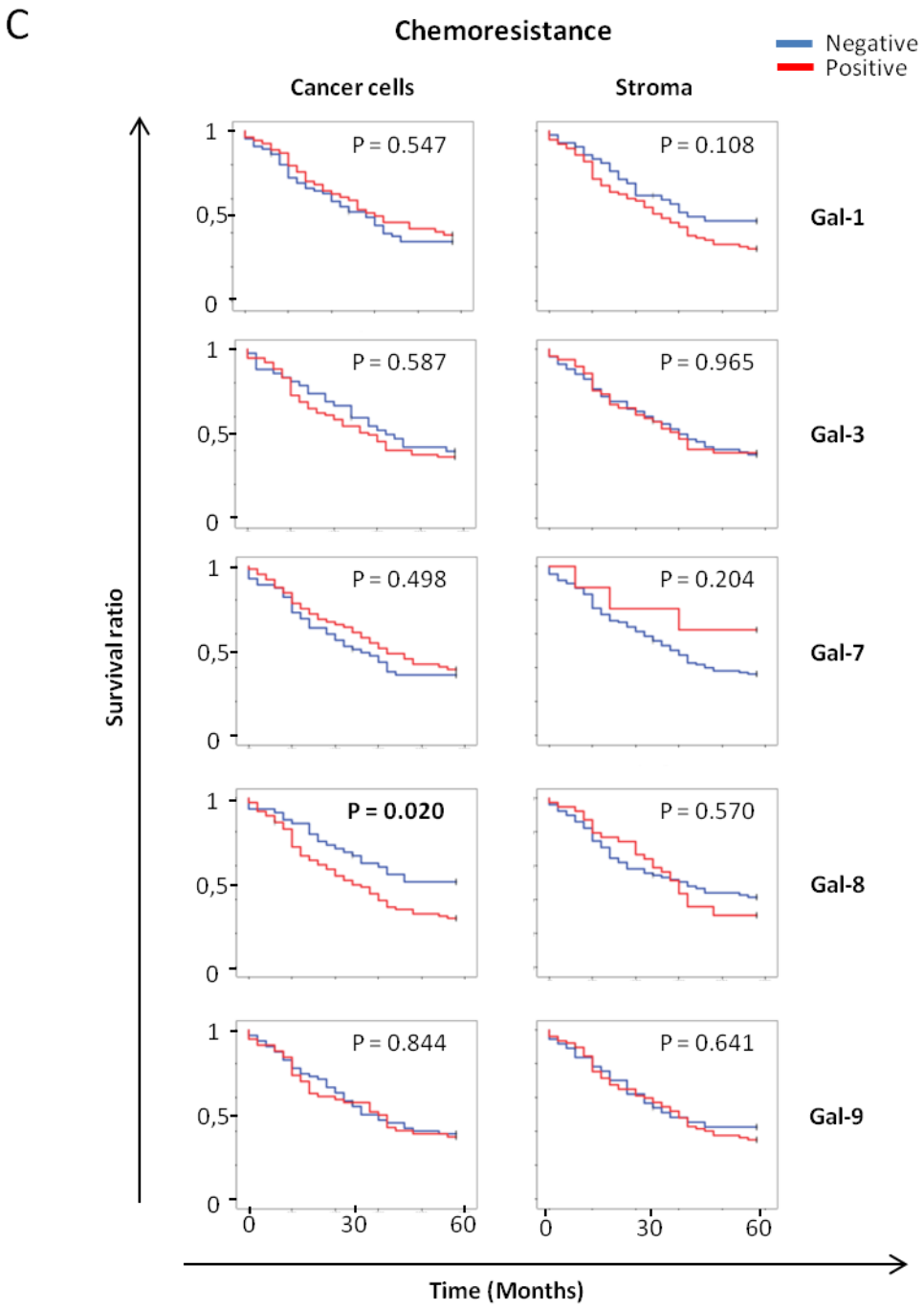
Figure S3 (Suite)

B



F

Figure S3 (suite)



CHAPITRE 5

DISCUSSION

Une étude préliminaire de l'expression de la galectine-7 dans plusieurs cancers nous avait permis de constater que l'expression de cette protéine est souvent altérée en situation de cancer (Annexe I). Plus précisément, nous avons pu observer une surexpression ou une absence de la protéine dans différents cancers. Deux types de cancers avaient attiré notre attention : le cancer de la prostate et le cancer de l'ovaire. Nous avons constaté que la galectine-7 est exprimée dans les cellules basales de la prostate alors qu'elle est absente de l'ovaire sain. En situation de cancer, nous avons observé une répression de la galectine-7 dans le cancer prostatique et sa surexpression dans les cancers ovariens. L'objectif de mon projet était d'étudier l'expression et la fonction de la galectine-7 dans ces deux cancers. Globalement, pour le cancer de la prostate nous avons démontré que : 1) l'expression de la galectine-7 est réprimée dans le cancer; 2) elle a une fonction anti-tumorale via la sensibilisation à l'apoptose et l'inhibition du potentiel invasif; 3) la fonction pro-apoptotique est CRD-indépendante alors que la fonction dans le potentiel invasif est CRD-dépendante. Pour ce qui est du cancer ovarien, nous avons démontré que : 1) la galectine-7 est exprimée dans le cancer de l'ovaire et est associée aux cancers agressifs; 2) elle stimule le potentiel invasif via la motilité et la sécrétion de MMP-9; 3) elle peut induire l'immunoévasion en provoquant l'apoptose des cellules immunitaires. Pour faire suite à ces deux études, nous nous sommes intéressés à l'expression des autres galectines. Il est important de mentionner que jusqu'à maintenant, la plupart des études sur le rôle des galectines dans le cancer se sont concentrées sur les galectines-1 et -3. On connaît encore très peu les profils d'expression et le rôle des autres galectines dans les différents cancers, si ce n'est d'une étude récente du groupe de Gabriel Rabinovich sur le cancer de la prostate [144]. Nous avons donc procédé à une étude exploratoire sur les autres galectines en nous concentrant sur le cancer de l'ovaire, puisqu'il est impératif de trouver de nouveaux biomarqueurs pour ce cancer et des nouvelles cibles thérapeutiques. Nos résultats nous ont ainsi permis d'identifier une signature d'expression des galectines qui pourrait servir d'outil pour mieux prédire la progression de la maladie et la réponse au traitement. Plus spécifiquement, nous avons pu établir que : 1) l'expression des galectines-1, -8 et -9 a un potentiel comme facteur prédictif; 2) la localisation cellulaire et la distribution tissulaire des galectines doivent être prises en compte pour évaluer avec précision le potentiel des galectines comme biomarqueurs; et 3) le «galectinome» pourrait être utilisé comme biomarqueur prédictif chez les patientes qui sont CA125 négatives.

Expression de la galectine-7 dans les tissus sains et cancéreux de la prostate

Lors de notre étude, nous avons vérifié l'expression de la galectine-7 dans le cancer de la prostate et avons pu déterminer que les tissus du cancer de la prostate n'expriment pas la galectine-7. Dans la majorité des cas, les tumeurs prostatiques sont des adénocarcinomes

acinaires et ont un phénotype de cellules luminales [232]. Les cellules basales sont absentes de ces tumeurs, ce qui explique sans doute en partie l'absence de galectine-7 dans les tissus cancéreux de la prostate et qui laisse croire qu'elles pourraient nuire à la progression tumorale si elles étaient présentes. De plus, les cancers prostatiques de type basal sont très rares, supportant l'idée que ces cellules pourraient afficher des fonctions anti-tumorales. L'origine du cancer de la prostate est encore méconnue, mais selon les dernières études, les adénocarcinomes pourraient être d'origine luminale ou basale [97]. Il a d'ailleurs été démontré que des cellules basales peuvent former des adénocarcinomes acinaires en réponse à des stimulations oncogéniques [97]. Ainsi, dans la situation où les adénocarcinomes proviendraient de cellules basales, la répression de la galectine-7 serait une étape importante de la progression tumorale. Comme il a été précédemment démontré, le promoteur de la galectine-7 peut être méthylé, ce qui inhibe l'expression du gène et subséquemment de la protéine [233]. Il pourrait s'agir du mécanisme par lequel l'expression de la galectine-7 est réprimée.

Localisation cellulaire de la galectine-7

La galectine-7 n'est pas la seule galectine dont l'expression est inhibée dans le cancer de la prostate, puisqu'un phénomène similaire est observé avec la galectine-3. Cette dernière possède une distribution nucléaire dans les cellules épithéliales de la prostate et son expression est réprimée au cours de la progression tumorale [143]. Dans les rares cas où la galectine-3 est exprimée, elle se retrouve dans le compartiment cytosolique et affiche des fonctions pro-tumorales [65]. En fait, Califice *et al.* ont pu démontrer que la localisation intracellulaire de la galectine-3 dicte ses fonctions puisque la protéine nucléaire est anti-tumorale et que la protéine cytoplasmique agit comme un oncogène [65]. Le cas de la galectine-7 semble être différent puisque la localisation nucléaire ne semble pas être essentielle à certaines de ses fonctions anti-tumorales.

Lorsque nous avons testé l'effet d'une mutation dans le CRD de la galectine-7, nous avons constaté que la localisation mitochondriale et nucléaire est dépendante du CRD alors que la localisation cytoplasmique et la sécrétion de la protéine ne semblent pas l'être, du moins en ce qui a trait aux cellules du cancer prostatique. Ces résultats suggèrent que la distribution nucléaire et mitochondriale de la galectine-7 sont dépendantes du CRD et qu'elles nécessitent sans doute des interactions avec des transporteurs glycosylés. Les transporteurs intracellulaires de la galectine-7 et les mécanismes de sécrétions sont encore toutefois inconnus.

Il est bien connu que la galectine-7, comme plusieurs autres protéines, peut afficher des fonctions pro- ou anti-apoptotiques, dépendamment du type cellulaire [27]. Ce qui est intéressant est qu'aujourd'hui nous pouvons affirmer que cet effet est indépendant de la localisation mitochondriale ou nucléaire. De plus, il est peu probable que la fonction pro-apoptotique de la galectine-7 soit dépendante de la protéine extracellulaire puisque plusieurs études ont démontré que les fonctions pro-apoptotiques des galectines extracellulaires sont dépendantes du CRD [21]. Notre étude sur le cancer de la prostate et une étude que nous avons récemment fait sur le cancer du sein (Annexe II) indiquent toutes deux que la modulation de l'apoptose se fait au niveau du cytosol. Dans notre étude sur le cancer du sein, nous avons pu démontrer que la galectine-7 a une fonction anti-apoptotique et que le mutant du CRD, qui est principalement cytosolique, affiche aussi des propriétés anti-apoptotiques [24]. Il a déjà été démontré que la galectine-7 se lie à Bcl-2 [44]. La famille Bcl-2 comprend plusieurs membres pro- et anti-apoptotiques, dont Bcl-2, Mcl-1, Bcl-xL, Bak et Bax [234]. Ceux-ci possèdent une homologie de séquence dans plusieurs domaines protéiques (BH1 à BH4). Ces domaines leur servent à interagir entre elles et permettent la régulation du leur transport entre le cytosol et la mitochondrie [234]. À ce jour, notre hypothèse est que la galectine-7 se lie de façon CRD-indépendante à certains membres de la famille Bcl-2 et perturbe ainsi la balance de leur localisation cytosolique ou mitochondriale. Dans notre modèle du cancer de la prostate, il est possible que la galectine-7 perturbe la translocation de Bcl-2 à la mitochondrie. Suivant un signal inducteur de mort cellulaire, l'activation des protéines pro-apoptotiques Bax et Bak serait donc favorisée.

Importance des interactions CRD-dépendantes

Nous avons observé quelques différences fonctionnelles entre la galectine-7 de type sauvage et la galectine-7 dont le CRD est muté. Premièrement, la protéine sauvage réduit la motilité des cellules cancéreuses prostatiques, inhibant par le fait même leur potentiel invasif. La forme mutée de la galectine-7 n'a aucun effet sur le potentiel invasif des cellules DU-145. Ainsi, il semblerait que le CRD soit impliqué dans la modulation de la motilité. Cette action n'est pas dépendante de la protéine extracellulaire, puisque l'ajout de galectine-7 recombinante ne module pas la motilité. Nous avons noté une forte présence des protéines sauvages et mutées au niveau des filopodes et de l'actine corticale. Deux équipes de recherche avaient précédemment démontré que la galectine-7 est exprimée dans les organes de motilité (pseudopodes et cils primaires) de cellules épithéliales [235, 236]. Il existe plusieurs protéines glycosylées qui sont associées aux structures de motilité [237, 238]. Par exemple, la cofiline est une protéine glycosylée associée au cytosquelette d'actine [238]. Il a été précédemment démontré dans un modèle de cancer du sein que la O-glycosylation de cette protéine est essentielle à sa localisation au niveau des filopodes et au

potentiel invasif des cellules du cancer du sein [238]. Il est possible que la galectine-7 participe à la motilité via son interaction avec certaines protéines glycosylées en association avec le cytosquelette d'actine et les structures de motilité.

Utilisation d'inhibiteurs du CRD dans le cancer

Nos expériences *in vivo* ont dévoilé une fonction inattendue de la galectine-7. Alors que la présence de la protéine de type sauvage réduit la croissance tumorale *in vivo*, la galectine-7 ayant un CRD défectueux la stimule. Ce résultat était surprenant à prime abord puisqu'aucune différence au niveau de la prolifération des cellules DU-145 n'avait été observée *in vitro* et que les deux protéines (sauvage et mutée) avaient des propriétés pro-apoptotiques. Notre hypothèse actuelle est que la mutation au niveau du CRD de la galectine-7 favorise ses fonctions CRD-indépendantes et mène à une réponse différente en présence de facteurs ou de cellules présentes dans l'environnement péritumoral. Les interactions CRD-indépendantes des galectines extracellulaires existent, mais sont très peu connues. Par exemple, la galectine-1 peut se lier au pré-récepteur des cellules B via des résidus hydrophobes et cette liaison permet l'oligomérisation du récepteur [48]. Il est difficile d'étudier les interactions CRD-indépendantes extracellulaires puisque la majorité des récepteurs membranaires et des cellules de la matrice extracellulaires sont glycosylés [1, 2]. Ainsi, il est possible que la galectine-7 possède de telles interactions dans le milieu extracellulaire et que la mutation du CRD augmente sa liaison à certains récepteurs ou molécules, favorisant la tumorigenèse. Par exemple, la galectine-7 mutée pourrait avoir plus de facilité à stimuler l'angiogenèse et ainsi favoriser la croissance tumorale. Il serait pertinent, dans une étude future, de mieux caractériser les tissus tumoraux prélevés sur les souris et ainsi vérifier l'indice de prolifération de cellules cancéreuses prostatiques ainsi que l'angiogenèse en présence de galectine-7 sauvage et mutée.

Globalement, ces résultats amènent à une réflexion importante face à l'utilisation d'inhibiteurs de galectines qui ciblent le CRD pour le traitement du cancer. Plusieurs compagnies développent actuellement des inhibiteurs de galectines qui ciblent le CRD. Le GCS-100 et le GM-CT-01 en sont des exemples. Ils sont composés de MCP (*modified citrus pectin*) et de polysaccharides, respectivement, et ils sont reconnus pour inhiber la galectine-3, bien qu'ils soient capables d'inhiber les fonctions du CRD de la galectine-1 et possiblement d'autres galectines. Ces inhibiteurs sont actuellement évalués dans des essais cliniques pour le traitement de différents types de cancers [211, 212, 239, 240]. Puisque les galectines ont une grande homologie dans la séquence de leur CRD, il est logique de croire que ces inhibiteurs auront des effets sur l'activité d'autres galectines présentes au niveau de la tumeur. Nos résultats et ceux du groupe de Rabinovich ont d'ailleurs permis de bien

mettre en évidence qu'il existe un répertoire important et varié de galectines au niveau de la cellule cancéreuse et du microenvironnement tumoral.

Expression de la galectine-7 dans le cancer ovarien

Nos études dans le cancer de l'ovaire ont mis en évidence l'association entre la présence de galectine-7 dans plusieurs sous-types histologiques du cancer ovarien. Nous avons aussi constaté que la galectine-7 est absente des tissus sains de l'ovaire et des trompes de Fallope et rarement exprimée dans les tumeurs bénignes. En fait comme le démontrent nos résultats *in silico* de la transformation de cellules ovariennes chez la souris, l'induction de l'expression de la galectine-7 semble être un événement qui se produit lors de la transformation cellulaire et qui perdure tout au long de la progression tumorale. Il est donc possible que la galectine-7 favorise la transformation, tout comme le font les galectine-1 et -3 via leur liaison à Ras [66, 241]. La liaison entre les galectines-1 et -3 et les membres de la famille Ras se fait via un domaine de liaison au prényle présent dans le CRD, mais est indépendante de la glycosylation [137]. Puisque les séquences du CRD sont similaires entre les galectines, il est possible que la galectine-7, et possiblement les autres galectines, possède également ce domaine et puisse donc stimuler la transformation.

Nous avons aussi pu démontrer que la galectine-7 est exprimée dans près de la moitié des cancers de types séreux de haut grade. Une hypothèse qui pourrait expliquer l'origine de l'induction de la galectine-7 serait la mutation du gène *TP53* qui code pour la protéine p53. Il a été démontré que dans plus de 95 % des cancers de l'ovaire de type séreux de haut grade, la protéine p53 est mutée [242]. Dans une étude précédente sur le cancer du sein, nous avions démontré que l'expression de la galectine-7 est induite par certaines formes mutées de la protéine p53 (Annexe V) [243]. Nous avons reproduit ces résultats dans les cellules du cancer de l'ovaire, supportant l'hypothèse que la présence de la protéine p53 mutée pourrait être responsable de l'expression anormalement élevée de galectine-7. Alternativement, il est aussi possible que cette expression soit induite suivant une hypométhylation globale du génome que l'on observe dans les cellules cancéreuses et qui serait responsable de l'expression de nombreux gènes pro-cancéreux [244].

Nos observations démontrant que l'expression de la galectine-7 au niveau protéique est plus souvent retrouvée dans des tumeurs de haut grade et dans les métastases comparativement aux tumeurs primaires sont compatibles avec nos analyses *in silico* de la banque de données TCGA qui suggère que la surexpression de la galectine-7 est associée à un taux de survie plus bas. À l'inverse, notre étude qui portait sur les cancers séreux de haut grade n'a révélé aucune différence entre le pronostic des patientes qui ont une tumeur positive pour la

galectine-7 et les patientes qui ont une tumeur négative. La différence entre ces résultats peut s'expliquer par les différentes cohortes de patientes. Dans notre première étude préliminaire, nous avons utilisé une micromatrice tissulaire qui comprenait des échantillons de différents sous-types histologiques et de différents grades, alors que les patientes de notre deuxième étude étaient toutes atteintes de cancers séreux de haut grade. Dans la banque de données de TCGA, il s'agissait aussi de patientes atteintes de ce sous-type histologique. Afin d'élucider l'importance de la présence de galectine-7 dans le cancer de l'ovaire, il serait important de confirmer nos résultats sur une cohorte de patientes plus grande et indépendante.

Rôle immunosuppressif de la galectine-7

Très peu d'études ont porté sur la présence et le rôle de la galectine-7 extracellulaire. Les indications suggérant qu'elle pourrait moduler la réponse immunitaire sont encore plus rares. Afin d'étudier cette question, nous avons fait appel à plusieurs modèles cellulaires qui nous ont permis de démontrer que la galectine-7 est relâchée dans le milieu extracellulaire par plusieurs types de cellules (kératinocytes, cancer du sein, de la prostate et de l'ovaire). Nos études sur les micromatrices tissulaires soulèvent aussi la possibilité que la galectine-7 soit relâchée par les cellules du stroma. Il est donc logique de croire que la galectine-7 pourrait se trouver en quantité suffisante pour moduler la réponse immune locale, comme il a été démontré pour la galectine-1. Nous avons d'ailleurs démontré que la galectine-7 peut induire l'apoptose des cellules T CD4+, T CD8+ ainsi que des monocytes. Ce potentiel immunosuppresseur de la galectine-7 est d'ailleurs appuyé par les résultats de deux autres études. Dans le premier cas, des cellules Jurkat ont été traitées avec de la galectine-7 recombinante et les auteurs ont pu observer l'apoptose des cellules immunes ainsi qu'une diminution de la sécrétion d'IL-2 et d'INF-γ [245]. Dans la deuxième étude, Rossi *et al.* ont démontré que la galectine-7 exogène permet d'éliminer les lymphocytes T CD4+ et T CD8+ et que les lymphocytes activés T CD8+ sont plus sensibles que les T CD4+ [246]. Dans un contexte tumoral, cela indique que la galectine-7 pourrait éliminer les cellules T infiltrant, dont les cellules T CD8+, réduisant la réponse anti-tumorale et menant donc à l'immunoévasion. Dans de futures recherches, il serait intéressant de vérifier l'effet de la galectine-7 sur les cellules Treg. Plusieurs études ont d'ailleurs démontré que les galectines, comme galectines-1, 3, 9 et 10, contribuent à l'activité suppressives des Treg [247].

Induction du potentiel invasif par la galectine-7

En ce qui concerne la motilité, nos résultats dans le cancer de l'ovaire contrastent avec ceux que nous avons obtenu dans nos études sur le cancer de la prostate. Alors que la galectine-7 réduit la motilité des cellules prostatiques, elle semble essentielle à la migration des

cellules du cancer de l'ovaire. Il est difficile d'expliquer une telle différence puisque les partenaires d'interaction de la galectine-7 sont méconnus. Une première hypothèse serait que la galectine-7 se lie à des protéines glycosylées associées au cytosquelette d'actine et que la disponibilité de ces protéines est différente selon le type cellulaire. Nous savons aussi que les galectines peuvent moduler certaines voies de signalisation, telle que la voie de wnt/β-caténine [27]. La double fonction de la galectine-7 pourrait provenir de l'activation ou de l'inhibition de voies de signalisation distinctes en fonction du type cellulaire. Par exemple, dans le cancer de l'ovaire, la galectine-7 pourrait interagir avec la protéine β-caténine via son CRD et la stabiliser à la membrane cellulaire. Ceci aurait pour effet de stabiliser l'E-cadhérine et ainsi favoriser la motilité. L'E-cadhérine est une protéine impliquée dans les jonctions cellulaires et il a été établi par des expériences chez la souris que son interaction avec la β-caténine est essentielle à la migration des cellules [248]. D'ailleurs, il a été démontré que la galectine-3 interagit avec la β-caténine, ce qui appuie l'hypothèse que la galectine-7 pourrait elle aussi interagir avec cette protéine [249-251].

Signature d'expression des galectines dans le cancer ovarien

En établissant le «galectinome» du cancer de l'ovaire, nous avons pu constater que l'expression de toutes les galectines est potentiellement dérégulée dans ce cancer, définissant l'hétérogénéité de cette maladie. Certaines galectines, telles que les galectines-4 et -7, qui sont absentes des tissus sains de l'ovaire et de la trompe de Fallope, sont parfois surexprimées dans le cancer ovarien. De plus, nous avons pu constater des profils d'expression différents en fonctions des sous-types histologiques, ce qui laisse entrevoir la possibilité d'utiliser une signature de galectines afin de distinguer les sous-types histologiques. Par exemple, selon nos résultats et ceux d'autres groupes de recherche, la galectine-4 semble exclusive au cancer de type mucineux [208]. Une étude indépendante, comprenant plus de cas pour chaque sous-type histologique serait nécessaire afin de d'établir une signature de galectine capable de prédire le sous-type.

Malgré la fréquence à laquelle les galectines sont retrouvées dans le cancer ovarien, il est trop tôt pour savoir si elles sont toutes impliquées dans la tumorigenèse de l'ovaire ou si leur expression n'est qu'une conséquence d'autres mécanismes moléculaires. Dans tous les cas, il est bien établi que les galectines peuvent influencer positivement ou négativement la progression tumorale et qu'elles peuvent parfois servir à prédire le pronostic des patients [27, 252, 253]. Notre étude dans le cancer ovarien et une autre étude que nous avons effectuée récemment dans le cancer du sein mettent en évidence l'importance de tenir compte de la localisation cellulaire et de la distribution tissulaire des galectines lorsqu'elles sont utilisées comme biomarqueurs. Dans le cancer de l'ovaire, nous avons pu observer une

association entre la présence de galectine-1 stromale et un haut taux de récidive, la présence de galectine-8 épithéliale et la résistance à la chimiothérapie et enfin, la présence de puncta périnucléaires de galectine-9 qui sont associés à un faible taux de survie. Dans l'étude que nous avons mené dans le cancer du sein (Annexe III), nous avons établi une signature de galectines qui prédit le pronostic des patientes et le type de cancer [222]. La présence des galectines-1, -3, et -9 dans le stroma d'une même tumeur permet d'identifier un cancer de type triple négatif ou Her2-positif [222]. De plus, la présence de galectine-1 stromale est associée à un mauvais pronostic alors que la galectine-8 nucléaire est associée à un bon pronostic et semble prévaloir sur la galectine-1. Ces deux études sont parmi les premières à s'intéresser à la signature des galectines et à distinguer leur localisation. Ces résultats indiquent qu'il est fort probable que les galectines participent à la progression tumorale et que leurs fonctions sont altérées en fonction de leur localisation et de la présence d'autres galectines. Nos résultats démontrent donc clairement qu'il est non seulement important de tenir compte de la localisation des galectines dans une cellule, mais également d'en définir leur répertoire, particulièrement pour la galectine-8 qui semble, dans les cancers ovariens et du sein, être associée avec un bon pronostic lorsqu'elle est exprimée sous sa forme stromale et nucléaire respectivement. Ces résultats démontrent aussi l'importance de développer des inhibiteurs de galectines qui sont spécifiques.

Puncta périnucléaires de la galectine-9

Nous avons observé la présence de galectine-9 sous forme de puncta périnucléaires dans les cellules cancéreuses de plusieurs tumeurs de l'ovaire. Malgré nos efforts, nous n'avons pas pu mettre en lumière la nature de ces structures. Nous croyons toutefois qu'il faudra porter une attention particulière à la possibilité que celles-ci soient des lysosomes. Weirsma *et al.* ont en effet récemment démontré, dans un modèle de cancer colorectal, que la galectine-9 est associée aux lysosomes et empêche la fusion entre les lysosomes et les autophagosomes [22]. Dans leur cas, la présence de galectine-9 au niveau des lysosomes était associée à un effet anti-tumoral, ce qui n'est pas surprenant puisqu'il est bien connu que l'autophagie peut avoir des fonctions pro- ou anti-tumorale, dépendamment du contexte cellulaire [254]. Il serait donc possible que la galectine-9 soit associée au lysosome dans les cellules du cancer de l'ovaire et module ainsi les niveaux d'autophagie, favorisant la progression tumorale.

Plusieurs études ont démontré l'association entre la présence de galectine-9 et un bon pronostic [22, 221, 255, 256]. Par exemple, dans le cancer gastrique, la répression de la galectine-9 est associée à un mauvais pronostic et il a été démontré que l'expression de la protéine permet de réduire la prolifération des cellules cancéreuses, appuyant une fonction

anti-tumorale [256, 257]. Il est logique de croire que l'association entre la galectine-9 et un bon ou un mauvais pronostic puisse dépendre des isoformes de la protéine exprimés par la cellule. La galectine-9 est composée de deux CRD reliés entre eux par une chaîne peptidique [14]. Il existe trois gènes qui codent pour la galectine-9 : *LGALS9*, *LGALS9B* et *LGALS9C* et chacun de ces gènes peut produire différents variants par épissage alternatif [258, 259]. Jusqu'à aujourd'hui, cinq variants ont été caractérisés, dont trois ont des différences au niveau de la région peptidique qui relie les deux CRD et deux ont un CRD tronqué en C-terminal [258]. Lors de la caractérisation de ces variants, Heusschen *et al.* ont démontré que différents types de cellules n'expriment pas les mêmes isoformes de galectine-9 [258]. De plus, il semblerait que les différents isoformes n'aient pas les mêmes fonctions. Dans leur étude, Zhang *et al.* ont observé que les trois isoformes les mieux caractérisés de la galectine-9 (petit, moyen et large) n'ont pas la même affinité pour le lactose. De plus, les isoformes moyens et petits induisent l'expression d'E-sélectine sur les cellules LoVo et favorise leur adhésion aux cellules endothéliales, alors que l'isoforme long n'a pas cet effet [259]. Il serait donc possible que la galectine-9 ait des fonctions pro-tumorales ou anti-tumorales dépendamment des isoformes qui sont exprimés et de leur localisation cellulaire. À ce jour, il n'existe malheureusement pas assez d'information sur les différents isoformes pour déterminer si cette hypothèse est valide. De plus, les anticorps commerciaux anti-galectine-9 qui sont actuellement disponibles ne permettent pas de distinguer les différents isoformes. Une solution serait d'étudier la présence des différents isoformes par d'autres méthodes telles que l'hybridation *in situ* en immunofluorescence. Cela permettrait de vérifier la localisation de chaque isoforme et d'associer leur présence au pronostic de patients atteints de cancer.

Les galectines comme outils de pronostic et de diagnostic

Plusieurs équipes de recherche se sont intéressées à l'utilisation de galectines comme biomarqueurs pour différentes pathologies. L'exemple le plus éloquent est celui de la galectine-3 dans les problèmes cardiaques. En fait, il a été démontré que la présence de galectine-3 dans le sang de patients qui ont souffert d'un arrêt cardiaque est associée à des complications qui mèneront probablement à une réhospitalisation et possiblement au décès [260]. Le remodelage ventriculaire et la fibrose myocardiaque sont deux événements qui provoquent les arrêts cardiaques et la galectine-3 participe à ces deux mécanismes par la stimulation de l'inflammation et de la fibrose [261]. Ainsi, les auteurs de plusieurs études sur le sujet suggèrent d'utiliser la galectine-3 comme biomarqueur et cible thérapeutique (revue dans [260, 261]).

Le cancer de l'ovaire étant difficile à diagnostiquer, il serait pertinent de pouvoir développer un test sanguin qui permet de vérifier la présence de plusieurs protéines associées à cette pathologie. Les galectines sont sécrétées par les cellules cancéreuses de l'ovaire et elles peuvent se retrouver dans la circulation sanguine [21]. Puisque nous avons pu constater des changements au niveau de l'expression des galectines dans le cancer ovarien, il serait pertinent de vérifier si cette dérégulation peut être détectée par un test sanguin. Par exemple, nous avons démontré que la galectine-7 est absente des tissus sains de l'ovaire et des trompes de Fallope, mais qu'elle est exprimée dans plus de la moitié des cancers ovariens. Étant sécrétée par les cellules cancéreuses, il serait envisageable de détecter sa présence dans un échantillon de sang par un test ELISA. Il existe cependant un problème qui pourrait être rencontré lors de l'utilisation d'un tel test à des fins cliniques. Différentes conditions peuvent en effet mener à une dérégulation de la balance des galectines présentes dans le sang ce qui pourrait affecter le nombre de faux positifs. Par exemple, il a été démontré que les niveaux de galectine-9 sériques augmentent lors d'une infection par le virus de l'influenza [262]. Afin de contourner ce problème, l'utilisation d'une signature de galectine en combinaison avec d'autres biomarqueurs associés au cancer ovarien pourrait être envisageable afin de réduire les risques de faux positifs. Barrow *et al.* se sont intéressés à déterminer une telle signature dans le sang de patients atteints de cancer du côlon et du sein. Ils ont déterminé les niveaux sériques de galectines-1, -2, -3, -4, -8 et -9 et ont pu associer l'expression des galectines-2, -3, -4, et -8 au cancer et ils ont noté que l'expression de ces galectines était encore plus élevée dans les cancers agressifs [229].

Aujourd'hui le seul biomarqueur pour le cancer de l'ovaire qui est utilisé au niveau clinique est la protéine sérique CA125. Malheureusement, ce biomarqueur est inadéquat pour la détection de cette maladie, car 50 % des patientes avec un cancer de bas stade n'expriment pas la protéine [164]. CA125 est donc utilisé pour faire le suivi de la progression tumorale pendant et après le traitement de chimiothérapie. Malheureusement, près de 20 % des patientes ayant un cancer avancé n'expriment également pas CA125 et ne peuvent donc pas bénéficier de cet outil [164]. Nous avons pu établir une signature de galectines qui permet de prédire le pronostic des patientes qui sont CA125 positives ou négatives. La présence de galectine-1 stromale, de galectine-8 épithéliale ou de puncta périnucléaires de galectine-9 permet de prédire les patientes qui sont à haut risque. Dans de futures études, il serait important de vérifier s'il est possible de les détecter au niveau sérique, car ce serait un outil intéressant, complémentaire à CA125, pour faire le suivi de la maladie pendant et après le traitement. Il existe peu d'options pour le traitement des patientes atteintes du cancer ovarien. Il est fort probable que les galectines jouent un rôle dans la réponse à la chimiothérapie ainsi que dans l'immunoévasion tumorale. Une meilleure compréhension de

l’implication des galectines dans la progression tumorale permettrait possiblement de les utiliser comme biomarqueurs et cibles thérapeutiques.

CHAPITRE 6

CONCLUSION GÉNÉRALE

En conclusion, nos résultats apportent de nouvelles informations quant aux fonctions pro- et anti-tumorales de la galectine-7 et mettent en évidence l'importance d'étudier le «galectinome» des tumeurs tout en prenant en considération leur distribution cellulaire et tissulaire. Notre étude est la première à démontrer que la galectine-7 a une fonction anti-tumorale dans le cancer de la prostate et à mettre en évidence l'importance des interactions CRD-indépendantes de la galectine-7 pour ses fonctions pro- et anti-tumorales. De plus, nos résultats sur le cancer ovarien ont permis d'ajouter la galectine-7 sur la liste des molécules inhibitrices de la réponse anti-tumorale et mettent en évidence une nouvelle cible thérapeutique potentielle contre le cancer de l'ovaire. Enfin, dans une ère où la médecine personnalisée prend de l'ampleur, l'identification d'une signature de protéine prédictive du pronostic des patientes atteintes du cancer de l'ovaire est un pas de plus vers le développement de nouveaux tests cliniques qui permettront de mieux cibler les besoins de chaque patiente.

En perspective, plusieurs avenues pourraient être envisagées pour la poursuite de ce projet à court et à moyen terme. Premièrement, il sera important de mieux définir les fonctions CRD-indépendantes de la galectine-7 afin de pouvoir éventuellement l'utiliser comme cible thérapeutique dans le cancer. Ensuite, il serait pertinent d'évaluer les fonctions immunosuppressives de la galectine-7 dans un modèle *in vivo*. Enfin, il serait nécessaire de confirmer les résultats obtenus concernant le «galectinome» de l'ovaire sur une plus grande cohorte de patientes, indépendante de celle que nous avons utilisée. De plus, il faudrait étudier l'expression et la distribution des galectines-2, et -10 à -16 dans le cancer ovarien afin de compléter notre modèle de signature d'expression des galectines. Pour permettre l'utilisation des galectines comme outils de diagnostic et/ou de pronostic, il serait pertinent d'étudier leur présence dans le sang des patientes.

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ANNEXES

ANNEXE I

EXPRESSION DE LA GALECTINE-7 DANS DIFFÉRENTS CANCERS

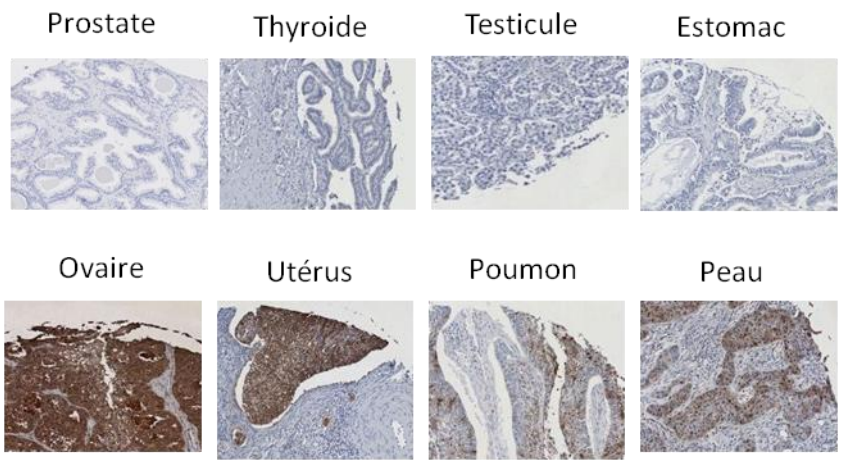

Figure AI.1 Expression de la galectine-7 dans le cancer. Analyse par immunohistochimie de l'expression de la galectine-7 dans le cancer de la prostate, de la thyroïde, des testicules, de l'estomac, de l'ovaire, de l'utérus, du poumon et de la peau.

ANNEXE II

LA GALECTINE-7 CYTOSOLIQUE INHIBE P53 ET INDUIT LA RÉSISTANCE À LA CHIMIOTHÉRAPIE DANS LES CELLULES DU CANCER DU SEIN

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Cytosolic galectin-7 impairs p53 functions and induces chemoresistance in breast cancer cells

Andrée-Anne Grosset[1,2], Marilyne Labrie[1], Donald Gagné[1], Maria-Claudia Vladoiu[1], Louis Gaboury[2], Nicolas Doucet[1] and Yves St-Pierre[1*]

Abstract

Background: Resistance to apoptosis induced by anti-cancer drugs is a major obstacle for the treatment of aggressive forms of breast cancer. Galectin-7 (gal-7) was recently shown to be specifically expressed in basal-like but not in luminal subtypes of human breast cancer.

Methods: We generated a mutant form of gal-7 (R74S). Arginine 74 is the structural equivalent of arginine 186 found in human galectin-3. Mutation R186S was previously shown to abolish the biological function of galectin-3.

Results: Mutation of arginine 74 induced only limited and local changes to the gal-7 fold. Recombinant forms of R74S and wtgal-7 were also equally effective at forming dimers in solution. Analysis of the thermodynamic parameters by isothermal titration calorimetry (ITC) indicated, however, that binding of lactose to gal-7 was inhibited by the R74S mutation. Using confocal microscopy and electron microscopy, we confirmed the expression of gal-7 in the cytosolic and nuclear compartments of breast cancer cells and the ability of gal-7 to translocate to mitochondria. The mutation at position 74, however, greatly reduced the expression of gal-7 in the nuclear and mitochondrial compartments. Interestingly, cells expressing mutated gal-7 were equally if not even more resistant to drug-induced apoptosis when compared to cells expressing wtgal-7. We also found that both wtgal-7 and R74S inhibited dox-induced PARP-1 cleavage and p53 protein expression. The inhibition of p53 correlated with a decrease in p21 protein expression and *CDKN1A* mRNA. Furthermore, analysis of nuclear and cytoplasmic fractions showed that both wild type and R74S mutant gal-7 inhibited p53 nuclear translocation, possibly by increasing degradation of cytosolic p53.

Conclusions: These findings pose a challenge to the paradigm that has guided the design of galectin-specific inhibitors for the treatment of cancer. This study suggests that targeting CRD-independent cytosolic gal-7 in breast cancer cells may be a valuable strategy for the treatment of this disease. Our study will thus complement efforts towards improving selectivity of targeted anticancer agents.

Keywords: Galectin-7, Localization, Apoptosis, p53, Breast cancer

Background

Members of the galectin family are characterized by their ability to bind β-galactosides via a highly conserved carbohydrate recognition domain (CRD). They play an important role in several physiological processes, including embryonic development, intercellular adhesion, host-pathogen interactions, cell migration, and immune response [1]. They are normally classified according to their structural organization. Galectins containing only one CRD are called prototype and include galectins 1, 2, 5, 7, 10, 11, 13, 14 and 15. Those with two distinct CRDs in tandem connected by a linker region (tandem-repeat type) are galectins 4, 6, 8, 9 and 12. Galectin-3 is the only member of the third group and is a chimera-type protein with one CRD connected to an unusual non-lectin domain rich in proline and glycine.

Historically, galectins have been known as small extracellular soluble that bind cell surface glycans, helping organizing membrane domains and regulating the signaling threshold and the receptor residency time [2]. Galectins, however, exhibits a wide range of subcellular

* Correspondence: yves.st-pierre@iaf.inrs.ca
[1]INRS-Institut Armand-Frappier, 531 Blv. des Prairies, Laval, Quebec H7V 1B7, Canada
Full list of author information is available at the end of the article

localizations, being found in both intracellular and extracellular compartments. Intracellularly, they have been reported to be exclusively/predominantly cytosolic, nuclear, mitochondrial, or distributed between the distinct subcellular compartments. Finally, even within a specific organelle, they appear to be distributed diffusely or to form aggregates or punctate structures. Such wide subcellular distribution significantly complicate galectin-targeted anticancer therapy since the pro- and the anti-tumoral functions of galectins differ according their subcellular localization [3].

Galectin-7 (gal-7) is a prototype galectin that forms homodimers [4]. Gal-7 is preferentially expressed in stratified epithelia, including epidermis, cornea, oral cavity, esophagus and rectal epithelium [5]. It is also expressed in mammary myoepithelial cells in tissues of normal individuals [6]. Its level of expression, however, is significantly altered in various types of cancer [7]. For example, gal-7 is expressed at higher levels in aggressive molecular subtypes of breast carcinoma, most notably in basal-like breast cancer with an ER/PR/HER-2 negative status [6]. Exogenous expression of gal-7 in breast cancer cell lines that express low or undetectable levels of gal-7 resulted in an increased metastatic behavior to the lung and bone and larger osteolytic lesions. Such pro-tumoral function of gal-7 has been largely attributed to its ability to protect cancer cells from pro-apoptotic signals [6,8]. Like other galectins, however, gal-7 is preferentially expressed intracellularly, most notably in cytosolic, nuclear and mitochondrial compartments [9-12]. Whether the resistance of breast cancer cells to apoptosis is dependent on the intracellular localization of gal-7 remains unknown. In the present work, we have addressed this question by generating a mutant form of gal-7 (R74S) with altered subcellular localization and tested its ability to mediate resistance of breast cancer cells to drug-induced cell death.

Methods

Tissue microarrays and immunohistochemistry

Representative specimens from our previous TMA analysis were immunostained for gal-7 using the Discovery XT automated immunostainer (Ventana Medical Systems, Tucson, AZ) [6]. Deparaffinized sections were incubated in Cell Conditioning 1 (pH 8.0) for antigen retrieval and then stained for 60 min with the anti-human gal-7 polyclonal antibody (R&D Systems, Minneapolis, MN) using a 1:150 dilution. The slides were counterstained with hematoxylin and bicarbonate. Each section was scanned at a high resolution using the Nanozoomer Digital Pathology System (Hamamatsu, Bridgewater, NJ). The study was approved by the research ethics committee of the research center at the Centre Hospitalier de l'Université de Montréal (approval No. SL 05.019).

Cell lines and reagents

The MCF-7 and MDA-MB-468 cell lines were provided by Dr. Peter Siegel (Rosalind and Morris Goodman Cancer Research Centre, McGill University, Montreal, QC, Canada) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 10 mM HEPES buffer and 1 mM sodium pyruvate. SKBR3 cells, obtained from Dr. Sylvie Mader (Institute for Research in Immunology and Cancer, University of Montreal, Montreal, QC, Canada), were grown in McCoy's 5A Medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 10 mM HEPES buffer at 37°C in a humidified atmosphere containing 5% CO_2. MCF10A and MCF12A protein extracts were provided by Dr. Isabelle Plante (INRS-Institut Armand-Frappier, Laval, QC, Canada). All cell culture products were purchased from Life Technologies (Burlington, ON, Canada). Cobalt chloride and lactose were purchased from Fisher Scientific (Ottawa, ON, Canada). MG-132 was from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Generation of stable transfectants expressing gal-7 and gal-7 R74S

To obtain stable MCF-7 breast carcinoma transfectants expressing gal-7, the cDNA encoding the human gal-7 (provided by Dr. Thierry Magnaldo) was cloned in srα eukaryotic expression vector (kind gift of Dr. François Denis) using *Spe*I and *Bam*HI restriction enzymes. The replacement of arginine 74 to serine (R74S) was introduced by oligo-directed site-specific mutagenesis using the forward (5′-GGC CGC GAG GAG TCC GGG CCG GGC GTT CCT- 3′) and reverse (5′ −GGC CGC GAG GAG TCC GGG CCG GGC GTT CCT- 3′) primers. Controls were generated using MCF-7 breast carcinoma cells transfected with the empty srα vector. Transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies). After 48 h of culture, transfected cells were allowed to grow in complete medium containing 1 μg/ml of puromycin. Individual colonies were expanded and gal-7 expression was monitored by Western blot analysis. All experiments were conducted with at least two independent clones expressing either wild type or mutant gal-7.

RNA isolation ant RT-PCR

Total cellular RNA was isolated from cells using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 μg of cellular RNA in a total reaction volume of 20 μL using the reverse transcriptase Omniscript (QIAGEN, Mississauga, ON, Canada). After reverse transcription, human *p53* (gene ID 7157, sense primer: 5′- CCA

GCC AAA GAA GAA ACC A -3′ and antisense primer: 5′- TAT GGC GGG AGG TAG ACT GA -3′), human *p21* (gene ID 1026, sense primer: 5′- CTG GAG ACT CTC AGG GTC GAA -3′ and antisense primer: 5′- GGA TTA GGG CTT CCT CTT GGA -3′) and *GAPDH* (gene ID 2597, sense primer: 5′- CGG AGT CAA CGG ATT TGG TCG TAT-3′ and antisense primer: 5′-CAG AAG TGG TGG TAC CTC TTC CGA -3′) cDNAs were amplified using the following conditions: 94°C for 3 min, followed by 25 to 35 cycles of the following: 94°C for 40 seconds, 60°C for 40 seconds, and 72°C for 40 seconds, followed by a final extension step at 72°C for 10 min. PCR was performed in a thermal cycler (Eppendorf, Mississauga, ON, Canada). The amplified products were analyzed by electrophoresis using 1.5% agarose gels and SYBR Safe (Life Technologies) staining and UV illumination.

Co-immunoprecipitation

MCF-7 stable transfectants expressing exogenous gal-7 and R74S mutant and MCF10A were transfected with vectors encoding wild type p53 (Origene, Burlington, MA). After 24 hrs, the cells were lysed in immunoprecipitation (IP) buffer containing 2% (v/v) CHAPS, 50 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM EDTA and protease inhibitors (Roche, Laval, QC, Canada). Equal amounts of whole cell protein extracts were used for each IP. Rabbit anti-p53 antibody (FL393; Santa Cruz Biotechnology, Santa Cruz, CA) or IgG control antibody (2 μg) were incubated 10 min at room temperature with Dynabeads Protein G (Life Technologies). The Dynabeads-antibody complex was incubated with proteins overnight at 4°C. After several washes in IP buffer, the protein complexes were resuspended in Laemmli loading buffer. Immunoprecipitated proteins were separated on a 15% SDS-PAGE gel and analyzed by Western blotting using anti-gal-7 and anti-p53 as described below.

Western blot analysis

Whole cell extracts were suspended using RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL) and protease inhibitors (Roche). Mitochondria and nuclear proteins were extracted using a kit (Thermo Fisher Scientific; Sigma-Aldrich) following the manufacturer's instructions. Protein concentrations were measured using a protein assay reagent (Bio-Rad Laboratories, Mississauga, ON, Canada). Equal amounts of proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were first blocked with 5% (v/v) milk in PBS/0.05% Tween 20 for 1 h and subsequently blotted overnight at 4°C with primary antibodies: goat anti-human gal-7 polyclonal antibody (1:1000; R&D Systems, Minneapolis, MN), rabbit anti-p53 (FL393; 1:1000; Santa Cruz Biotechnology), rabbit anti-p21 (1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-poly (ADP-ribose) polymerase (PARP)-1 (p25) monoclonal antibody (1:10000; Epitomics, Burlingame, CA), rabbit anti-COX IV polyclonal antibody (1:1000; Cell Signaling Technology), mouse anti-lamin A/C monoclonal antibody (1:1000; Cell Signaling Technology), rabbit anti-β-tubulin monoclonal antibody (1:10000; Cell Signaling Technology), and mouse anti-β-actin monoclonal antibody (1:20000; Sigma-Aldrich). Secondary antibodies consisted of horseradish peroxidase conjugated donkey anti-goat (R&D Systems), anti-rabbit or anti-mouse (GE Healthcare, Buckinghamshire, England). Detection was performed by the enhanced chemiluminescence method (GE Healthcare).

Immuno-electron microscopy

Cells were fixed in a 0.1% (v/v) gluteraldehyde and 4% (v/v) paraformaldehyde solution and embedded in the low viscosity embedding Spurr media. Ultrathin sections were cut, placed on nickel grids and incubated in sodium metaperiodate. Samples were blocked in 1% (v/v) BSA for 5 min, incubated 60 min in a goat anti-human gal-7 polyclonal antibody (1:150) and 60 min in a rabbit anti-goat 10 nm gold-conjugated secondary antibody (1:20, Electron Microscopy Sciences, Hatfield, PA). Each section were counterstained with uranyle acetate and lead citrate and visualized using a Hitachi 7100 transmission electron microscope.

Apoptosis detection by flow cytometry

The percentage of apoptotic cells was measured by two-color flow cytometry using Alexa Fluor 488 annexin V conjugate (Life Technologies) and propidium iodide (PI). Briefly, 1.75×10^5 cells were treated with 150 μM cobalt chloride overnight at 37°C without serum. Cells were then harvested, stained and analyzed by flow cytometry using a FacsCalibur (BD Biosciences, San Jose, CA).

Production of recombinant gal-7

Each of the DNA fragments coding for gal-7 and R74S was cloned into pET-22b(+) using *Nde*I and *Hin*dIII restriction enzymes. Recombinant proteins were expressed in *E. coli* BL21(DE3) at 37°C following addition of 1 mM IPTG at an $OD_{600\ nm}$ = 0.6-0.7 and an incubation of 4 h. Bacterial pellets were resuspended in lysis buffer (0.7 mg/mL lysozyme, 10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail), incubated for 1 h at 37°C and centrifuged for 30 min at 15 000 *g* (4°C). The supernatant was then filtered and applied to a lactose-agarose column and the protein was eluted in one mL fractions with 150 mM lactose. Fractions were analyzed by SDS-PAGE. Gal-7 and R74S were dialyzed against 20 mM potassium phosphate at pH 7.2 for all subsequent characterization experiments. ^{15}N-labeled samples were prepared by

growing *E. coli* BL21(DE3) in M9 minimal medium as previously described [13].

Solution NMR experiments

Proteins were at a concentration of 535 μM for gal-7 and 200 μM for gal-7 R74S. A 10% (v/v) D_2O solution was added to the protein samples for NMR spin-lock purposes. Protein concentration was determined by UV–vis spectrophotometry using an extinction coefficient of 8030 M^{-1} cm^{-1} at 280 nm [14]. ^{1}H-^{15}N HSQC spectra were acquired at 800 MHz on a Varian (Agilent) NMR spectrometer equipped with a triple resonance probe and pulsed-field gradients. All spectra were acquired at 310 K as calibrated with a standard methanol sample. The ^{1}H-^{15}N HSQC experiments were conducted with 256 t_1 and 8192 t_2 points with proton and nitrogen spectral widths of 3000 and 8000 Hz, respectively. Spectra were processed using NMRPipe [15] and further analysed using Sparky [16]. The ^{1}H-^{15}N composite chemical shift differences (Δδ) were calculated between wild-type and mutant enzymes according to the following equation [17]: $\Delta\delta\ (ppm) = [(\Delta\delta_{HN}^2 + \Delta\delta_N^2/25)/2]^{½}$. Only chemical shift variations showing Δδ >0.02 ppm were considered significant.

Isothermal titration calorimetry (ITC)

Lactose was reconstituted in a 20 mM potassium phosphate buffer at pH 7.2. Gal-7 and R74S were dialyzed in the same buffer after purification. All experiments were performed in a Nano ITC microcalorimeter (TA Instruments, New Castle, DE) at 25°C with a stirring rate of 250 rpm. Pre-equilibrated solutions of 200 μM protein and 6 mM ligand were used for each assay. A control experiment was performed by titrating lactose into protein-free buffer. Each experiment consisted of 20 injections of 2 μL ligand into protein, with an interval of 130 seconds between injections. All experiments were performed at least in triplicate. Data was analyzed and fitted using the NanoAnalyze software v2.3.6 (TA Instruments).

FITC conjugation and gal-7 binding assay

Briefly, 10 μl of a 2 mg/ml fluorescein isothiocyanate (FITC)/DMSO solution was added to 300 μl of 1.7 μg/μl recombinant wtgal-7 or R74S in a 0.1 M NaHCO3 pH 9.2 solution and incubated for 2 hrs at room temperature on a roller. FITC-conjugated wtgal-7 or R74S was then purified using a PD-10 sepharose column (GE healthcare) and eluted with PBS containing 0.01% [v/v] sodium azide (PBA). To measure FITC-wtgal-7 or R74S binding to cell surface, 2.5×10^5 cells were incubated for 30 min with the indicated concentrations. Cells were washed 2 times with PBA and resuspended in 500 μl PBA. Samples were analyzed by FACSCalibur (BD Biosciences).

Results

Subcellular localization of gal-7 in human breast cancer cells

Analysis of gal-7 expression in normal mammary epithelium shows positive gal-7 staining in both the nuclear and cytoplasmic compartments of myoepithelial cells (Figure 1A). A similar pattern of expression was observed in tissue sections obtained from patients with breast cancer, most notably in sections from patients with basal-like breast cancer, where gal-7 is preferentially expressed [6]. The expression of gal-7 in cytosolic and nuclear compartments was also confirmed

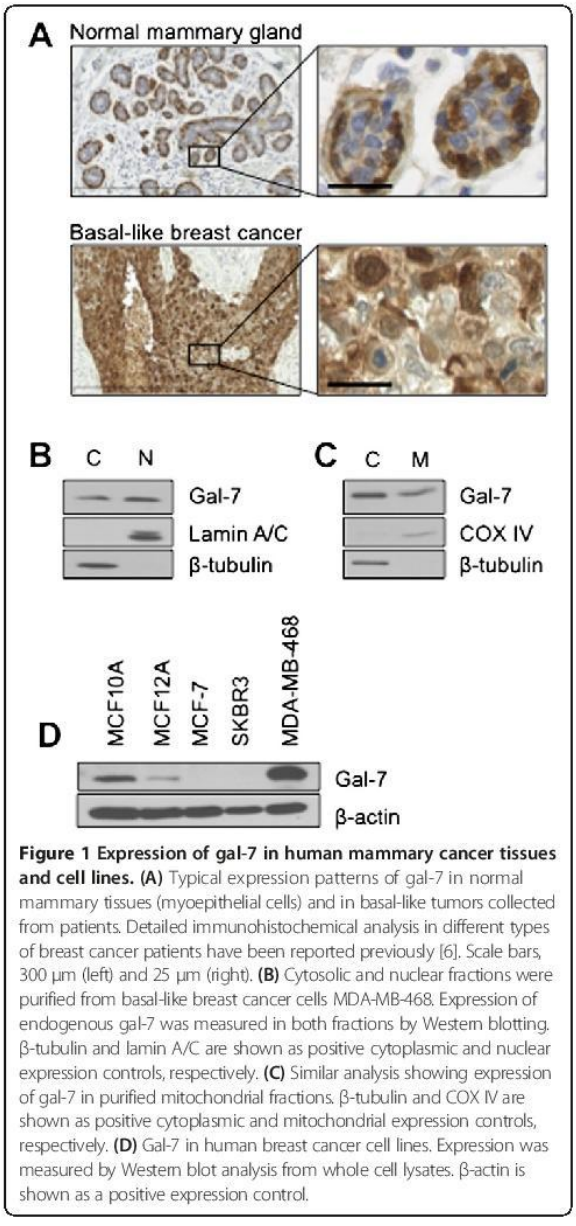

Figure 1 Expression of gal-7 in human mammary cancer tissues and cell lines. (A) Typical expression patterns of gal-7 in normal mammary tissues (myoepithelial cells) and in basal-like tumors collected from patients. Detailed immunohistochemical analysis in different types of breast cancer patients have been reported previously [6]. Scale bars, 300 μm (left) and 25 μm (right). **(B)** Cytosolic and nuclear fractions were purified from basal-like breast cancer cells MDA-MB-468. Expression of endogenous gal-7 was measured in both fractions by Western blotting. β-tubulin and lamin A/C are shown as positive cytoplasmic and nuclear expression controls, respectively. **(C)** Similar analysis showing expression of gal-7 in purified mitochondrial fractions. β-tubulin and COX IV are shown as positive cytoplasmic and mitochondrial expression controls, respectively. **(D)** Gal-7 in human breast cancer cell lines. Expression was measured by Western blot analysis from whole cell lysates. β-actin is shown as a positive expression control.

by western blot analysis of subcellular fractions isolated from MDA-MB-468 cells, which constitutively express gal-7 (Figure 1B-D). We also confirmed the ability of gal-7 to translocate to mitochondria in breast cancer cells, as recently observed in human colon carcinoma cells [11].

Generation of the R74S mutant

Site-directed mutagenesis was used to generate mutants of gal-7. A special attention was paid to arginine 74, the structural equivalent of arginine 186 in human galectin-3. Mutation R186S was previously shown to abolish the biological function of galectin-3 [18]. The replacement of arginine 74 to serine (R74S) was thus introduced in the human gal-7 gene by oligo-directed site-specific mutagenesis. To verify the integrity and structural perturbations caused by the R74S mutation, we used solution NMR spectroscopy, which provides a fast and highly sensitive assessment of structural perturbations caused by point mutations in proteins. For this purpose, the wild-type gal-7 (wtgal-7) and variant R74S were isotopically labeled, overexpressed in *E. coli* BL21(DE3) and purified to homogeneity. Their two-dimensional heteronuclear single quantum coherence spectra (^{1}H-^{15}N HSQC) were then acquired and overlaid (Figure 2A). Our ^{1}H-^{15}N HSQC spectral analysis showed that the R74S mutation induced only limited and local changes to the gal-7 fold (Figure 2A-D). Recombinant forms of R74S and wtgal-7 were also equally effective at forming dimers in solution (Figure 2E). Analysis of the thermodynamic parameters of the proteins by isothermal titration calorimetry (ITC) indicated, however, that binding of lactose to gal-7 was partially inhibited by the R74S mutation, with a K_d value of 720 μM for the R74S variant relative to 378 μM for the wild-type protein. A typical titration profile is shown in Additional file 1: Figure S1. This finding was corroborated by our flow cytometric analysis showing that binding of recombinant R74S to glycan receptors on the surface of Jurkat T cells was significantly lower than that observed with the wild-type protein (Additional file 2: Figure S2).

Functional characterization of the R74S mutant

Because MCF-7 cells have been extensively used as a model system for human breast cancer, we have used this cell line to express and further characterize the R74S mutant. The relevance of MCF-7 model for our studies was first established by expressing the wild-type form of gal-7 (wtgal-7). Western blotting analysis of stable transfectants showed that wtgal-7 was present in cytosolic, nuclear, and mitochondrial extracts of MCF-7 cells transfected with an expression vector encoding wtgal-7 (Figure 3A-B), a pattern similar to that found in MDA-MB-468 cells. Immunogold immunohistochemistry by electron microscopy (EM) confirmed the presence of gal-7 in these subcellular compartments (Figure 3C-F). The gold beads labeled mitochondria on the outer membrane and inside the organelle. In all cases, gal-7 was expressed in clusters, mostly being found inside the

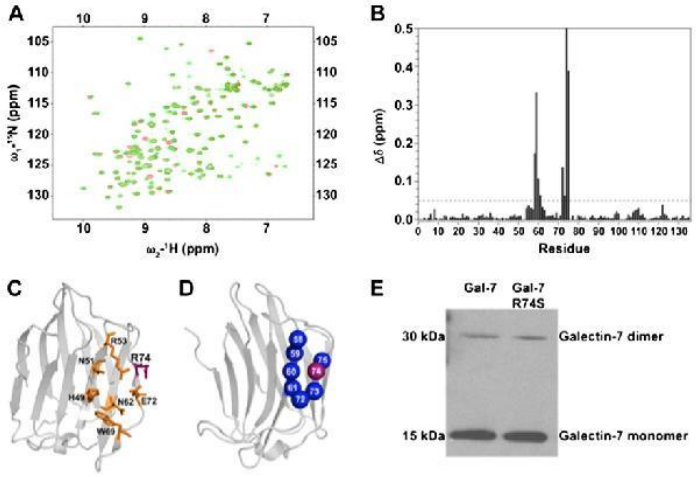

Figure 2 Structural analysis of wild-type gal-7 and the R74S mutant. (A) Superimposed ^{1}H-^{15}N HSQC spectra of wild-type (green) and R74S (red) gal-7 at 310 K and 800 MHz. **(B)** ^{1}H-^{15}N chemical shift differences Δδ (ppm) caused by the R74S mutation mapped on the primary sequence of gal-7. The ^{1}H-^{15}N weighted average composite chemical shift differences (Δδ) were calculated between WT and variant R74S according to the following equation [17]: $\Delta\delta\ (\text{ppm}) = [(\Delta\delta_{HN}^2 + \Delta\delta_{N}^2/25)/2]^{1/2}$. **(C)** Three-dimensional structure of gal-7 showing the general β-sheet topology of the carbohydrate recognition domain (CRD) and the position of the carbohydrate binding site, as delineated by residues H49, N51, R53, N62, W69, E72 and R74. **(D)** Mapping of ^{1}H-^{15}N chemical shift variations (Δδ) between wtgal-7 and variant R74S on the 3D structure of gal-7 [PDB: 3ZXF]. Residues with chemical shift variations Δδ >0.05 ppm are plotted on the structure of gal-7 (in blue). The position of the R74 residue is shown in purple. **(E)** Immunoblots showing soluble monomeric and dimeric forms of recombinant gal-7 in a native gel.

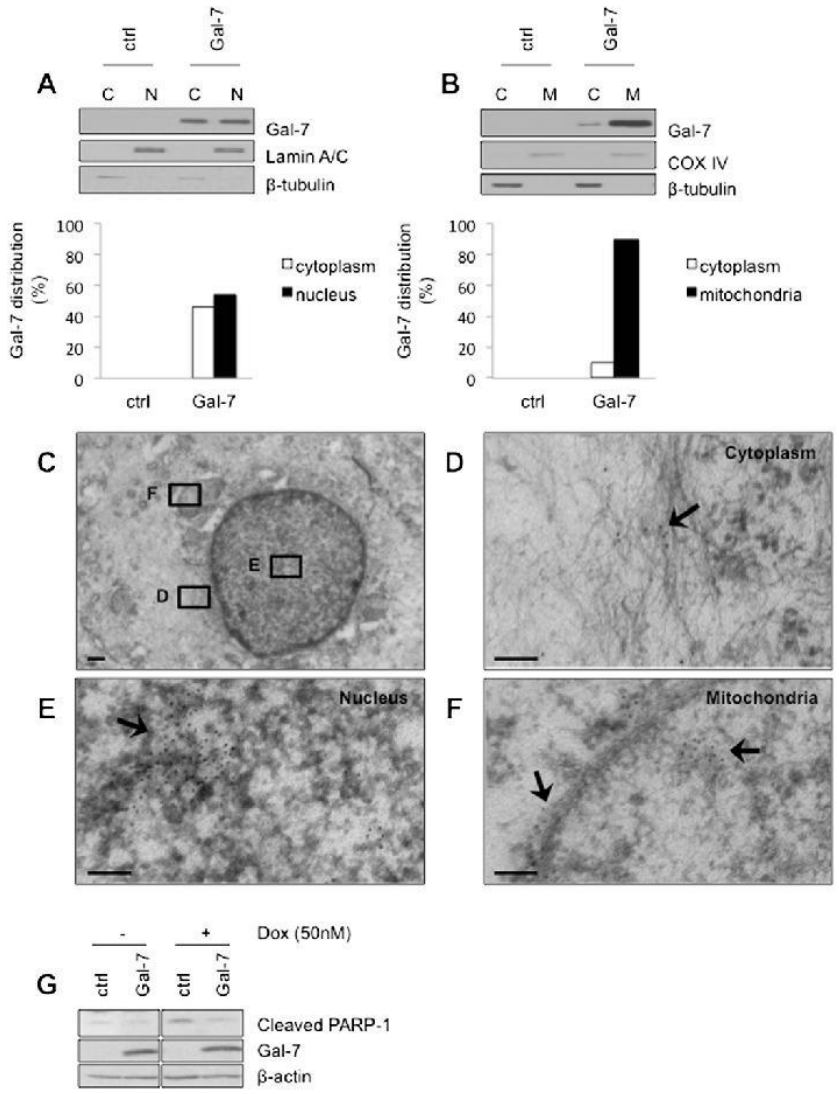

Figure 3 Subcellular distribution of gal-7 in MCF-7 cells. (A) Cytosolic and nuclear fractions were purified from control (srα) cells and MCF-7 cells expressing gal-7. Expression of gal-7 was measured in both fractions by Western blotting. β-tubulin and lamin A/C are shown as positive cytoplasmic and nuclear expression controls, respectively. **(B)** Similar analysis showing expression of gal-7 in purified mitochondrial fractions. β-tubulin and COX IV are shown as positive cytoplasmic and mitochondrial expression controls, respectively. **(C-F)** Subcellular localization of gal-7 in MCF-7 as measured by electron microscopy. Scale bars, 500 nm **(C)** and 100 nm **(D-F)**. **(G)** Effect of gal-7 on induction of apoptosis by doxorubicin. Stable transfectants of MCF-7 cells expressing gal-7 were treated with 50 nM doxorubicin for 72 hrs at 37°C. Cells were then harvested and PARP-1 cleavage levels were assayed by Western blotting.

mitochondria (Additional file 3: Figure S3). MCF-7 expressing wtgal-7 was also more resistant to apoptosis induced by doxorubicin (dox) as compared to control MCF-7 cells transfected with a (empty) control vector (Figure 3G). These results corroborated our previous data using the mouse 4T1 breast cancer cells [6]. We thus examined whether the R → S mutation at position 74 induced a change in the subcellular localization of gal-7. Our Western blot analyses showed that this mutation greatly reduced the expression of gal-7 in the nucleus and mitochondria (Figure 4A-B). This effect was confirmed by EM studies (Figure 4C-F). Interestingly, however, we found that MCF-7 cells expressing wild-type and R74S forms of gal-7 were equally resistant to apoptosis induced by cobalt chloride, a hypoxia mimicking agent (Figure 5A-B), and by other anticancer drugs,

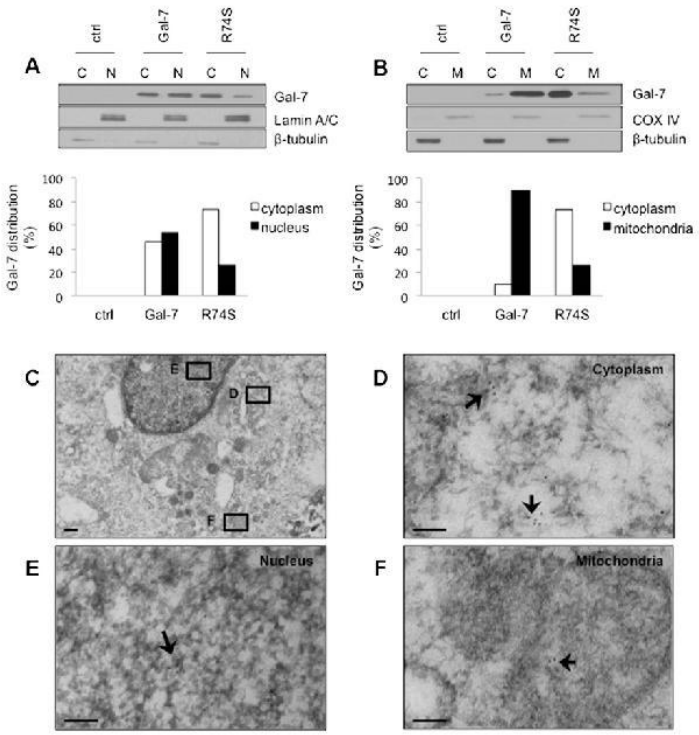

Figure 4 Subcellular distribution of the R74S mutant. The effect of the R74S mutation on the **(A)** nuclear and **(B)** mitochondrial localization of gal-7, as measured by Western blot analysis of subcellular fractions. Membrane blots were incubated with anti-gal-7, anti-lamin A/C, anti-COX-IV and anti-β-tubulin. Lamin A/C, COX-IV and β-tubulin were used as nuclear, mitochondrial and cytosolic markers, respectively. **(C)** Whole MCF-7 cell and distribution of galectin-7 R74S mutant in **(D)** cytoplasm, **(E)** nucleus and **(F)** mitochondria, as measured by electron microscopy. Scale bars, 500 nm **(C)** and 100 nm **(D-F)**.

such as etoposide and doxorubicin (dox) (Figures 5C and 6A). In fact, MCF-7 cells expressing R74S were more resistant to apoptosis induced by etoposide and dox.

Gal-7 reduces p53-induced p21 expression

Because MCF-7 cells express a wild-type form of p53 and that DNA damage response induced by dox is known to increase the cyclin-independent kinase inhibitor p21 via a p53-dependent pathway, we took this opportunity to examine the expression of p21 and p53 in MCF-7 cells expressing wtgal-7 and R74S. We found that both wtgal-7 and R74S inhibited dox-induced PARP-1 cleavage and p53 protein expression (Figure 6A-B). The inhibition of p53 correlated with a decrease in p21 protein expression and *CDKN1A* mRNA (Figure 6B-C). Again, the inhibition by R74S was stronger than that observed with the wild-type form of gal-7. Furthermore, analysis of nuclear and cytoplasmic fractions showed that wild type and R74S mutant gal-7 inhibited p53 nuclear translocation (Figure 7A). Treatment of cells with MG-132, a well-known proteasome inhibitor, restored the p53 expression, suggesting that both forms of gal-7 promote degradation of cytosolic p53 (Figure 7B). This possibility is supported by our data showing that both forms of gal-7 co-precipitate with p53 (Figure 7C-D). The ability of endogenous gal-7 to co-precipitate with p53 was further confirmed using MCF10A cells (Additional file 4: Figure S4).

Discussion

There is an increasing interest in the development of galectin-specific inhibitors for the treatment of cancer. Because galectins exert both intracellular and extracellular functions, a better understanding of their subcellular localization in cancer cells is critical to promote the development of new anti-cancer therapies directed at these proteins.

Gal-7 is highly expressed at both the mRNA and protein levels in tissues of patients with aggressive forms of cancer, including basal-like breast cancer subtype [6,19,20]. Experimentally, gal-7 has been shown to increase the metastatic behavior of cancer cells while its suppression reduces their metastatic behavior [6,21,22]. Like other members of the galectin family, however, gal-7 has been shown to have a dual role in cancer. While it promotes

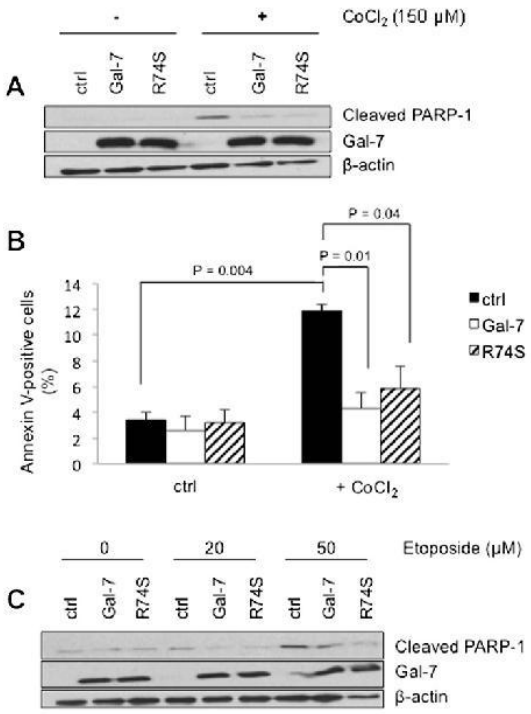

Figure 5 Anti-apoptotic function of gal-7 is not altered by R74S mutation. Effect of wild-type and mutated gal-7 on **(A)** PARP-1 cleavage and **(B)** Annexin V positive cells induced upon treatment with 150 µM $CoCl_2$. **(C)** Western blot analysis of PARP-1 cleavage for increasing doses of etoposide for 24 hrs at 37°C. Controls included stable MCF-7 cells transfected with the empty (srα) expression vector.

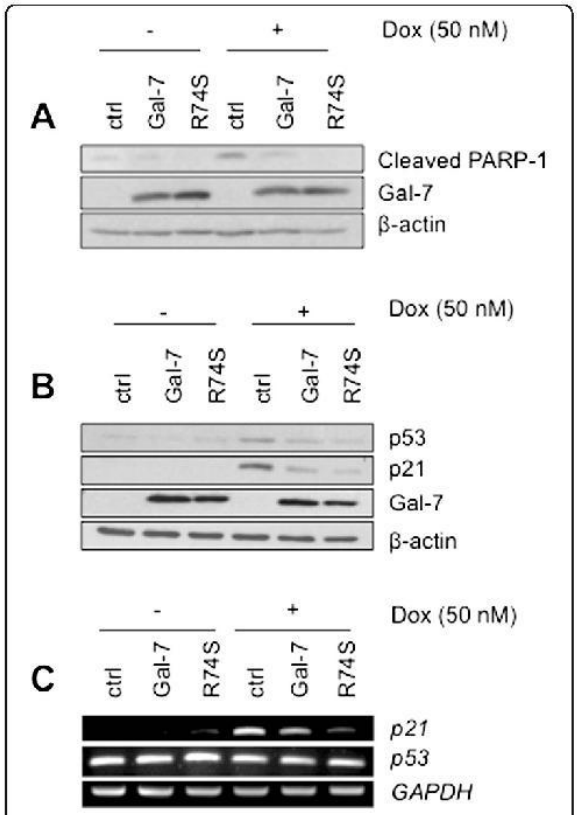

Figure 6 Gal-7 and its R74S variant suppress PARP-1 cleavage, p53 expression and p21 transcription induced by doxorubicin. **(A)** Stable transfectants of MCF-7 cells expressing wild type or R74S mutant gal-7 were treated with 50 nM doxorubicin for 72 hrs at 37°C and immunoblotted with cleaved PARP-1-specific antibody. **(B)** Western blot analysis showing p53, p21 and gal-7 protein expression following doxorubicin treatment for 24 hrs. **(C)** mRNA levels of *p53* and *p21* were assayed by semi-quantitative RT-PCR. β-actin and GAPDH were used as loading controls.

cancer progression in many types of cancer, it may exert an anti-tumor activity in other types of cancer, such as urothelial carcinoma and colon cancer. In all cases, the role of gal-7 in apoptosis was associated with its intracellular localization, as shown by the strong cytosolic and nuclear immunoreactivity with anti-gal-7 antibodies [6,23]. Our model system with the R74S mutant will thus be useful to determine whether translocation to mitochondria and nucleus modulates the ability of gal-7 to modulate apoptosis in other cancer cell types.

Because R74 is located in the vicinity of the CRD, it is not surprising that a mutation at this position reduces the affinity to lactose or the binding to cell surface glycoproteins. It may also affect the fine specificities of ligand recognition in the ligand binding groove, as suggested by the tridimensional structure of gal-7 [4]. We expect, however, that the R74S mutation will not affect the protein-protein interactions that gal-7 displays with proteins such as Bcl-2 and Smad3 [9,11]. Similarly to galectin-3 that utilizes synexin for its translocation to the perinuclear mitochondrial membranes, gal-7 might also require the aid of similar transport proteins for its translocation [24]. As such, the cytosolic presence of the R74S mutant is potentially due to the loss of the interaction between gal-7 and its transport proteins resulting in its pronounced cytosolic localization. Alternatively, the mutation at the R74 promoting the cytosolic sequestration of gal-7 may allow enhanced binding to cytosolic proteins increasing as such various cytoplasmic signaling pathways. Nevertheless, future studies will be needed to determine the specific mechanism by which gal-7 translocates to mitochondria and to the nucleus.

Our results suggest that gal-7 may be involved in the regulation of p21 expression. These results may thus provide a new mechanism underlying the functions of gal-7 in apoptosis and warrant further investigation. Specifically, we found that the R74S mutation does not alter the proliferation rate of breast cancer cells (Additional file 5: Figure S5).

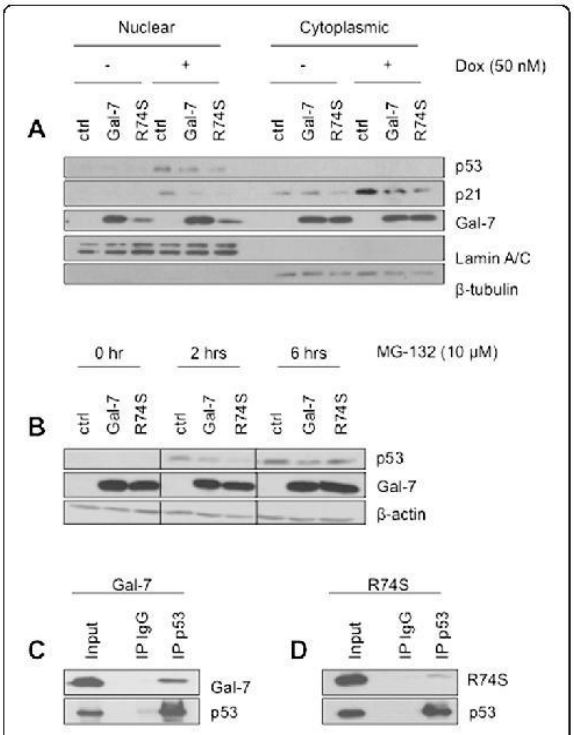

Figure 7 Decreased of p53 nuclear translocation through proteasomal degradation induced by cytoplasmic gal-7. (A) Cytosolic and nuclear fractions were purified from control (sra) cells and MCF-7 cells expressing wild type or mutated gal-7 after doxorubicin treatment. Expression of p53, p21 and gal-7 was measured in both fractions by Western blotting. β-tubulin and lamin A/C are shown as positive cytoplasmic and nuclear expression controls, respectively. **(B)** Cells were treated with 10 μM MG-132 for 0, 2, and 6 hrs. Total cellular extracts were subjected to Western blot analysis for p53, gal-7 and β-actin. Immunoprecipitation (IP) experiments showing p53 interaction with **(C)** wild type and **(D)** R74S mutant of gal-7 in MCF-7 cells. Whole-cell lysates were made from MCF-7 cells transfected with a construct expressing p53. Lysates were immunoprecipitated with anti-p53 or a control IgG antibody, and immunoblot analysis was performed with anti-gal-7.

Rather, our data obtained using the proteasome inhibitor and the co-immunoprecipitation of gal-7 with p53 suggests that gal-7 may help to stabilize cytosolic p53, possibly by modulating its interaction with MDM2. Whether gal-7 directly binds to p53 or belongs to the p53 multimolecular complex is currently unknown. Although glycosylation of p53 has been reported [25-27] and that some p53-interacting proteins are glycosylated [28], our observation that R74S also co-immunoprecipitates with p53 suggests that such interaction could be CRD-independent. Such CRD-independent function for galectins is not uncommon, especially for intracellular galectins [3]. Another possibility that may explain lower levels of nuclear p53 protein and reduced p21 activation is that gal-7 may be part of a complex network of interrelated mechanisms that regulate the nucleo-cytoplasmic transport of p53 following cellular stress. These possibilities are currently under investigation.

Conclusions

In the present work, we have shown that: 1) a mutation at position 74 inhibited translocation of gal-7 to the mitochondria and the nucleus, sequestering gal-7 to the cytosolic compartment; 2) such decrease of gal-7 expression in the nucleus and mitochondria does not impair the ability of gal-7 to drug-induced apoptosis; in fact, the R74S mutant protected even more cells from apoptosis induced by anti-cancer drugs, and 3) sequestration of gal-7 to the cytosol impaired the translocation of p53 to the nucleus and the upregulation of p21. Taken together, these results suggest that targeting cytosolic gal-7 in breast cancer cells may be a valuable strategy for the treatment of this disease.

Additional files

Additional file 1: Figure S1. Isothermal calorimetric titration of gal-7 with lactose. (Top) Typical ITC experiment carried out by adding 2 μl aliquots of 6 mM lactose to 200 μM wtgal-7 or R74S. (Bottom) Heat released per mole of lactose injected as a function of the sugar / protein molar ratio. The titration was obtained at 25°C in 20 mM potassium phosphate buffer, pH 7.2.

Additional file 2: Figure S2. Binding of recombinant wtgal-7 and R74S to Jurkat T cells. Increasing concentrations of recombinant FITC-labeled wtgal-7 or R74S were added to Jurkat T cells. Binding was measured by flow cytometry after 30 min of incubation at 4°C.

Additional file 3: Figure S3. Clusters of gal-7 in MCF-7 cells observed by electron microscopy. Transmission electron micrographs showing gal-7 clusters (arrows) in (A) the cytoplasm, (B) nucleus, and (C) mitochondria of MCF-7 stable transfectants expressing gal-7. In (D), quantitative assessment of the number of immunogold particles per clusters as measured from transmission electron micrographs.

Additional file 4: Figure S4. Interaction of endogenous gal-7 with p53 in MCF10A cells. Immunoprecipitation (IP) experiments showing p53 interaction with endogenous gal-7 in MCF10A cells. Whole-cell lysates were made from MCF10A cells transfected with a construct expressing p53. Lysates were IP with anti-p53 or a control IgG antibody, and immunoblot analysis was performed with anti-gal-7.

Additional file 5: Figure S5. Cellular proliferation of MCF-7 is not significantly affected by gal-7 and R74S variant. Stable transfectants of MCF-7 cells expressing empty vector (ctrl), wild type or R74S mutant gal-7 were seeded at 5000 cells per well in E-Plates 96 and observed for a period of 72 h. Dynamic proliferation assay was achieved via xCELLigence real-time cell analyzer.

Abbreviations

gal-7: Galectin-7; wtgal-7: Wild-type galectin-7; CRD: Carbohydrate recognition domain; dox: Doxorubicin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: AAG, YSP, ND and LG. Performed the experiments and analyzed the data: AAG, ML, MCV and DG. Wrote the paper: AAG and YSP. All authors read and approved the final manuscript.

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Author details
[1]INRS-Institut Armand-Frappier, 531 Blv. des Prairies, Laval, Quebec H7V 1B7, Canada. [2]IRIC | Université de Montréal, 2950 Chemin de Polytechnique, Montreal, Quebec H3T 1J4, Canada.

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ANNEXE III

UNE SIGNATURE DE GALECTINES CONTRIBUE À L'HÉTÉROGÉNÉITÉ DU CANCER DU SEIN ET APPORTE DE NOUVELLES INFORMATIONS PRONOSTICS ET DE NOUVELLES CIBLES THÉRAPEUTIQUES

Galectin signatures contribute to the heterogeneity of breast cancer and provide new prognostic information and therapeutic targets

Andrée-Anne Grosset[1,2], Marilyne Labrie[1], Maria Claudia Vladoiu[1], Einas M Yousef[2], Louis Gaboury[2], Yves St-Pierre[1]

[1]INRS-Institut Armand-Frappier, 531 Blv des Prairies, Laval, Quebec H7V 1B7, Canada

[2]IRIC | Université de Montréal, 2950 Chemin de Polytechnique, Montreal, Quebec H3T 1J4, Canada

Correspondence to: Yves St-Pierre, **e-mail:** yves.st-pierre@iaf.inrs.ca

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ABSTRACT

Because of their ability to induce local immunosuppression and to confer cancer cells with resistance to apoptosis, members of the galectin family are emerging as a new class of actionable targets in cancer. Unfortunately, we have yet to obtain a clear picture of the galectin signatures in cancer cells and the surrounding tumor microenvironment. The aim of this study was to provide the first detailed analysis of the galectin signature in molecular subtypes of breast cancer. Expression signatures of galectins were obtained at the mRNA and protein levels. A particular attention was paid to stromal versus epithelial staining and to subcellular compartmentalization. Analysis of the stromal signature showed that gal-1, -3, -9-positive stroma were preferentially found in triple-negative (TN) and HER2 subtypes. In cancer cells, gal-1, -3, -8, and -9 showed a dual expression pattern, being found either in the cytosol or in the cytosol and the nucleus. TN patients with gal-8-positive nuclei had significantly better disease-free survival (DFS), distant-disease-free survival (DDFS), and overall survival (OS). In contrast, high expression of nuclear gal-1 correlated with poor DDFS and OS. TNBC patients who were positive for both nuclear gal-1 and gal-8 had 5-year DFS and DDFS of 100%, suggesting a dominance of the gal-8 phenotype. Overall, the results indicate that specific galectin expression signatures contribute to the phenotypic heterogeneity of aggressive subtypes of breast cancer. Our data also suggest that galectins have clinical utility as indicators of disease progression and therapeutic targets in aggressive molecular subtypes of breast cancer.

INTRODUCTION

Gene profiling studies have greatly helped at better classifying breast cancer into at least four generally recognized molecular subtypes. Clinically, these molecular subtypes are identified based on the immunohistochemical expression of the estrogen (ER) and progesterone (PR) receptors and of Human Epidermal Growth Factor Receptor 2 (HER2), a member of the epidermal growth factor receptor family. These molecular subtypes include luminal A and B, HER2-positive, and triple negative breast cancer (TNBC; ER-, PR-, and HER2-negative tumor) [1, 2]. Because they lack actionable targets, TNBC and HER2-positive subtypes are thus untreatable with hormone therapies and have a very poor prognosis. The heterogeneity of HER2 and TNBCs at the molecular and cellular levels represents, however, a formidable obstacle to the development of new treatment modalities of these aggressive subtypes [3, 4]. Such challenge is further complicated by the complexity of the tumor microenvironment (TME) which plays a critical role in the disease progression [5].

Carbohydrate-dependent interactions are critical in many physiological processes as well as in pathological abnormalities, most notably in cancer. Outside the cells, these interactions are well known to facilitate intercellular communications, increasing the stability of growth receptors via lattice formation, and modulating the immune response following binding to cell surface receptors [6]. Inside the cells, they modulate signaling

cascades, direct trafficking of proteins or contribute to the regulation of gene expression by binding to transcription factors or proteins involved in mRNA splicing [7].

In cancer, such carbohydrate-dependent interactions are mediated in a large part by galectins, an evolutionarily ancient family of soluble proteins that bind N-linked and O-linked beta-galactosides via a conserved Carbohydrate Recognition Domain (CRD). Galectins were first isolated from chick muscle and calf heart and lungs and have since been named in the order of discovery [8, 9]. The 15 members of the family are generally classified according to the number and structure of their CRD. Galectins are therefore divided into tandem, dimeric and chimeric galectins. Dimeric galectins (galectins-1, -2, -5, -7, -10, -11, -13, -14 and -15) have two identical CRD subunits while tandem ones (galectins-4, -6, -8, -9 and -12) have two distinct CRD subunits. Galectin-3 is the only chimeric galectin discovered in mammals thus far. Galectins-5 and -6 are found only in rodents. There is compelling evidence, however, that prototypic galectins might have non-carbohydrate binding partners and functions (reviwed in reference 7). These CRD-independent functions represent a paradigm shift in our understanding of galectin functions.

Our knowledge on the role of galectins in cancer and as biomarkers of disease progression has attracted the interest of many, most notably because these small molecular weight proteins undergo significant changes in their pattern of expression during progression of cancer. Their role in cancer progression, however, is not lacking in subtlety [10]. While cancer progression in accelerated by some galectins, others clearly inhibit tumor growth and/or formation of metastasis. Moreover, there is increasing evidence that galectins function as alarmins [11]. In response to aggression, they are released via a non-classical secretion pathway in the extracellular space where they play a critical role in controlling the immune response. Such complexity in their behavior represents a true challenge when developing galectin inhibitors or use their expression pattern as predictive biomarkers. Moreover, while most studies have examined one galectin at a time, focusing largely on galectin-1 and galectin-3, it is now well established that normal and tumor cells express more than one galectins, and that multiple galectins could be released in the tumor microenvironment (TME) [12, 13]. Defining a galectin signature for specific subtypes is thus critical to identify new therapeutic targets in concert with companion diagnostics and/or molecular signatures to guide therapeutic decisions. In the present work, we have examined the expression of seven galectins in breast cancer tissues by immunohistochemistry and correlated their expression with the different molecular subtypes of breast cancer.

RESULTS

In silico analysis of galectin mRNA expression in breast cancer tissues

We first used the prognostic module of the Breast Cancer Gene-Expression Miner v3.1 (bc-GenExMiner) public database to investigate galectin expression at the mRNA level among breast cancer molecular subtypes. For each subtype, an expression map containing the percentage of patients with low, intermediate, and high gene expression for *gal-1, -2, -3, -4, -7, -8,* and *-9* was retrieved. Gene expression values were being beforehand split in order to form three equal groups so that "high expression" represents the 1/3 of the patients with highest expression of *a gene* and "low expression" is the lower 1/3 of the patients. For example, a representative schematic diagram for *lgals1* shows that *lgals1* expression measured in 1260 samples is at its highest in 41% of patients with basal-like breast and HER2 subtypes of breast cancer (Figure 1A). The diagrams for other members of the galectin family are shown in Supplementary Figure S1. Overall, we found that the relative expression of galectins among the different molecular subtype had a similar distribution, although the percentage of patients with the highest expression of *lgals8*, and to a lesser extent *lgals3*, were lower in patients with a basal-like subtype (Figure 1B).

We next performed an mRNA survival analysis for each galectin using the Breastmark *RNA expression database and algorithm that facilitate* investigation of prognostic markers in the context of disease-free survival (DFS), distant disease-free survival (DDFS) and overall survival (OS) [14]. An initial search was performed for breast cancer as a whole, independently of the lymph node status and across the molecular subtypes classified according the PAM50 molecular classifier [15]. Generation of Kaplan-Meier surviving plots showed no significant differences of DFS between groups of patients with high or low expression level of genes encoding *gal-2, -3, -4, -7, -8,* and *-9* (Supplementary Figure S2). The only notable difference was seen in patients with higher expression of mRNA level of *lgals1*. These patients had a significantly lower ($p = 0.016$) DFS than patients with negative/low levels of *lgals1*, consistent with previous observations that galectin-1 expression correlates with a poor prognosis in breast cancer [16]. High expression of *lgals1* is indeed a poor prognostic factor for both lymph node (LN)-positive and negative breast cancer (Table 1).

We next focused on aggressive molecular subtypes for which new prognostics are needed. In the case of HER2 molecular subtype, we found that patients with high *lgals3* gene expression had a significantly ($p = 0.031$) lower OS than patients with lower levels of *lgals3* (Figure 2).

In contrast, high *lgals2* expression was associated with a good DFS, although the difference fell just short of the traditional definition of statistical significance (0.064). A similar trend was observed for *lgals2* and *lgals9* in patients with triple-negative breast cancer (Figure 3). High expression levels of *lgals2* ($p = 0.031$), *lgals4* ($p = 0.061$) and *lgals9* ($p = 0.008$) were all good prognostic factors for LN-negative patients (Table 2). *Lgals9* ($p = 0.004$) was also a good prognostic factor for patients with luminal B subtype. Similar results were obtained using the ssp2006 as a classifier [17] (*Data not shown*). Overall, these results indicate that expression of galectins at the mRNA level can be either a good or bad prognostic markers for patients with aggressive subtypes of breast cancer.

Galectin protein signature in normal and cancerous breast tissues

We next studied the expression of galectins at the protein level by immunohistochemistry (IHC) using tissue microarrays (TMAs) constructed from 213 human breast cancer tumor tissues representative of each molecular

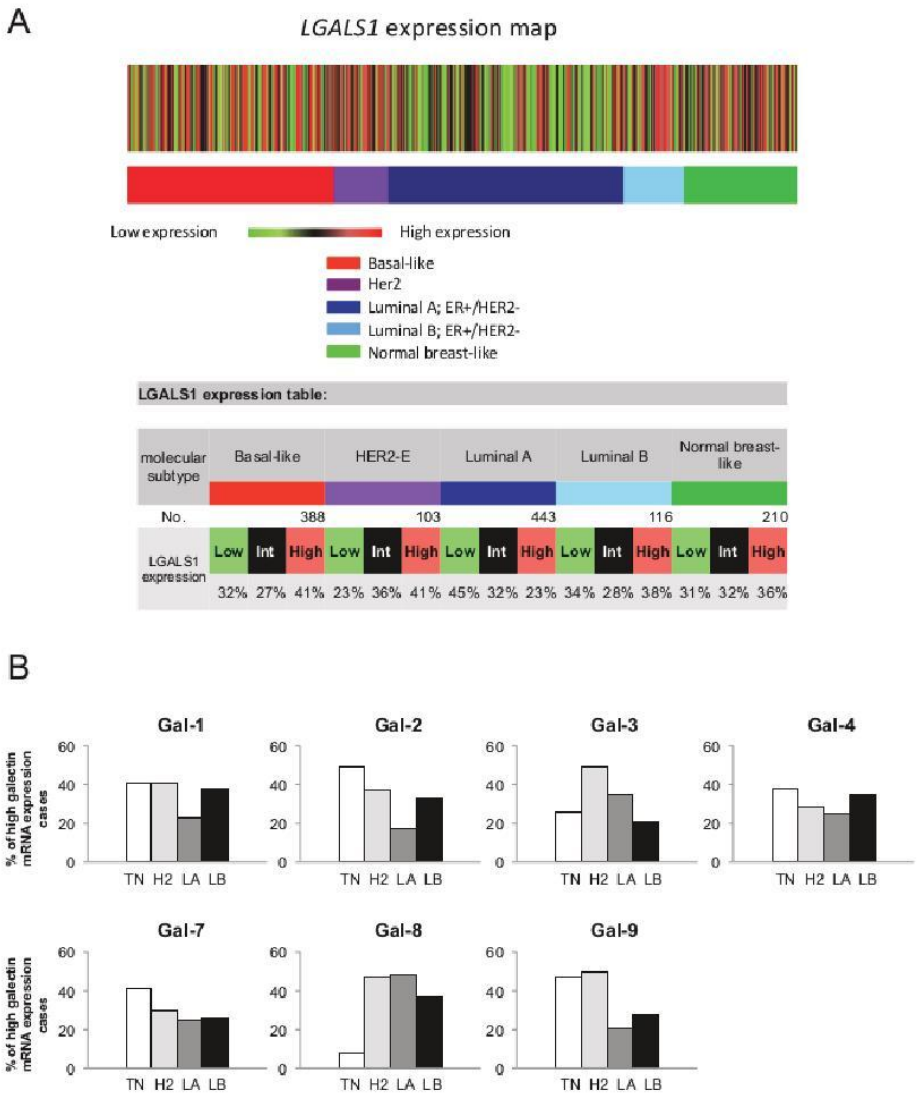

Figure 1: Gene expression map of *lgals1* in PAM50 molecular subtypes of breast cancer using bc-GenExMiner database. (**A**) The map shows the percentage of patients with low, medium and high expression of *lgals1* mRNA in each molecular subtype: Basal-like (ER-, HER-2-), HER-2E (HER-2 enriched), Luminal A (ER+, HER-2-, low proliferation) and Luminal B (ER+, HER-2-, high proliferation). The number of patients for each subtypes is shown. (**B**) The distribution for *lgals1, lgals2, lgals3, lgals4, lgals7, lgals8*, and *lgals9* in each molecular subtypes.

Table 1: High mRNA expression as a poor prognotic factor for DFS using the pam50 classifier

Gene	Across		LN-pos		LN-neg		Lum A		Lum B		HER2		Basal	
	n	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*
Lgals1	2652	**0.016**	744	**0.049**	1183	**0.044**	823	0.410	1013	0.614	286	0.452	424	**0.047**
Lgasl3	2601	**0.031**	714	0.546	1161	**0.100**	811	0.663	998	0.313	275	**0.031**	412	0.637
Lgals7	455	0.245	173	0.163	173	0.864	153	**0.038**	146	0.200	66	0.965	72	0.220
Lgals8	2497	0.726	672	0.218	1105	0.613	783	0.699	950	0.604	273	0.294	407	0.794

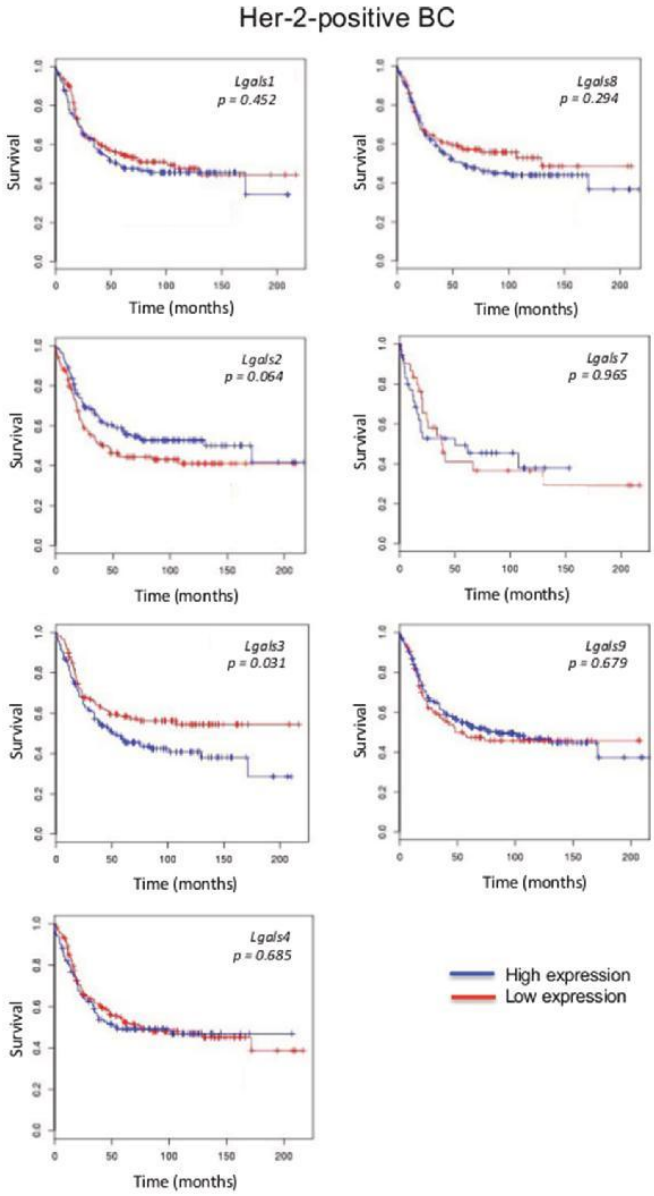

Figure 2: Prognostic role of galectin genes in HER2 breast cancer. Kaplan-Meier estimates of overall survival in HER2 patients expressing low or high galectin expression. These figures were generated using BreastMark public database.

subtypes of breast cancer defined based on ER/PR/HER2 status and with clinical data. We first validated the specificity of the IHC reactivity of the commercial antibodies using information from the Human Protein Atlas [18, 19] and a review of the literature (Supplementary Table S1). Representative positive IHC staining of tissues for each selected antibody showed a complete agreement with the predictions (Supplementary Figure S3). Once validated, the antibodies were used to examine expression of galectins in normal breast tissues. Our results showed that gal-2 and gal-4 were only weakly expressed in normal breast tissues, with very weak cytoplasmic staining in luminal cells (Figure 4). Also in normal tissues, we found a moderate/high cytoplasmic and/or nuclear staining for all galectins except gal-7, which showed its typical cytoplasmic and nuclear staining in myoepithelial cells [20] (Table 3). Gal-9 staining revealed a cytoplasmic staining in luminal epithelial cells. Some isolated stromal and epithelial cells were also strongly reactive in the nucleus. These patterns of expression in normal breast tissue were, for most galectins, significantly altered in breast cancer tissues. High levels of galectin expression were observed across all molecular subtypes, except for gal-7, which staining was restricted to HER2 and

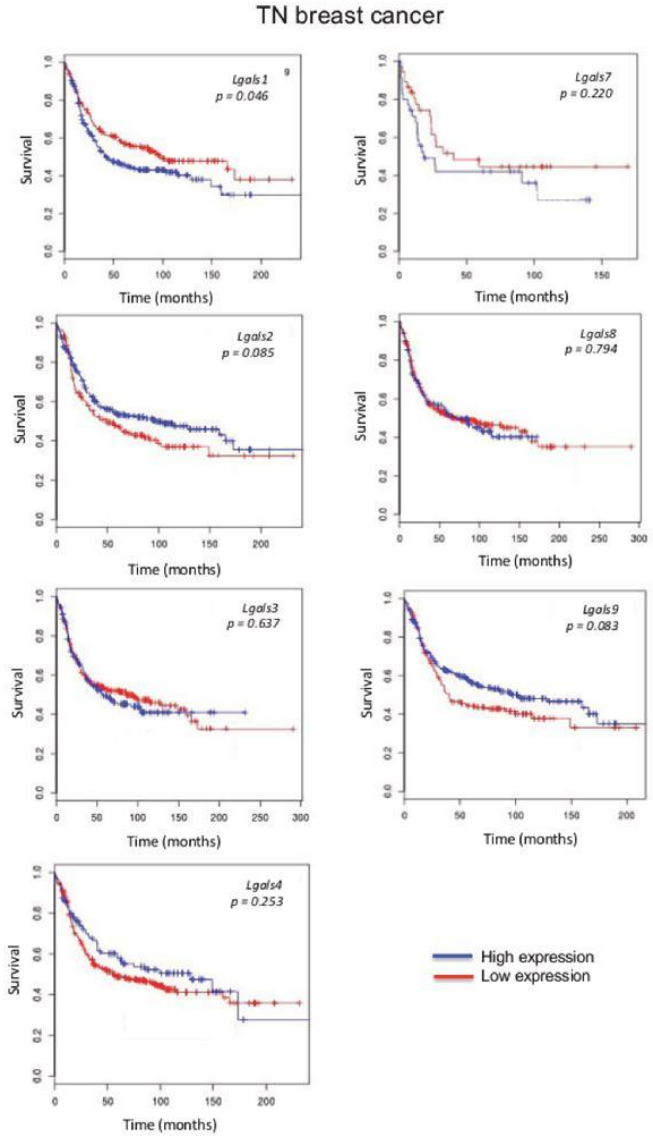

Figure 3: Prognostic role of galectin genes in triple-negative breast cancer. Kaplan-Meier estimates of OS in TNBC patients expressing low or high galectin expression. These figures were generated using BreastMark public database.

Table 2: High mRNA expression as a good prognotic factor for DFS using the pam50 classifier

Gene	Across		LN-pos		LN-neg		Lum A		Lum B		HER2		Basal	
	n	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*	*n*	*P*
Lgals2	2952	0.558	719	0.998	1155	**0.031**	807	0.795	987	0.732	275	0.064	419	**0.085**
Lgals4	2652	0.281	744	0.537	1183	**0.061**	823	0.449	1013	0.353	286	0.685	424	0.253
Lgals9	2652	0.350	744	0.393	1183	**0.008**	823	0.562	1013	**0.004**	286	0.679	424	**0.083**

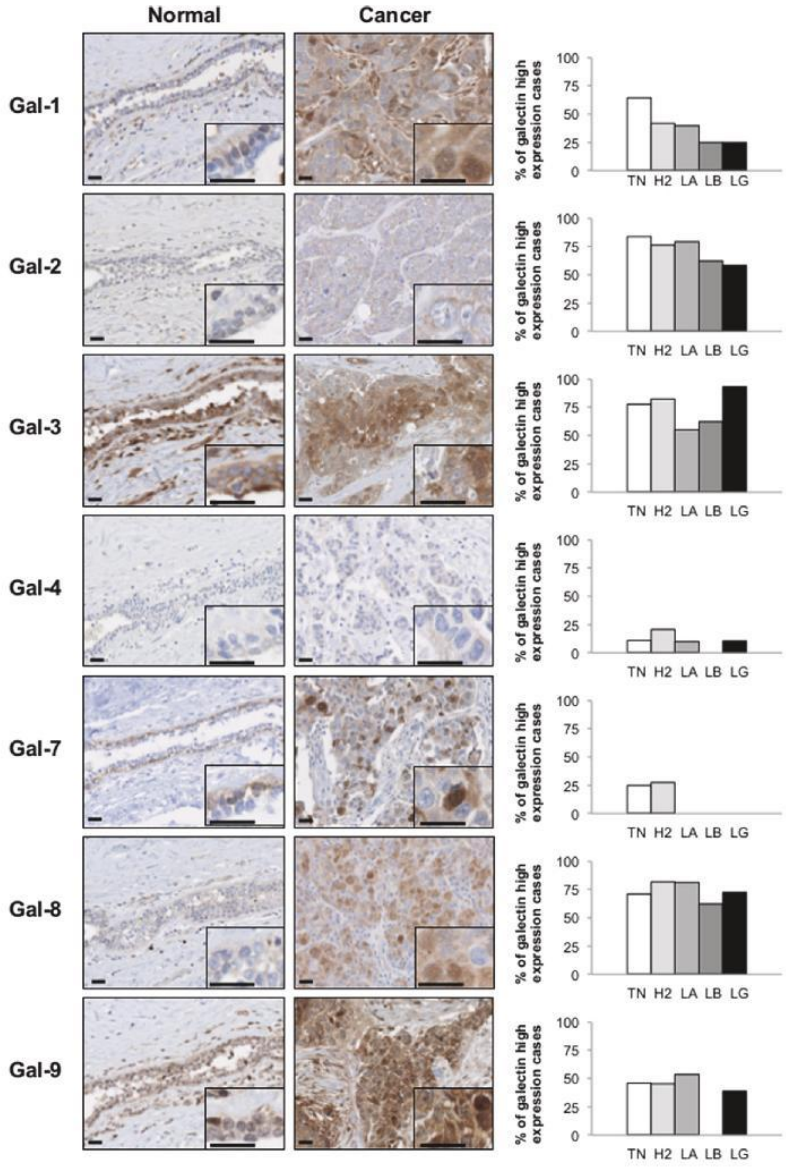

Figure 4: Galectins expression in normal mammary gland and breast cancer tissues. *Left panels*, representative images of galectin expression in normal mammary and breast cancer tissues. The histograms on the right represent the percentage of molecular subtypes expressing high levels for each galectin: TN (triple-negative; ER-, PR-, HER-2-), H2 (HER-2 positive; ER-, PR-, HER-2+), LA (Luminal A; ER+, HER-2-), LB (Luminal B; ER+, HER-2+). LG (low grade). All scale bars 25 μm.

Table 3: Galectins distribution, cellular localization and intensity in normal mammary gland

	Breast epithelial cells			Stroma	
	Luminal cells	Myoepithelial cells	Intensity	Localization	Intensity
Gal-1	-	c, n	moderate	e, c, n	high
Gal-2	c	-	weak	e, c	moderate
Gal-3	c	c	high	e, c, n	high
Gal-4	c	-	weak	c	weak
Gal-7	-	c, n	moderate	-	-
Gal-8	c	c	moderate	e, c	moderate
Gal-9	c	c, n	moderate	e, c, n	high

Abbreviations: c, cytoplasmic; n, nuclear; e, extracellular.

triple-negative breast cancer (TNBCs). Gal-1-positive staining was also preferentially expressed in TNBCs subtype whereas expression of gal-2, -3, -4, -8, and -9 showed an almost equal distribution in all subtypes, although we could not find any gal-9 positive luminal B samples (Table 4). High levels of gal-1 also showed a significant correlation with Ki-67-positive ($p = 0.048$) staining. Generation of Kaplan-Meier surviving plots showed that high expression levels of gal-3 ($p = 0.0548$) and gal-7 ($p = 0.0786$) were associated with a worse DFS across the molecular subtypes although the differences barely missed the commonly acceptable statistical significance of $p = 0.05$ (Figure 5). In the case of gal-3, however, this difference was statistically significant ($n = 68$, $p = 0.0327$) for TNBC (*data not shown*). Moreover, high expression levels of gal-3 in TNBC patients correlated ($p < 0.05$) with recurrence (Supplementary Table S2).

Cellular localization of galectins in breast cancer cells

Because previous studies have shown that galectin functions depends on its subcellular localization [21, 22], we examined the subcellular distribution of galectins in breast cancer cells and its association with tumor progression. We found that gal-1, -3, -8, and -9 have a dual expression patterns in breast cancer cells. Their expression is either restricted to the cytoplasmic compartments or found in the cytoplasm and the nucleus in the same cell (Figure 6). We found no evidence of nuclear localization of gal-2 and gal-4, in contrast to gal-7, which is almost always found in both nuclear and cytoplasmic compartments in the same cell (Figure 4).

Prognostic values of subcellular galectins

In the case of gal-1 and gal-2, their respective nuclear and membrane localization correlated with TNBCs (Table 5). In contrast, gal-8 nuclear expression was preferentially found in low grade breast tumor and significantly ($p < 0.0001$) less frequent in TNBCs. Membrane-associated gal-2 ($p = 0.039$) also correlated with high expression of Ki-67. In fact, in TNBC, patients with gal-8-positive nuclear staining had significantly better DFS ($p = 0.0243$), DDFS ($p = 0.0019$), and OS ($p = 0.0292$) (Figure 7). Such correlation was also observed independently of the molecular subtype (Supplementary Figure S4). In contrast, high expression of nuclear gal-1 correlated with a worst DDFS ($p = 0.0080$) and OS ($p = 0.0294$) in TNBC (Figure 8). Interestingly, patients who were positive for both nuclear gal-1 and nuclear gal-8 had an actual 5-year DFS and DDFS of 100%.

Stromal galectin expression in stromal cells of the tumor microenvironment

There is increasing evidence that the tumor microenvironment is a key contributor to tumor progression [5]. We have thus examined whether galectins are expressed in stromal cells of the tumor microenvironment. We found that gal-1, -3, and -9 are commonly found in cells surrounding the tumor, while expression of other galectins were mostly associated with epithelial cancer cells (Figure 9A). In many cases, staining for gal-1, -3, or -9 were found in both epithelial and stromal cells (approximately 50% in the case of gal-1 and gal-9 and 75% in the case of gal-3). In other cases, we could clearly distinguish two clear patterns of staining with these galectins, i.e. that gal-1, -3, and -9 expression were either strictly found in epithelial tissues or in stromal cells. Gal-1, -3, -9-positive stroma were preferentially found in tumours from TN and HER2 patients (Figure 9B) and correlated with EGFR-positive, Ki67-positive, and mutated p53 (Table 6). Overall, we found that 22 patients were positive for all three galectins (Table 7). All were high grade tumors or were classified as TNBC/HER2 molecular subtypes. We also found a statistically

Table 4: Histoclinical correlations of breast cancers according to galectins expression in cancer cells

	Gal-1 %(n)		Gal-2 %(n)		Gal-3 %(n)		Gal-4 %(n)		Gal-7 %(n)		Gal-8 %(n)		Gal-9 %(n)	
	Neg/ Low	High	Neg/ Low	High	Neg/ Low	High	Neg/ Low	High	Neg/ Low	High	Neg/ Low	High	Neg/ Low	High
Molecular subtype	**P = 0.001**								***P* < 0.0001**					
Triple neg	**14 (26)**	**25 (47)**	6 (12)	33 (62)	9 (16)	30 (56)	36 (66)	4 (8)	**29 (54)**	**10 (18)**	12 (21)	29 (52)	21 (39)	18 (33)
HER-2	**10 (18)**	**7 (13)**	4 (8)	14 (26)	3 (6)	15 (28)	15 (27)	4 (7)	**13 (24)**	**5 (9)**	3 (6)	15 (27)	10 (18)	8 (15)
Luminal A	**26 (48)**	**13 (24)**	11 (21)	26 (49)	11 (20)	27 (50)	33 (62)	4 (7)	**39 (71)**	**0 (0)**	8 (15)	29 (52)	20 (37)	18 (33)
Luminal B	**3 (6)**	**1 (2)**	2 (3)	3 (5)	2 (3)	3 (5)	4 (8)	0 (0)	**4 (8)**	**0 (0)**	2 (3)	3 (5)	4 (8)	0 (0)
SBR-EE Grade	***P* = 0.006**		***P* = 0.02**		***P* = 0.017**				***P* = 0.005**					
I (low)	**14 (24)**	**5 (8)**	**8 (13)**	**11 (18)**	**1 (2)**	**16 (28)**	15 (26)	2 (3)	**18 (31)**	**0 (0)**	5 (8)	12 (21)	11 (19)	7 (12)
III (high)	**39 (67)**	**42 (72)**	**17 (29)**	**65 (112)**	**23 (39)**	**60 (102)**	74 (127)	9 (15)	**66 (113)**	**16 (27)**	21 (36)	62 (104)	46 (78)	36 (62)
Age, yr														
≤ 45	9 (16)	11 (20)	5 (9)	15 (27)	4 (8)	15 (27)	16 (30)	4 (7)	16 (30)	4 (7)	3 (5)	16 (29)	12 (22)	8 (15)
> 45	44 (81)	36 (66)	19 (35)	62 (114)	20 (37)	61 (111)	72 (132)	8 (15)	69 (126)	11 (20)	22 (39)	59 (107)	43 (79)	36 (66)
Tumor volume														
≤ 10 cm^3	31 (52)	26 (44)	14 (23)	42 (72)	12 (20)	44 (75)	47 (80)	8 (14)	49 (84)	7 (12)	13 (21)	45 (75)	31 (52)	25 (43)
> 10 cm3	21 (36)	22 (37)	10 (17)	35 (59)	14 (24)	30 (52)	41 (70)	4 (7)	37 (62)	7 (12)	13 (21)	30 (51)	23 (39)	21 (35)
Lymph node metastasis														
Negative	32 (58)	32 (58)	13 (23)	51 (93)	15 (26)	49 (87)	57 (102)	6 (11)	56 (101)	7 (13)	15 (27)	48 (85)	35 (62)	28 (50)
Positive	20 (35)	16 (28)	11 (19)	25 (46)	9 (16)	28 (50)	31 (56)	6 (11)	29 (52)	7 (13)	9 (15)	28 (49)	20 (35)	17 (31)
ER	***P* = 0.004**								***P* < 0.0001**					
Neg/Low	**27 (50)**	**34 (62)**	12 (22)	51 (94)	12 (22)	50 (92)	55 (101)	8 (15)	**47 (86)**	**15 (27)**	17 (31)	45 (82)	34 (62)	27 (50)
High	**26 (48)**	**13 (24)**	12 (22)	26 (48)	13 (23)	26 (47)	34 (62)	4 (7)	**39 (71)**	**0 (0)**	8 (14)	30 (54)	22 (40)	17 (31)
PR	***P* = 0.034**								***P* = 0.029**					
Neg/ Low	**43 (79)**	**43 (79)**	20 (38)	66 (123)	22 (40)	65 (119)	76 (141)	10 (19)	**72 (132)**	**15 (27)**	22(40)	65 (118)	49 (90)	37 (67)
High	**10 (19)**	**4 (7)**	3(6)	10 (19)	3 (5)	11 (20)	12 (22)	2 (3)	**14 (25)**	**0 (0)**	3(5)	10 (18)	7 (12)	8 (14)
HER-2														
Neg/ Low	41 (74)	38 (69)	17 (32)	60 (110)	19 (35)	58 (105)	69 (126)	8 (15)	68 (123)	10 (18)	20 (36)	57 (102)	41 (74)	37 (66)
High	13 (24)	8 (15)	6 (11)	17 (31)	5 (9)	18 (33)	19 (35)	4 (7)	18 (32)	5 (9)	5 (9)	18 (32)	14 (26)	8 (15)

Ki-67	*P* = 0.048								*P* = 0.032					
Neg/ Low	**36 (66)**	**24 (44)**	17 (31)	43 (79)	14 (25)	46 (85)	50 (92)	9 (17)	**54 (99)**	**6 (11)**	16 (29)	43 (76)	35 (63)	25 (46)
High	**18 (32)**	**22 (40)**	7 (12)	34 (63)	11 (20)	29 (53)	38 (70)	3 (5)	**31 (56)**	**9 (16)**	9 (16)	32 (58)	21 (38)	19 (35)

Fisher's exact test and chi-square test.

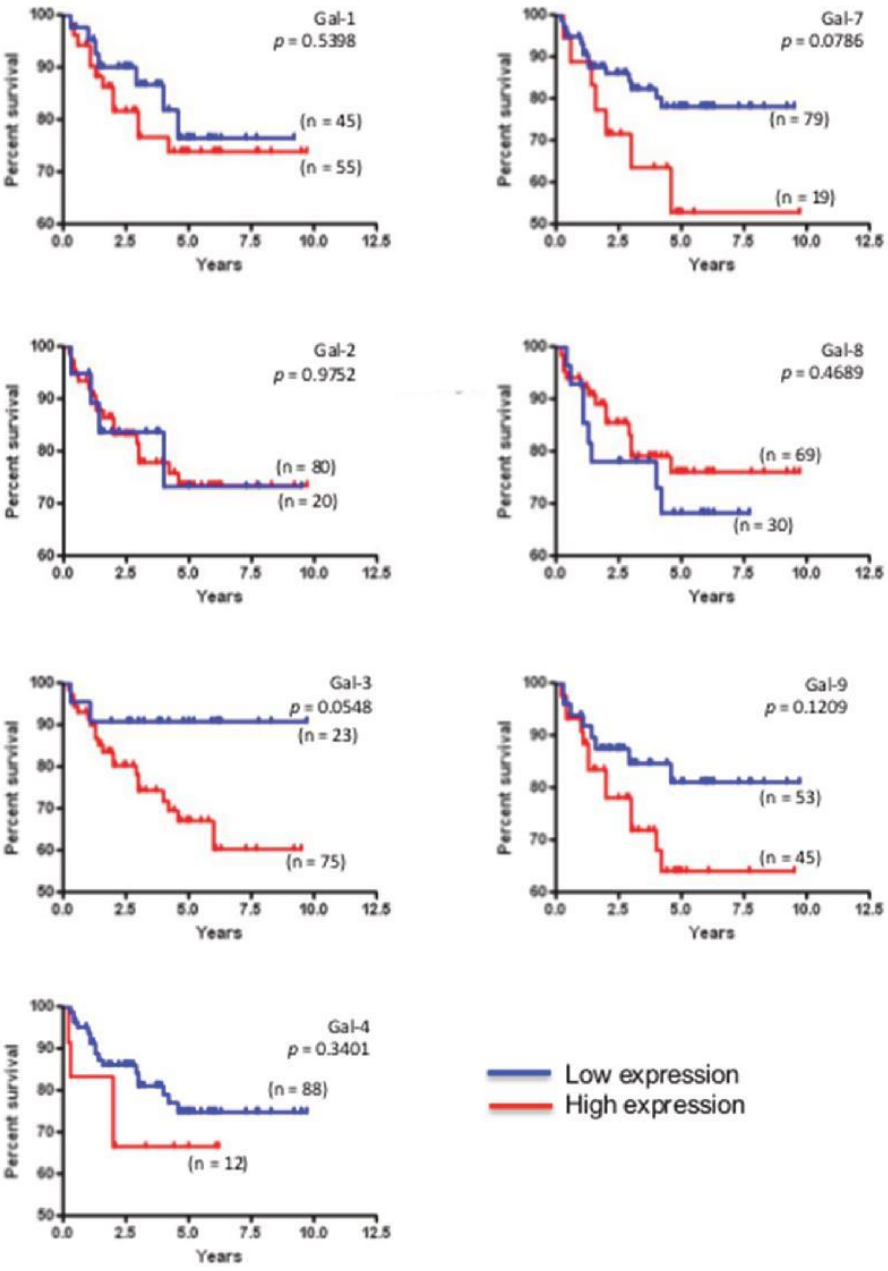

Figure 5: Prognostic potential of galectin in across molecular subtypes of breast cancer. Kaplan-Meier estimates of 5-year DFS in breast cancer patients with low or high galectin expression independently of the molecular subtype.

significant positive correlation between expressions of these galectins (Figure 10).

DISCUSSION

Triple-negative breast cancer is among the most aggressive breast cancer subtypes. To date, there is no clinically available targeted therapy for patients diagnosed with TNBC and approximately 30% of TNBC patients eventually experience distant relapse. The heterogeneity of TNBC makes predicting treatment difficult and remains a major obstacle for the development of TNBC-specific therapeutic targets. In this study, we report that specific galectin expression signatures at the mRNA and protein levels contribute to the phenotypic heterogeneity of TNBC and segregate subsets of aggressive breast cancer into clinically meaningful subtypes.

Gene or protein expression signatures of cancer tissues are generally obtained from whole tumor homogenates, thus reflecting the expression from all cell types present in the tumor. Given the critical role stromal cells in cancer progression, several groups have thus looked at defining signatures that reflects expression profiles of for both cancer and stromal cells [23]. Such strategy has shown, for instance, that the molecular signature of an immune response is an important

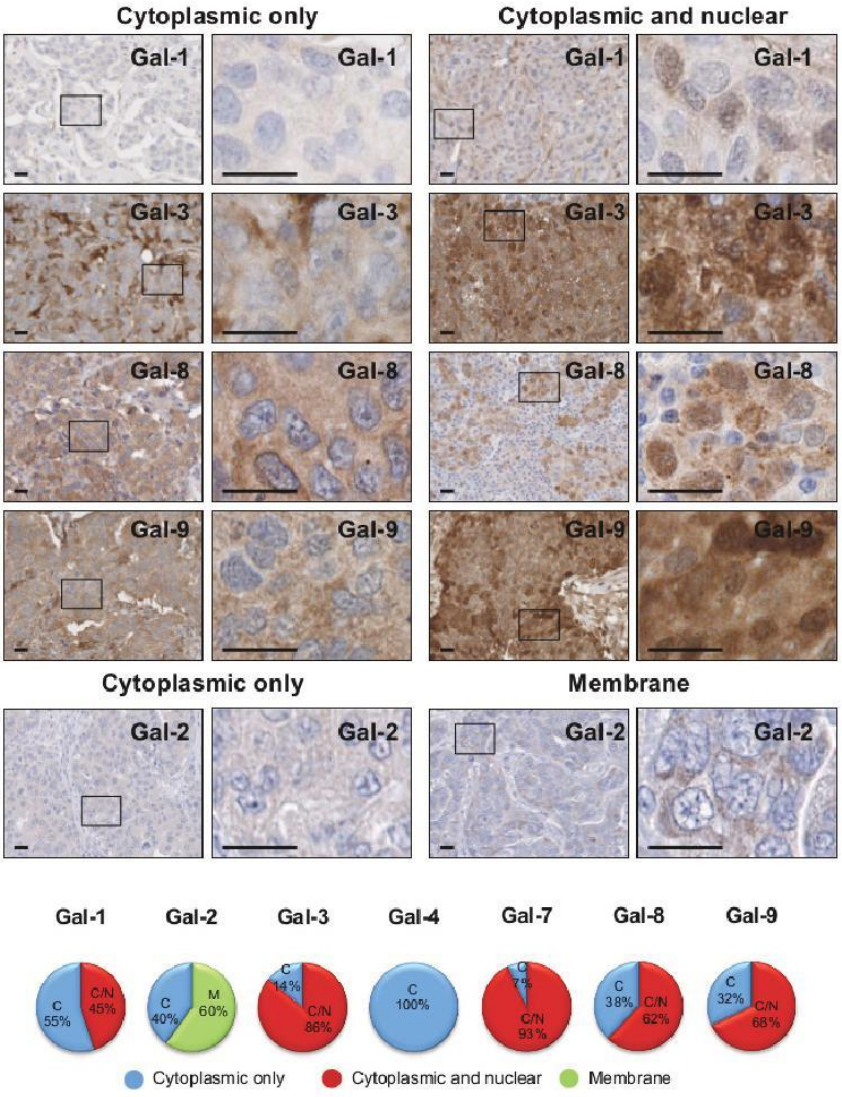

Figure 6: Subcellular localization of galectins in breast cancer tissues. Representative IHC stainings of galectin expression in breast cancer tissues. All scale bars, 25 μm. The lower circle graphs show the percentage of galectin-positive staining in the cytosol only (C), at the membrane (M) and in the cytosol and nuclei (C/N).

Table 5: Histoclinical correlations of breast cancers according to galectins localization in cancer cells

Characteristics	Gal-1 % (n)		Gal-2 % (n)		Gal-3 % (n)		Gal-8 % (n)		Gal-9 % (n)	
	C	C/N	C	M	C	C/N	C	C/N	C	C/N
Age, yr										
≤ 45	14 (12)	9 (8)	9 (13)	10 (14)	3 (4)	17 (23)	10 (14)	11 (15)	5 (4)	14 (11)
> 45	41 (35)	36 (31)	31 (43)	50 (71)	11 (15)	69 (96)	27 (37)	52 (70)	27 (22)	54 (44)
Tumor volume							***P* = 0.002**			
≤ 10 cm^3	33 (27)	21 (17)	24 (32)	31 (40)	8 (10)	51 (65)	**16 (20)**	**44 (55)**	14 (11)	41 (32)
> 10 cm^3	24 (19)	22 (18)	14 (19)	31(40)	7 (9)	34 (43)	**22 (28)**	**18 (23)**	17 (13)	28 (22)
Lymph node metastasis					***P* = 0.043**					
Negative	37 (32)	30 (26)	25 (34)	42 (59)	**6 (8)**	**58 (79)**	22 (29)	42 (56)	21 (17)	41 (33)
Positive	18 (15)	15 (13)	16 (22)	17 (24)	**8 (11)**	**28 (39)**	16 (22)	20 (27)	11 (9)	27 (22)
SBR-EE Grade			***P* < 0.0001**				***P* = 0.024**			
I (low)	6 (5)	4 (3)	**12 (15)**	**2 (3)**	1 (1)	21 (27)	**2 (3)**	**15 (18)**	1 (1)	15 (11)
III (high)	46 (37)	44 (35)	**28 (36)**	**58 (76)**	14 (18)	64 (84)	**35 (44)**	**48 (60)**	30 (22)	54 (40)
Molecular subtypes	***P* = 0.019**		***P* < 0.0001**				***P* < 0.0001**			
Triple negative	**22 (19)**	**33 (28)**	**7 (10)**	**37 (52)**	7 (10)	33 (46)	**24 (33)**	**14 (19)**	17 (14)	23 (19)
HER-2 positive	**9 (8)**	**6 (5)**	**11 (15)**	**8 (11)**	4 (5)	16 (23)	**6 (8)**	**14 (19)**	6 (5)	12 (10)
Luminal A	**22 (19)**	**6 (5)**	**20 (29)**	**14 (20)**	3 (4)	33 (46)	**7 (10)**	**31 (42)**	9 (7)	32 (26)
Luminal B	**1 (1)**	**1 (1)**	**2 (3)**	**1 (2)**	0 (0)	4 (5)	**0 (0)**	**4 (5)**	0 (0)	0 (0)
ER expression	***P* = 0.007**		***P* = 0.007**				***P* = 0.0003**			
Neg/ Low	**33 (28)**	**40 (34)**	**21 (30)**	**45 (64)**	11 (15)	55 (77)	**30 (41)**	**30 (41)**	23 (19)	38 (31)
High	**22 (19)**	**6 (5)**	**19 (27)**	**15 (21)**	3 (4)	31 (43)	**7 (10)**	**33 (44)**	9 (7)	30 (24)
PR expression			***P* = 0.042**							
Neg/ Low	50 (43)	42 (36)	**32 (45)**	**55 (78)**	12 (17)	73 (102)	35 (47)	52 (71)	28 (23)	54 (44)
High	5 (4)	3 (3)	**8 (12)**	**5 (7)**	1 (2)	13 (18)	3 (4)	10 (14)	4 (3)	14 (11)
HER-2 expression			***P* = 0.023**							
Neg/ Low	43 (36)	39 (33)	**27 (38)**	**51 (72)**	10 (14)	66 (91)	31 (42)	45 (60)	26 (21)	56 (45)
High	11 (9)	7 (6)	**13 (18)**	**9 (13)**	4 (5)	20 (28)	6 (8)	18 (24)	6 (5)	12 (10)
EGFR expression	***P* = 0.008**						***P* = 0.044**			
Neg/ Low	**49 (42)**	**28 (24)**	35 (49)	46 (65)	12 (16)	66 (90)	**26 (35)**	**54 (72)**	26 (21)	53 (42)
High	**6 (5)**	**17 (14)**	6 (8)	13 (19)	2 (3)	20 (28)	**11 (15)**	**9 (12)**	6 (5)	15 (12)
Ki-67 expression			***P* = 0.039**				***P* = 0.002**			
Neg/ Low	33 (28)	19 (16)	**27 (38)**	**29 (41)**	8 (11)	54 (74)	**15 (20)**	**42 (56)**	16 (13)	41 (33)
High	21 (18)	26 (22)	**13 (19)**	**31 (44)**	6 (8)	33 (45)	**23 (31)**	**20 (27)**	16 (13)	27 (22)

Fisher's exact test and chi-square test.

prognostic marker in breast cancer and in other cancer forms [23, 24]. Overall, we found that breast cancer stroma was rarely positive for gal-2, -4, -7, and 8. In contrast, gal-1, -3, and -9-positive stroma were frequent, most notably in aggressive molecular subtypes. Interestingly, when released in the extracellular space, these galectins have been shown to contribute induce immune tolerance in various physiological and pathological processes. Such a role as alarmins for galectins has been well documented in pre-eclempsia for several members for the galectin family, including gal-1, -3, and -9 which have been shown to be up-regulated preeclamptic placentas [11, 25–28]. Galectins are also well known for key role in modulating local and systemic anti-tumor responses in cancer [29]. This has been particularly well described for gal-1, induces apoptosis of IFN-γ–producing cells and skews the tumor microenvironment toward a Th2 cytokine profile [30–33]. Gal-1 also contributes to the infiltration of

IL-10-producing Treg1 cells to promote the tumor evasion [34]. In fact, we found that in some patients ($n = 22$), all three galectins are expressed in the stroma. Such triple-positive signature was exclusively found in high grade BC and in TNBC (77%) or HER2 (23%) molecular subtypes. Not surprisingly, 75% (16/22) were expressing high levels of Ki-67-positive cells. These results suggest that stromal expression of gal-1, -3, -9 is associated with the most aggressive forms of breast cancer. This possibly explains why absence of gal-3 in preclinical mouse models of breast cancer does not alter tumor progression [35]. Future analyses with a higher number of patients will be needed to determine whether TNBC/HER2 patients that do express all three galectins in their stroma have a worst prognosis as patients than patients who do not express any or less than three of these galectins. It will also be interesting to identify stromal cells that express galectins and whether they do contribute to the presence of galectins in the extracellular space. Although our IHC staining does not allow to determine which galectins are released in the extracellular space and which cells are responsible for this secretion, historically, the presence of extracellular galectins has been attributed to cancer cells. We cannot exclude the possibility, however, that normal and/or

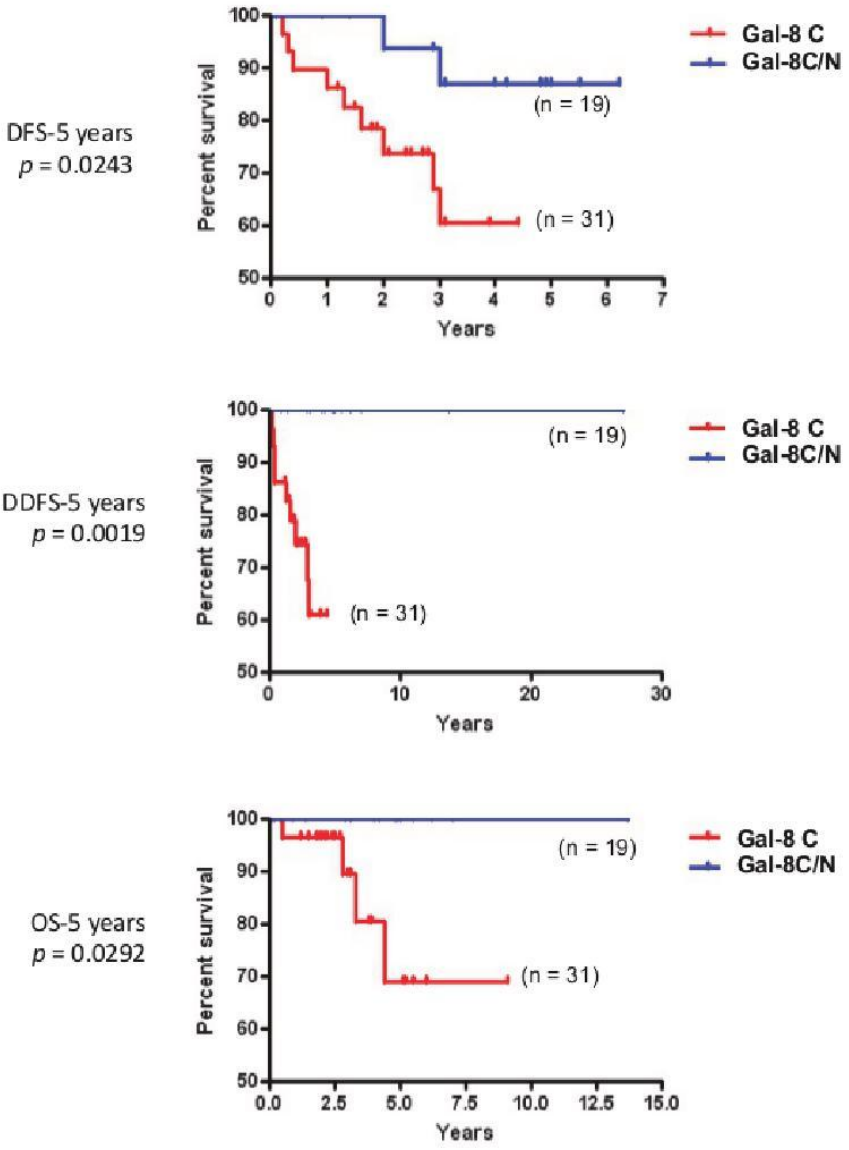

Figure 7: Prognostic potential of nuclear galectin-8 in TNBC. Kaplan-Meier estimates of 5-year DFS, DDFS, and OS in TNBC breast cancer patients expressing cytosolic (C) or cytosolic and nuclear (C/N) galectin-8.

cancer-associated stromal cells release soluble galectins. The presence of circulating levels of galectins in normal individuals certainly support sthis possibility. However, given the increasing evidence that intracellular galectins have many cellular functions and the strong cytosolic and nuclear staining that we observed in both cancer and stromal cells, we need to pay a particular attention to their role inside the cells. The emerging evidence that galectins have critical CRD-independent and intracellular functions certainly calls for a refocusing of our efforts on the development of new galectin-specific antagonists.

It is now well established that it is important to distinguish between stromal and tumor cell signatures to help in defining the heterogeneity of TNBCs and to identify new predictive tools and therapeutic targets. In our study, we have pushed this reasoning one step further by examining the subcellular compartmentalization of a galectins. Our approach was motivated by previous reports showing that members of the galectin family are well known for their heterogeneous pattern of expression and their wide range of biological functions, most notably as modulator of the immune response [29]. Overall, we found that galectins can be potential biomarkers of good and bad prognosis. Gal-1, -3, and -9 expressions in stroma or in tumor cells were all associated with a bad prognosis. Expression of gal-7 was also associated with a bad prognosis, as we previously reported [20]. In contrast to gal-1, -3, and -9, gal-7-positive staining was strictly found in the cytosol and nucleus of epithelial cells of approximately 25% of TNBC and HER2 molecular subtypes. It was not found in luminal A or B subtypes. In the case of gal-2, positive staining was found in all molecular subtypes but its membrane-associated form was also associated with a bad prognosis, as shown by its preferential association with TNBCs and Ki-67-positive staining. Gal-8 was the only galectin that we found was associated with a good prognosis. This association was observed when we took into consideration its nuclear localization in epithelial cells, which correlated with negative/low Ki-67 staining. Overall, these signatures are clearly different from that recently reported in the case of prostate cancer [36]. The authors found that gal-1 was the most abundantly expressed galectin in prostate cancer tissue. In contrast, other galectins (including gal-3, -4, -9, and 12) were expressed at lower levels whereas expression of gal-8 remained unchanged. Although the authors have not examined whether changes in subcellular localization or in the stromal cells occurred, these differences suggest that galectin signatures in cancer are tissue-specific and contributes to the heterogeneity of cancer (Figure 11).

Our results showing that nuclear gal-8 is associated with a good prognosis and that nuclear gal-1 is associated with a negative prognosis are eloquent examples of the importance of taking into account the subcellular localization of proteins with a wide range of subcellular localization. Interestingly, in patients that expressed both

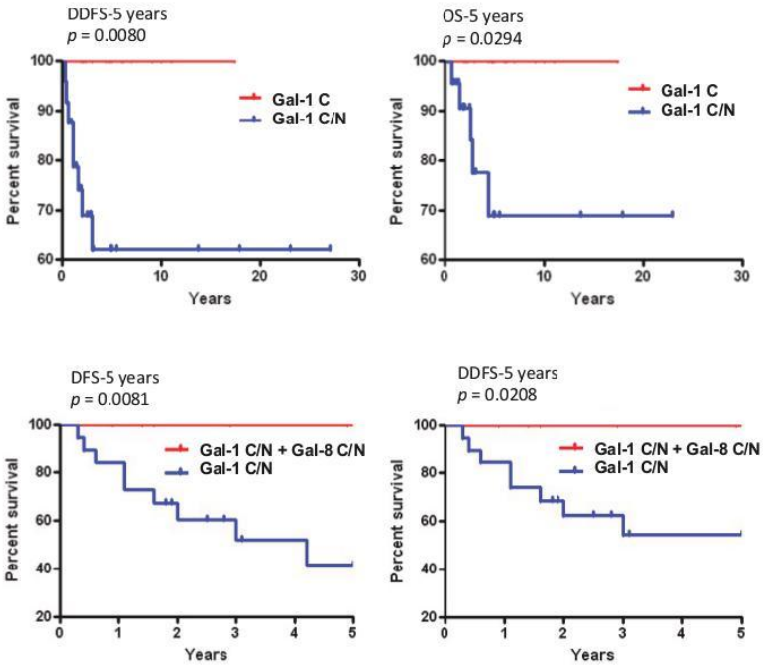

Figure 8: Prognostic potential of nuclear galectin-1 and -8. *Upper histograms*, Kaplan-Meier estimates of 5-year DDFS and OS in TNBC breast cancer patients expressing cytosolic (C) or cytosolic and nuclear (C/N) galectin-1. *Lower histograms*, Kaplan-Meier estimates of 5-year DDFS and DFS in breast cancer patients expressing nuclear galectin-1 and/or galectin-8.

nuclear gal-1 and gal-8, the phenotype of gal-8 was clearly dominant. Despite having nuclear gal-1, the 5-year survival rate of patients expressing nuclear gal-8 was 100%. Such dominance for gal-8 also suggests that both galectins have distinct (and contradictory) nuclear functions and that nuclear galectins undergo profound changes during cancer progression. These results uncover the clinical significance of nuclear gal-8 suspected from previous observations in normal and breast tissues [37]. Historically, gal-8 has been mostly recognized "matricellular extracellular protein" that mediates cell-matrix adhesion following binding to cell-surface integrins [38]. Although our approach is not sensitive enough to confirm the presence of gal-8 outside the cell surface, our data showing strong nuclear and cytosolic gal-8 is consistent with other reports that have shown that gal-8 shuttles between the nucleus and the cytosol in cancer cells [39]. Although the molecular mechanism regulating gal-8 (and gal-1) trafficking in the nucleus is currently unknown, it will be interesting to test whether karyopherins are involved. These proteins have

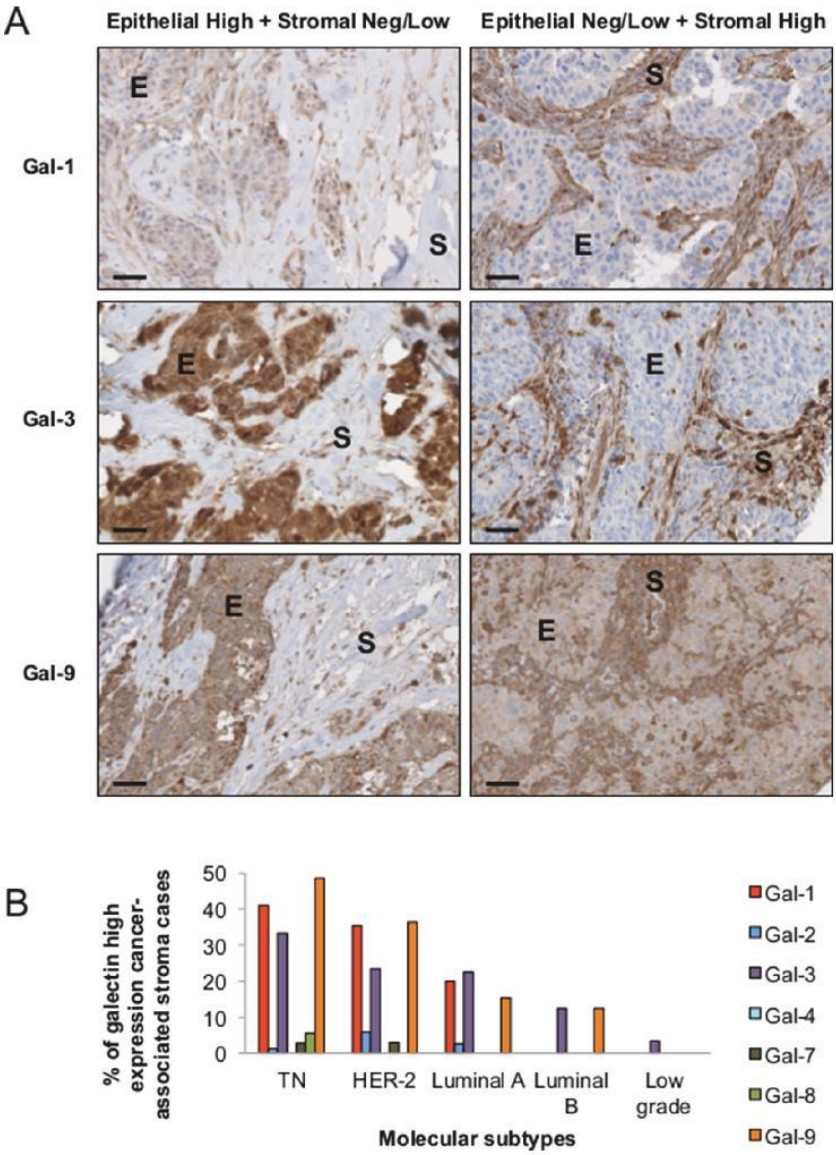

Figure 9: Stromal expression of galectins. (A) Representative IHC staining of galectin-1, -3, and -9 expression in epithelial (E) and stromal (S) cells in breast cancer tissues. All scale bars, 50 μm. (**B**) Bar histograms showing the percentage of cases with high levels of expression of stromal galectin-1, -3, and 9 in molecular subtypes. (C) Correlation between galectin-1, -3, and -9 expression in breast cancer tissues measured across the molecular subtypes.

Table 6: Histoclinical correlations of breast cancers according to galectins expression in cancer-associated stroma

Characteristics	Gal-1 % (n)		Gal-3 % (n)		Gal-9 % (n)	
	Neg/Low	High	Neg/Low	High	Neg/Low	High
Age, yr						
≤ 45	16 (30)	3 (6)	15 (28)	4 (7)	15 (27)	6 (10)
> 45	57 (104)	24 (43)	61 (112)	20 (36)	54 (98)	26 (47)
Tumor volume						
≤ 10 cm^3	43 (72)	14 (24)	42 (71)	14 (24)	39 (66)	17 (29)
> 10 cm^3	29 (49)	14 (24)	35 (59)	10 (17)	29 (49)	15 (25)
Lymph node metastasis						
Negative	48 (85)	17 (31)	48 (85)	16 (28)	42 (74)	21 (38)
Positive	25 (45)	10 (18)	29 (51)	8 (15)	26 (47)	11 (19)
SBR-EE Grade	***P* < 0.0001**		***P* = 0.003**		***P* < 0.0001**	
I (low)	**19 (32)**	**0 (0)**	**17 (29)**	**1 (1)**	**18 (30)**	**1 (1)**
III (high)	**54 (92)**	**28 (47)**	**60 (102)**	**23 (39)**	**51 (87)**	**31 (53)**
Molecular subtypes	***P* = 0.0001**		*P* = 0.0516		***P* < 0.0001**	
Triple negative	**23 (43)**	**16 (30)**	26 (48)	13 (24)	**19 (35)**	**20 (37)**
HER-2 positive	**11 (20)**	**6 (11)**	14 (26)	4 (8)	**11 (21)**	**7 (12)**
Luminal A	**35 (64)**	**4 (8)**	33 (60)	5 (10)	**34 (63)**	**4 (7)**
Luminal B	**4 (8)**	**0 (0)**	4 (7)	1 (1)	**4 (7)**	**1 (1)**
ER expression	***P* < 0.0001**		***P* = 0.031**		***P* < 0.0001**	
Neg/ Low	**38 (70)**	**23 (42)**	**44 (81)**	**18 (33)**	**34 (63)**	**27(49)**
High	**35 (65)**	**4 (7)**	**33 (60)**	**5 (10)**	**34 (63)**	**4 (8)**
PR expression	***P* = 0.017**				***P* = 0.0005**	
Neg/ Low	**60 (111)**	**26 (47)**	**65 (119)**	**22 (40)**	**55 (101)**	**31 (56)**
High	**13 (24)**	**1 (2)**	**12 (22)**	**2 (3)**	**14 (25)**	**1 (1)**
HER-2 expression						
Neg/ Low	58 (105)	21 (38)	58 (106)	19 (34)	53 (96)	24 (44)
High	15 (28)	6 (11)	18 (33)	5 (9)	16 (28)	7 (13)
EGFR expression	***P* = 0.016**		***P* = 0.007**		***P* < 0.0001**	
Neg/ Low	**63 (114)**	**19 (34)**	**65 (119)**	**15 (28)**	**61 (110)**	**19 (35)**
High	**10 (18)**	**8 (15)**	**11 (20)**	**8 (15)**	**7 (13)**	**12 (22)**
Ki-67 expression	***P* = 0.027**		***P* = 0.007**		***P* = 0.001**	
Neg/ Low	**48 (87)**	**13 (23)**	**50 (92)**	**10 (18)**	**47 (85)**	**13 (24)**
High	**25 (46)**	**14 (26)**	**26 (48)**	**14 (25)**	**22 (40)**	**18 (33)**
p53 expression	***P* = 0.024**		***P* = 0.05**		***P* = 0.013**	
Neg/ Low	**57 (103)**	**16 (29)**	**59 (107)**	**14 (26)**	**53 (96)**	**18 (33)**
High	**17 (30)**	**11 (20)**	**18 (33)**	**9 (17)**	**16 (29)**	**13 (24)**

Fisher's exact test and chi-square test.

been shown to regulate nucleus-cytoplasm transport of galectin-3 [40]. Gal-8, however, is possibly not the only members of the galectin family to be associated with a good prognosis in breast cancer. Preliminary *in silico* analysis using the bc-GenExMiner database shows that high expression of *lgals12, lgals13*, and *lgals14* correlates with a good prognosis in LN-negative and luminal B patients (Supplementary Figure S5). Future work will be needed, however, to determine their expression patterns and the good prognostic potential at the protein level.

Table 7: Histoclinical correlations of breast cancers according to galectins expression in cancer-associated stroma

Characteristics	Gal-1 / Gal-3 / Gal-9 stromal expression		*P*
	Negative *n* = 104	Triple positive *n* = 22	
Age, yr			0.562
≤ 45	18% (22)	2% (3)	
> 45	65% (81)	15% (19)	
Tumor volume			0.466
≤ 10 cm^3	48% (55)	9% (10)	
> 10 cm^3	34% (39)	10% (11)	
Lymph node metastasis			1.000
Negative	52% (63)	12% (14)	
Positive	30% (36)	7% (8)	
SBR-EE grade			**0.003**
I (low)	24% (28)	0% (0)	
III (high)	58% (67)	18% (21)	
Molecular subtype			< **0.0001**
Triple negative	23% (29)	13% (17)	
HER-2 positive	12% (15)	4% (5)	
Luminal A	42% (53)	0% (0)	
Luminal B (HER-2+)	5% (7)	0% (0)	
ER expression			< **0.0001**
Neg/ Low	41% (51)	17% (22)	
High	42% (53)	0% (0)	
PR expression			**0.023**
Neg/ Low	66% (83)	17% (22)	
High	17% (21)	0% (0)	
HER-2 expression			1.000
Neg/ Low	64% (80)	14% (17)	
High	18% (22)	4% (5)	
EGFR expression			**0.0003**
Neg/ Low	72% (89)	9% (11)	
High	10% (13)	9% (11)	
Ki-67 expression			**0.0005**
Neg/ Low	57% (71)	5% (6)	
High	26% (32)	13% (16)	
p53 expression			**0.003**
Neg/ Low	65% (81)	8% (10)	
High	18% (22)	10% (12)	

Fisher's exact test and chi-square test.

Our study is the first study that provides a detailed analysis of the galectin protein signature in molecular subtypes of breast cancer. This signature is clearly different from the mRNA signatures obtained from *in silico* analyses of public databases [41]. For example, our analysis using the bc-GenExMiner database shows that gal-1, -2, -3, -4, -7, -8, and -9 were all expressed at the mRNA level in breast tumor tissues and had a relatively similar distribution among the molecular subtypes. While such databases clearly helps in our understanding of breast

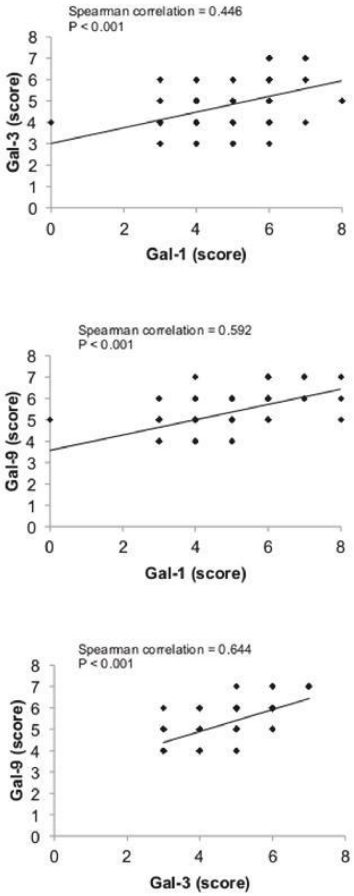

Figure 10: Correlation between galectin-1, -3, and -9 expression in breast cancer tissues. Correlations were measured across the molecular subtypes.

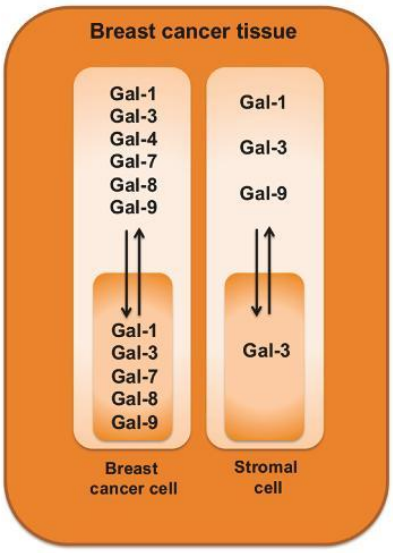

Figure 11: Schematic diagram highlighting galectin expression patterns in breast cancer tissues.

cancer and facilitate the identification of novel intrinsic subtypes, caution should be exercised when evaluating the prognostic or therapeutic potential of a given gene, especially genes encoding multifunctional proteins like galectins. A case in point is the relevance of measuring cytoplasmic versus nuclear gal-8 or gal-1 staining. This is a critical issue given that a considerable amount of efforts are underway for the development of galectins inhibitors for the treatment of cancer.

MATERIALS AND METHODS

In silico analysis

The prognostic module of bc-GenExMiner v3.1 (Breast Cancer Gene-Expression Miner v3.1) [42] was used to correlate survival with each member of galectin family. Gene expression maps represent the percentage of patients with low, intermediate and high gene expression according to molecular subtypes. Kaplan-Meier survival curves (disease free, distance disease free and overall survival) of 4738 patients were obtained from the algorithm BreastMark [14] and the classifier PAM50 [15].

Patients and tumor materials

A cohort of 213 patients diagnosed with primary breast cancer between 2003 and 2008 at the Centre Hospitalier de l'Université de Montréal (CHUM) was used for the study. Tumors were selected on the basis of the histological diagnosis according to the classification of the Modified Scarff-Bloom-Richardson-Elston-Ellis grading system (SBR-EE) [43]. The cohort consisted of both low-grade and high-grade ductal carcinomas and of carcinomas with medullary features. Estrogen receptor status was positive in all low-grade carcinomas. This study was approved by the research ethics committee (CÉR) of the research centre at the CHUM (study SL05.019), in accordance with the Tri-Council Policy Statement on Research with Human Subjects. Consents directly from patients were not required in this study as per Ethics Board guidelines.

Tissue microarrays and immunohistochemistry

Formalin-fixed paraffin-embedded material from each primary tumor sample was used to construct tissue microarrays with an automated arrayer design to construct high-density tissue micro-array blocks (ATA-27 Beecher Instruments, Sun Prairie, WI). To that end, triplicate 1 mm cores from each tumor and control tissues were punched out and arrayed into six recipient blocks. For immunohistochemical analysis, three-micrometer thick sections were prepared from each TMA. Immunostaining reactions for each galectin were carried out using the Discovery XT automated immunostainer (Ventana Medical Systems, Tucson, AZ). Deparaffinized sections were incubated in cell conditioning pH 8, except for anti-galectin-2 (pH 6), for antigen retrieval and then with primary antibodies for 1 to 3 hrs: mouse monoclonal anti-galectin-1 (1:50; Novocastra, Leica Biosystems, Newcastle Upon Tyne, United Kingdom), rabbit polyclonal anti-galectin-2 (1:100; Proteintech Group, Chicago, IL, USA), rabbit monoclonal anti-galectin-3 (1:1000; Abcam, Cambridge, MA, USA), goat polyclonal anti-galectin-4 (1:200; Santa Cruz, Santa Cruz, CA, USA), goat polyclonal anti-galectin-7 (1:1000; R&D Systems, Minneapolis, MN, USA), rabbit polyclonal anti-galectin-8 (1:50; Abcam) and rabbit polyclonal anti-galectin-9 (1:100; Abcam). The slides were counterstained with hematoxylin and bicarbonate. Each section was scanned at a high resolution using the Nanozoomer Digital Pathology (Hamamatsu, Bridgewater, NJ). The validation of antibodies specificity was assessed using a tissue microarray of 21 different human normal tissues. Positive and negative controls were evaluated according to publications. The omission of the primary antibody was also used as a negative control. All antibodies were validated by their respective company and anti-galectin-1, 2, 3, 4 and 7 were used in previous publications [20, 44–49].

Evaluation of immunohistochemical staining

The percentage of staining was scored from 0 to 4 according to the percentage of positive cells displaying galectins expression within a sample (0 (0–9%); 1 (10–25%); 2 (26–50%); 3 (51–75%); 4 (76–100%). The intensity of staining was also scored from 0 to 4, with a score of 0 representing no staining and a score of 4 representing the strongest staining observed. Histological scores were calculated by adding both scores and a strong expression was defined by a score of 6 to 8. This scoring system is somewhat comparable to the Allred score [48, 49].

Statistical analysis

Kaplan-Meier curves and relationship between proteins expression were assessed using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA). For Fisher's exact test, chi-square test and spearman analysis, SPSS Statistics (IBM Corporation, Armoncon, NY) was used. A P value of 0.05 or less was considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

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ANNEXE IV

LES GALECTINES INTRACELLULAIRES DANS LE CANCER : NOUVELLE CIBLES THÉRAPEUTIQUES POTENTIELLES

Maria Vladoiu, Marilyne Labrie, Yves St-Pierre (2016). Intracellular galectins in cancer cells: Potential new targets for therapy (Review). International journal of Oncology (4) : 1001-1014 Published on: 01 avril 2014 doi : 10.3892/ijo.2014.2267

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Intracellular galectins in cancer cells: Potential new targets for therapy (Review)

MARIA C. VLADOIU, MARILYNE LABRIE and YVES ST-PIERRE

INRS-Institut Armand-Frappier, Laval, QC H7V 1B7, Canada

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Abstract. Dysregulation of galectin expression is frequently observed in cancer tissues. Such an abnormal expression pattern often correlates with aggressiveness and relapse in many types of cancer. Because galectins have the ability to modulate functions that are important for cell survival, migration and metastasis, they also represent attractive targets for cancer therapy. This has been well-exploited for extracellular galectins, which bind glycoconjugates expressed on the surface of cancer cells. Although the existence of intracellular functions of galectins has been known for many years, an increasing number of studies indicate that these proteins can also alter tumor progression through their interaction with intracellular ligands. In fact, in some instances, the interactions of galectins with their intracellular ligands seem to occur independently of their carbohydrate recognition domain. Such findings call for a change in the basic assumptions, or paradigms, concerning the activity of galectins in cancer and may force us to revisit our strategies to develop galectin antagonists for the treatment of cancer.

Contents

1. Introduction
2. Where do we find galectins inside the cells?
3. Intracellular functions of galectins in cancer
4. CRD-independent functions for intracellular galectins?

1. Introduction

Galectins represent a family of evolutionarily conserved animal lectins that are widely distributed from lower invertebrates to higher vertebrates. They were initially described in the electric eel, *Electrophorus electricus*, as low molecular weight, β-galactoside binding proteins (1). Since then, galectins have been numbered according to the order of their discovery. The 15 family members are now classified according to their structure and number of carbohydrate recognition domain (CRD). The prototype subfamily of galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15) consists of a single CRD with a short N-terminal sequence. The tandem-repeat type subfamily (galectin-4, -6, -8, -9 and -12) has two non-identical CRDs joined by a short linker peptide sequence. There is also a chimerical form of galectin (galectin-3) that contains one CRD connected to a non-lectin domain.

One of the first clues that galectins were involved in cancer was published more than 25 years ago when it was observed that they were differently regulated in normal and cancer tissues. Since then, a large number of studies have focused on the role of galectins in cancer and excellent reviews on the role of galectins have been published (2-5). Historically, studies on the role of galectins in cancer have mostly focused on their ability to bind membrane-anchored cell surface receptors via their CRD. Their dimeric form (or multimeric in the case of galectin-3) induces crosslinking of the receptors and formation of a lattice that triggers a cascade of transmembrane signaling events. For example, binding of galectin-3 protects EGF and TGF-β receptors from negative regulation via constitutive endocytosis and increases sensitivity of tumor cells to growth factors (6). Binding to cell surface receptors can also induce apoptosis. This is particularly relevant in the case of galectin-1, which is capable of inducing apoptosis of T-cells and potentially create an immunosuppressive tumor microenvironment (7). Alternatively, binding to cell surface receptors can facilitate intercellular adhesion (to promote homo- and heterotypic aggregation) or adhesion of tumor cells to extracellular matrix proteins. Exhaustive efforts have thus been deployed for the identification of highly selective and potent galectin inhibitors. Despite decades of research, the progression in this field has been relatively slow. In most cases, these inhibitors are peptides or high molecular weight, naturally occurring polysaccharides that are used to specifically block the binding of extracellular galectins to carbohydrate structures on cell surface receptors. While targeting extracellular galectins is warranted, such inhibitors are largely if not completely ineffective at targeting intracellular galectins. Indeed, most galectins preferentially exist in intracellular

Correspondence to: Professor Yves St-Pierre, INRS-Institut Armand-Frappier, University of Quebec, 531 Boul. Des Prairies, Laval, QC H7V 1B7, Canada
E-mail: yves.st-pierre@iaf.inrs.ca

Key words: galectin, cancer, subcellular localization

compartments, consistent with the fact that they do not harbor a signal sequence and are transported outside the cells via a non-classical secretory pathway, possibly via galectin-rich vesicles or exosomes. A better understanding of their intracellular functions in cancer cells is thus critical to help develop new anticancer therapies directed at these proteins.

2. Where do we find galectins inside the cells?

The answer to this question is rather simple: almost anywhere (Fig. 1). They can be detected in various intracellular compartments of both normal and cancerous cells. Frequently, modifications in the subcellular localization occur when cells undergo cell-transformation into malignant phenotypes (4). It is noteworthy to mention that galectins expression is also modulated during some of these cell transformation processes, hence their presence/absence in those subcellular localizations is not exclusive to protein translocation (8). Up to now, however, our knowledge of intracellular galectins has mostly been obtained while studying galectin-3. As we gain more and more knowledge on other members of the galectin family, we find overwhelming evidence that most if not all galectins are often expressed inside the cells. Here we describe the intracellular localization of various galectins with their respective cancer tissues and/or cell lines (Table I).

Galectin-1 is observed in the nuclear compartment of transfected HeLa cells (9) and the inner plasma membrane of colorectal adenocarcinoma cells (HCT116) (10). Moreover, its presence is also seen in the cytosol of neuroblastoma and small cell lung carcinoma tissues, testicular interstitial and cervical carcinoma cell lines (MA-10 and HeLa), hypopharyngeal (HSCCs) and laryngeal (LSCCs) squamous cell carcinoma tissues, human melanoma cell lines (A375 and A2058) and colorectal cancer tissues including adenomas, carcinomas and metastases from patients (9,11-15). Although fewer studies have been conducted on galectin-2, the available data indicate its presence in the nucleus of genetically engineered human colon cancer cells that have ectopic stable expression (16) in addition to gastric carcinoma tissues, epidermoid carcinoma, osteosarcoma and glioblastoma cell lines (A-431, U-2 OS and U-251MG) (17,18). Its presence has also been reported in the cytosol of gastric carcinoma tissues and in mitochondria of epidermoid carcinoma, osteosarcoma and glioblastoma cell lines (A-431, U-2 OS and U-251MG) (17,18). In the case of galectin-3, one of the most investigated members of the galectin family, its presence is detected in the nucleus of aggressive endometrial adenocarcinoma, melanoma cell lines, malignant thyroid carcinomas (follicular adenoma, Hürthle cell adenoma and papillary carcinoma) (19-21). Galectin-3 is also found in the cytosol of colonic adenomas/carcinomas tissues, follicular/papillary thyroid carcinomas, endometrial adenocarcinoma, human melanoma cell lines (MlDo and M4Be), malignant thyroid carcinoma (follicular adenoma, Hürthle cell adenoma and papillary carcinoma) and in tongue squamous cell carcinoma tissues (19-24). Additionally, galectin-3 is found in the mitochondria of colorectal adenocarcinoma cell line (SNU-769B), in endosomal compartments of breast adenocarcinoma cell line (SKBR3), and in apical membrane regions of human colon adenocarcinoma cell lines (T84 and HCT116) (10,25-27). Galectin-4 is detected in the cytosol of human breast ductal carcinoma tissues (28,29) and pancreatic adenocarcinoma cell line (Pa-Tu-8988S) (29) as well as inside the basal plasma membrane of human colon adenocarcinoma cells (T84) (27). Galectin-7, which has recently attracted more interest in cancer because its preferential expression in epithelial tissues and carcinomas, is seen in the nucleus of many cancer cells, including hypopharyngeal (HSCCs) and laryngeal (LSCCs) squamous cell carcinomas tissues, colon carcinoma cells (DLD-1), cervical adenocarcinoma (HeLa), epithelial ovarian cancer tissues and oral epithelial dysplasia tissues (13,30-32). Galectin-7 is also observed in the cytosol of the colon carcinoma cell line DLD-1, cervical adenocarcinoma cells (HeLa), epithelial ovarian cancer and oral epithelial dysplasia tissues (17,30-32). Like galectin-3, it is also detected in mitochondrial fractions, most notably in the case of human colorectal carcinoma and cervical adenocarcinoma cell lines (HCT116, HeLa) and the HaCaT keratinocyte cell line (33). Galectin-8 expression is detected in the cytosol, nucleus and mitochondria of tumor-associated epithelial cells from human prostate and breast tissues (34). Intracellular galectin-9 is observed in the cytosol of human melanoma cell lines (MM-BP and MM-RU) and the MCF-7 breast carcinoma cell line (35,36). Galectin-10 is observed in the nuclei and cytosol of epidermoid carcinoma cells and in the cytoplasmic compartments of glioblastoma and osteosarcoma. In the human promyelocytic leukemia HL-60 cell line, it is found in the nucleus, cytosol and mitochondria (37) while its localization is associated with the inner plasma membrane of many glioblastoma cell lines (A-431, U-2 OS and U-251MG) (17). Galectin-12 is observed in the cytosol and mitochondria of osteosarcoma and glioblastoma cell lines (U-2 OS and U-251MG) (17).

Although there are no reports yet that other galectins are present inside cancer cells, there are indications that this may well be the case given their presence inside normal cells. For example, galectin-12, a close structural homolog of galectin-7, has been found in the nucleus and mitochondrial fractions of adipocytes (38-40). The fact that galectin-12-deficient mice have abnormal mitochondrial activity is particularly interesting considering the key role of mitochondria in energy metabolism of cancer cells (41,42). Galectin-10 is also found inside human regulatory T-cells and other inflammatory cells (43) while galectin-13 is found in the perinuclear area of syncytiotrophoblasts (44). Computational predictions of where galectins resides in a cell show that it is logical to assume that many galectins will be present within several intracellular compartments. For example, using pSORT, a commonly used tool to predict intracellular localization of proteins, we found that all galectins have a strong preference for cytoplasmic, nuclear and mitochondrial compartments (Table II) (45,46). We have obtained similar results using other computational tools (unpublished data).

3. Intracellular functions of galectins in cancer

The main challenge in studying the galectin functions in neoplasms remains their opposing functions in tumor progression. Depending on the type of cancer, one galectin

Table I. Intracellular localization of galectins in different cancers.

Localization	Galectin	Cancer cell line/tissue from patients	(Refs.)
Nuclear	Galectin-1	Cervical adenocarcinoma	(9)
	Galectin-2	Colorectal carcinoma	(16)
		Epidermoid carcinoma, osteosarcoma and glioblastoma	(17)
		Gastric carcinoma	(18)
	Galectin-3	Adenocarcinoma of the endometrium	(19)
		Melanoma	(20)
		Thyroid carcinoma (follicular/Hürthle cell/papillary)	(21)
	Galectin-7	Hypopharyngeal/laryngeal squamous cell carcinoma	(13)
		Colorectal carcinoma and cervical adenocarcinoma	(30)
		Epithelial ovarian cancer	(31)
		Oral epithelial dysplasia	(32)
	Galectin-8	Tumor-associated epithelial cells from prostate and breast carcinoma	(34)
	Galectin-10	Epidermoid carcinoma	(17)
		Human promyelocytic leukemia (HL-60)	(37)
Cytoplasmic	Galectin-1	Cervical adenocarcinoma	(9)
		Neuroblastoma and small cell lung carcinoma	(11)
		Testicular (interstitial cell) carcinoma	(12)
		Hypopharyngeal/laryngeal squamous cell carcinoma	(13)
		Melanoma	(14)
		Colorectal carcinoma	(15)
	Galectin-2	Gastric carcinoma	(18)
	Galectin-3	Colorectal adenoma and carcinoma	(22)
		Follicular and papillary thyroid carcinoma	(23)
		Adenocarcinoma of the endometrium	(19)
		Melanoma	(20)
		Thyroid carcinoma (follicular/ Hürthle cell/papillary)	(21)
		Squamous cell carcinoma of the tongue	(24)
	Galectin-4	Ductal breast carcinoma	(28)
		Pancreatic adenocarcinoma	(29)
	Galectin-7	Colon carcinoma and cervical adenocarcinoma	(30)
		Epithelial ovarian cancer	(31)
		Epidermoid carcinoma and osteosarcoma	(17)
		Oral epithelial dysplasia	(32)
	Galectin-8	Tumor-associated epithelial cell from prostate and breast carcinoma	(34)
	Galectin-9	Melanoma	(35)
		Breast carcinoma	(36)
	Galectin-10	Epidermoid carcinoma and glioblastoma	(17)
		Human promyelocytic leukemia (HL-60)	(37)
	Galectin-12	Osteosarcoma and glioblastoma	(17)
Mitochondrial	Galectin-2	Epidermoid carcinoma, osteosarcoma and glioblastoma	(17)
	Galectin-3	Colorectal adenocarcinoma	(25)
	Galectin-7	Colorectal carcinoma and cervical adenocarcinoma	(33)
	Galectin-8	Tumor-associated epithelial cell from prostate and breast carcinoma	(34)
	Galectin-10	Human promyelocytic leukemia (HL-60)	(37)
	Galectin-12	Osteosarcoma and glioblastoma	(17)
Endosomal compartments	Galectin-3	Breast adenocarcinoma	(26)
Plasma membrane	Galectin-1	Colorectal adenocarcinoma	(10)
	Galectin-3	Colorectal adenocarcinoma	(10,27)
	Galectin-4	Colorectal adenocarcinoma	(27)
	Galectin-10	Epidermoid carcinoma, osteosarcoma and glioblastoma	(17)

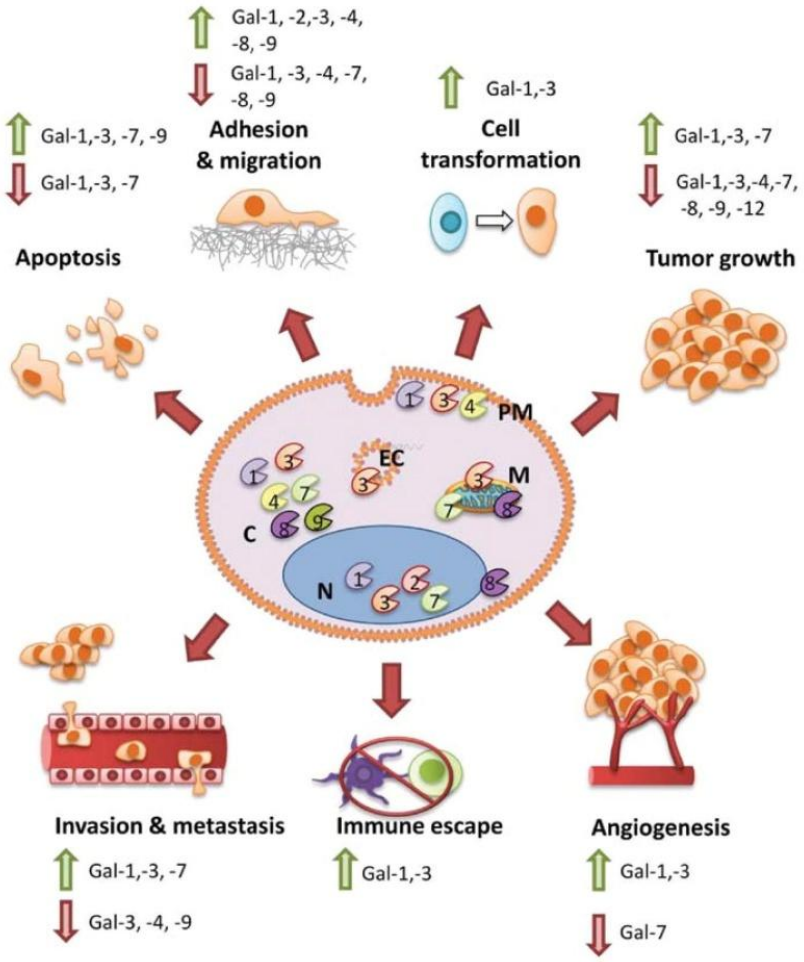

Figure 1. Pro- and anti-tumoral functions of galectins in cancer. Galectins are found in the cytoplasm (C), mitochondria (M), nucleus (N), endosomal compartments (EC) and inner plasma membrane (PM). They are capable of modulating many aspects of tumor progression such as cell adhesion and migration, immune escape, cell transformation, apoptosis, angiogenesis, tumor growth, invasion and metastasis.

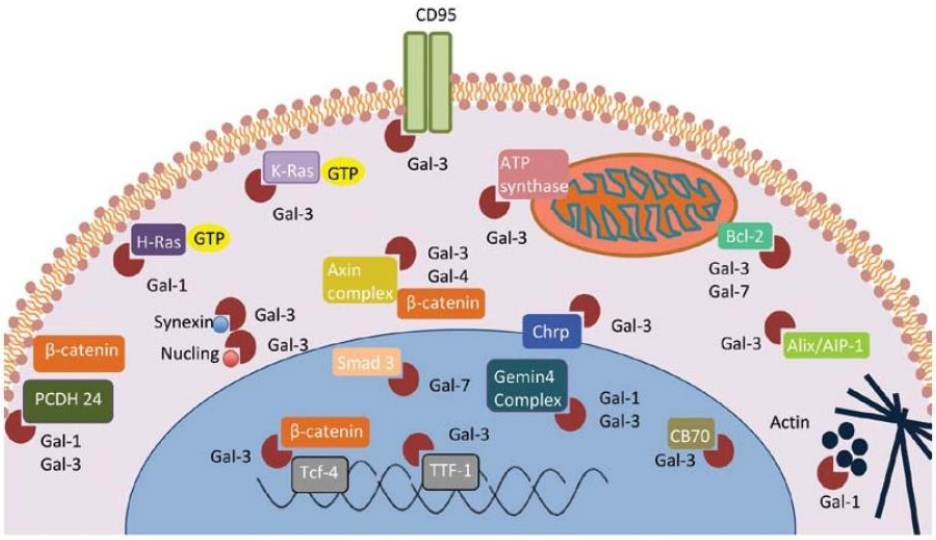

Figure 2. Intracellular binding partners of various galectins. Galectins have numerous binding partners with respect to their inner compartmentalization.

Table II. Predicted intracellular localization of galectins.

Cellular compartment	Galectin (%)									
	1	2	3	4	7	8	9	10	12	13
Cytoplasmic	65	52	26	65	65	70	65	52	39	61
Nuclear	22	26	48	17	17	17	17	13	13	17
Mitochondrial	4	9	9	13	17	9	4	4.3	44	13
ER	4	-	-	-	-	4	4	-	-	4
VSS	4	-	-	4	-	-	4	-	-	-
Vacuolar	-	4	-	-	-	-	-	-	-	4
Cytoskeletal	-	4	-	-	-	-	-	22	4.3	-
Peroxisomal	-	4	-	-	-	-	-	9	-	-

ER, endoplasmic reticulum; VSS, vesicles of secretory system.

can either have pro/antitumoral properties (5,47,48) (Fig. 1). This characteristic of galectins can be explained by the large diversity of binding partners (Fig. 2) and by the expression pattern of these partners, which varies contingent to the cell type. Another proposed hypothesis supporting the dual functionalities of galectins in cancer is based on the distinct compartmentalization of the proteins within the cells. In fact, it was shown that intracellular localization of galectins differs according to the cell type and tumor progression stage. Supporting this hypothesis, Califice *et al* (47) demonstrated that overexpression of galectin-3 in LnCap prostate cancer cells in the cytoplasm induces invasion behavior, anchorage-independant growth, tumor growth and angiogenesis and reduces apoptosis, while nuclear overexpression results in the opposite biological activities. Hence, it is of great interest to take a closer look at the intracellular localization of these galectins and the impact it has on their biological functions with regards to cancer progression. Here, we discuss the main findings on the possible roles of intracellular galectins in cancer. A detailed report of their functions and their putative ligands is found in Tables III and IV.

Cell transformation. A positive correlation between the expression of galectin-1 and -3 and malignant transformation has been established using different cellular models (49-51). Although the mechanisms involved are not completely clear, it potentially involves interactions with membrane-bound H-Ras and K-Ras (52-54). Interestingly, Ras-transformed NIH-3T3 cells have increased expression of galectin-1 and galectin-3 compared to control cells (55). This induction is not necessarily a consequence of Ras pathway activation but rather a secondary effect of cell transformation. Hebert *et al* demonstrated that Ras transfected cells that have a transformed phenotype, express galectin-3 while Ras transfected cells that have not achieved cell transformation do not (56). Another possibility for galectin-induced malignant transformation might be via their association with the spliceosome. Indeed, galectin-1 and -3 are found in Gemin4 (C50)/SMN/Gemin2 complex and play an important role in spliceosome assembly (57). This association suggests that those galectins might regulate the processing of pre-mRNA during malignant transformation.

Apoptosis. Apoptosis regulation by galectins is probably one of their most studied intracellular functions. Several studies have shown that galectins either positively or negatively regulate apoptosis in various cancer cell models. Galectin-1 for example, increases apoptosis of LnCap prostate cancer cells, CoLo201 colon cancer cells, Leydig tumor cells and B-cell lymphomas (12,58-61). Conversely, it reduces apoptosis in gliomas, cervical and lung cancer (62-64). Galectin-3 has also been shown to modulate apoptosis. In myeloid leukemia, neuroblastoma, colorectal, breast, prostate, thyroid, bladder, pancreatic, gastric and some B-cell lymphoma cancer cells it has been shown to have anti-apoptotic functions (47,65-80). In contrast, it seems to induce apoptosis in other B-cell lymphomas (81). Galectin-7 displays a dual functionality in apoptosis as well since it reduces chemosensitivity in melanomas, breast and lymphoid cancer cells, yet it sensitizes colon, urothelial and cervical cancer cells to cell death (82-87). This role of galectin-7 in melanoma cells is clearly distinct from that of galecin-9 which rather promotes death of melanoma cells (35,88).

The underlying mechanisms of galectin's regulation of apoptosis are not fully understood. Nonetheless, many binding partners implicated in cell fate have been identified. Galectin-3 and -7 have been shown to interact *in vitro* and *in vivo* with the anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein (33,89,90). The domain of galectin-7 protein implicated in this binding has not yet been identified. Still, the NWGR motif present at the N-terminus of galectin-3 protein shows a strong homology with the BH1 motif of Bcl-2, which appears to be essential for its anti-apoptotic functions (90). Due to a strong homology between the different pro- and anti-apoptotic members of the Bcl-2 family, galectins might also be able to interact with other members of the family. The modulation of either their stability or their localization would explain the dual role of galectins in apoptosis. The members of the Bcl-2 family are probably not the only galectin-binding partners implicated in apoptosis regulation. Synexin, a calcium and phospholipid-binding protein has been shown to drive the perinuclear translocation of galectin-3, which is essential to its anti-apoptotic function (91). Galectin-3 also interacts with the intracellular domain of the CD95 receptor, also known as FAS receptor

Table III. Intracellular functions of galectins in different cancers.

Galectin	Cancer type	Effect	(Refs.)
Galectin-1	Thyroid	Expression associated with malignant transformation	(50,157)
	Prostate	Increases adhesion, reduces growth rate and induces apoptosis of LnCaP cells, provoque tumor immune evasion and increases tumor vascularization. Stimulate heterotypic cell-cell adhesion	(58,108,141,158,159)
	Breast	Induces angiogenesis, tumor immune evasion and progression	(138,140,160,161)
	Colorectal	Associated with malignant progression, reduces cell migration and induces cell adhesion to ECM and apoptosis of Colo201 cells	(59,110,162)
	Cervical	Induces radioresistance, proliferation and invasion	(62,115)
	Lung	Promote chemoresistance, migration and invasion	(63,109)
	Ovarian	Increases proliferation and invasion	(64)
	Gliomas	Increases cell growth, invasion, angiogenesis and chemotherapy resistance	(51,112,113,137,163-165)
	B-cell lymphoma	Decreases viability and cell growth	(60,61)
	Melanoma	Induces cell aggregation	(166)
	Neuroblastoma	Reduces cell growth, induces immunoevasion	(93,142)
	Leydig tumor cells	Regulates positively or negatively cell proliferation and apoptosis	(12)
	Hepatic	Increases migration and invasion	(111)
	Pancreas	Promotes proliferation, invasion and immune evasion	(114,142)
Galectin-2	Breast	Increases adhesion	(116)
	Colon	Increases adhesion	(116)
Galectin-3	Colorectal	Increases metastasis formation, reduces apoptosis and induces tumor immune evasion	(65,66,125,143,167)
	Breast	Induces cell cycle arrest in response to anoikis, increases adhesion, tumor growth and protects from apoptosis	(67,68,98,168-170)
	Prostate	Induces chemoresistance, cell proliferation, angiogenesis, migration and invasion	(47,69,120,126,171)
	Thyroid	Promotes anchorage-independent growth and motility, regulate cell cycle and cell transformation, promotes chemoresistance	(70-72,172-174)
	Liver	Promotes metastasis formation	(124)
	Lung	Increases adhesion, motility, invasion and tumor immune evasion	(117)
	B-cell lymphoma	Increases resistance to fas-induced apoptosis, chemoresistance or induces apoptosis	(73,81,175,176)
	Myeloid leukemia	Reduces chemosensitivity	(74,75)
	Gliomas	Decreases cell motility and adhesion	(121)
	Melanoma	Increases metastasis formation, tumor immune evasion and angiogenesis	(122,123,139,177,178)
	Bladder	Protects cells against TRAIL-induces apoptosis	(76)
	Ovarian	Reduces cell proliferation and increases apoptosis resistance	(179,180)
	Pancreas	Increases invasion and proliferation, reduces chemosensitivity	(94,95,181,182)
	Gastric	Increses cell motility and chemoresistance	(78,118,119)
	Tongue	Increases cell proliferation, migration and invasion	(96,97)
	Neuroblastoma	Reduces apoptosis	(79)
	Renal	Reduces chemosensitivity	(80)
Galectin-4	Colorectal	Promotes adhesion, reduces cell migration and motility, induces cell cycle arrest	(100,101,183)
	Pancreas	Reduces migration and metastasis formation	(29)

Table III. Continued.

Galectin	Cancer type	Effect	(Refs.)
Galectin-7	Breast	Increases invasion, reduces chemosensitivity	(82)
	Lymphoma	Increases metastasis formation	(127-129)
	Ovarian	Increases cell proliferation	(31)
	Neuroblastoma	Reduces cell growth	(104)
	Colon	Increases chemosensitivity and reduces cell growth, anchorage-independent cell growth and angiogenesis	(83)
	Urothelial	Increases chemosensitivity	(85)
	Cervical	Increases invasive behavior *in vitro*, reduces invasion and chemoresistance	(86,87,130)
	Melanoma	Increases chemoresistance	(84)
	Gastric	Reduces cell proliferation, migration and invasion	(103)
Galectin-8	Glioblastoma	Stimulates cell migration	(131)
	Colon	Reduces tumor growth and cell migration	(106)
Galectin-9	Melanoma	Induces cell aggregation and apoptosis	(35,88,136)
	Breast	Increases cell aggregation and reduces adhesion	(36)
	Oral	Increases adhesion	(132,133)
	Colon	Increases adhesion *in vitro* but reduces metastasis formation *in vivo*	(134-136)
	Myeloma	Reduces cell growth and induces apoptosis	(105,184)
Galectin-12	Cervical	Reduces cell growth	(107)
	T-cell leukemia	Reduces cell growth	(107)

(FasR) or apoptosis antigen 1 (APO-1 or APT), leading to opposing apoptogenic mitochondrial activity (92).

Proliferation. Given their role in apoptosis, it is not surprising that galectins play a central role in the control of cell proliferation in tumors. This has been well documented in the case of galectin-1, which reduces proliferation of B-cell lymphomas, neuroblastoma and LnCap prostate cancer cells while it increases cell division of glioma, cervical, ovarian and pancreatic cancer cells (12,51,58,61,62,64,93). A similar case exists for galectin-3, which displays, once more dual functionalities in cell proliferation. For instance, galectin-3 increases proliferation of breast, prostate, pancreatic and tongue tumors (47,94-98). This might be due to the interaction of galectin-3 with the APC/Axin/β-catenin complex in the nucleus. This interaction increases the transcriptional activity of Tcf-4 transcription factor and subsequently elevates c-myc and cyclin D1 expression (99). In contrast, cytoplasmic galectin-3, along with galectin-1, bind to protocadherin-24, allowing cytoplasmic localization of β-catenin, while decreasing Wnt signaling (10). Ectopic expression of galectin-4 has also been shown to induce cell cycle arrest and to reduce cell migration/motility while sensitizing cells to camptothecin-induced apoptosis in colorectal cancer (100,101). The data from Satelli *et al* (101) suggest that galectin-4 induces downregulation of β-catenin, Dvl2, TCF1, TCF4, c-Myc, LRP6 and cyclin D1 expression levels while upregulating p21, p15 Naked 1 and Ephrin B1 (101,102). An interaction between galectin-4 and APC/Axin/β-catenin is also observed that possibly restricts the translocation of the complex to the nucleus. This results in a downregulation of Wnt signaling and a decrease in proliferative potential of colon cancer cells. In contrast, galectin-7 seems to exhibit an increased proliferative activity in ovarian cancer cells, whereas it reduces the proliferation rate of neuroblastomas, colon and gastric cancer cells (31,103,104). Galectin-8 and -9 have been shown to reduce colon and myelomas tumor growth, respectively (105,106). Galectin-12, for its part, reduces the proliferation of T-leukemia and cervical cancer cells (107). Such contradictory roles for galectins in cell proliferation suggest that extreme precaution must be taken in order to target intracellular galectins in cancer.

Adhesion, migration and invasion. The metastatic behavior of cancer cells is initiated by dysregulation in cell adhesion, migration and invasion abilities. Alterations of interactions between extracellular transmembrane receptors and galectins are often seen in malignancies and late stages of carcinomas. For instance, galectin-1 increases adhesion of colorectal and prostate cancer cells (59,108) and stimulates migration of hepatic and lung cancer cells, while reducing colorectal cell migration (63,109-111). It also increases the invasive behavior of gliomas, lung, ovarian, hepatic, pancreatic and cervical cancer cells (51,63,64,109,111-115). The ability of galectins to increase adhesion and migration has been well documented in the case of galectin-2 and most notably in the case of galectin-3 (47,67,97,116-120). Specifically, galectin-3 reduces glioma cell migration (121). In general, however, galectin-3

Table IV. Intracellular ligands of galectins.

Galectin	Binding partners	CRD/non-CRD binding	Effect	(Refs.)
Galectin-1	H-Ras		Increased membrane anchorage of Ras and GTP bound state resulting in cell transformation	(52)
	Gemin4 (C50)/SMN/ Gemin2 complex		Supply functional snRNPs to the H/E complex in the pathway of spliceosome assembly	(57)
	Protocadherin-24		Localization of β-catenin to the cell membrane resulting in decreased Wnt signaling	(10)
	Monomeric actin	CRD	Polymerization-depolymerization of actin in platelet aggregation	(185,186)
Galectin-3	ATP synthase		Inhibition of ATP synthase activity and cell cycle progression to G0/G1 phase	(25)
	Protocadherin-24		Localization β-catenin to the cell membrane resulting in decreased Wnt signaling	(10)
	CD95 (APO-1/Fas)	Non-CRD	Induction of apoptogenic activity at the mitochondria	(92)
	Nucling		Increase sensitivity to apoptosis	(187)
	Synexin		Decrease sensitivity to apoptosis	(91)
	CBP70	CRD	ND	(188)
	β-catenin/TCF complex	NH2 and COOH termini	Induction of transcriptional activity of Tcf-4 with an increase in c-Myc + cyclin D1 expression	(99)
	Axin/β-catenin/APC	Consensus sequence (S92XXXS96)	Promotion GSK-3β-dependent phosphorylation of galectin-3/β-catenin resulting in a decrease in Wnt signaling	(147)
	TTF-1		Upregulation of transcriptional activity of TTF-1 contributing to cellular proliferation	(189)
	K-Ras		Increase Raf-1/PI3K signaling and attenuated ERK signaling	(53,54)
	Bcl-2	Non-CRD (NWGR motif)	Apoptosis-suppressing activity and increase mitochondrial integrity and decrease caspase activation	(89,90)
	Alix/AIP-1		Facilitation of pro-apoptotic signaling (Ca^{2+} dependent)	(190-192)
	Gemin4 (C50)/SMN/ Gemin2 complex		Supply functional snRNPs to the H/E complex in the pathway of spliceosome assembly	(57)
	Chrp	CRD	ND	(193,194)
Galectin-4	β-catenin/APC/Axin		Increase Naked 1 which destabilizes Dsh/Dvl proteins resulting in a decreased Wnt signaling	(101,102)
Galectin-7	Bcl-2		Sensitize mitochondria to apoptosis signals	(33)
	Smad 3		Decrease expression of TGF-β responsive genes resulting in an anti-fibrotic effect on liver tissue	(195)

is mostly associated with increased invasive behavior in most cancer cell types tested (94,95,117,122-126), supporting the view that targeting this galectin might be a promising avenue for the treatment of many types of cancer. Whether this is also true for other galectins has to be determined. On the contrary, galectin-4 was found to promote adhesion of colorectal cells and to reduce migration and metastasis formation of colorectal and pancreatic cancer cells (29). Further, conflicting functionalities are once again displayed in the case of galectin-7 dependent on the cell type. Particularly, galectin-7 reduces migration of gastric cancer cells and invasion of urothelial and gastric cancer cells (85,103) while it is associated with increased invasion of other types of cancer, including breast cancer and T-cell lymphoma (82,127-130). Galectin-8 also seems to have different abilities to modulate migration, most notably in glioblastoma and colon cancer

cells (106,131). A similar scenario exists for galectin-9, which increases adhesion of melanoma, oral and colon cancer cells, but reduces adhesion of melanoma and breast cancer cells and metastasis formation of colon cancer cells (35,36,132-136). How galectin positively or negatively modulates the invasive behavior of cancer cells remains largely unknown. There are some indications that galectins may increase the secretion of extracellular proteases, remarkably in the case of galectin-7, which induces the upregulation of matrix metalloproteinase-9 (MMP-9) gene expression, possibly through the p38 mitogenic-activated protein kinase (MAPK) (128,130). Unlike apoptosis, however, the identification of the intracellular binding partners that are involved in the modulation of the invasive behavior of cancer cells remains unknown. In contrast, extracellular galectins and their respective binding partners have been fairly well characterized.

Other functions of galectins. Angiogenesis is also among the functions associated with galectin activity. For example, galectin-1 increases glioma, prostate and breast tumor vascularisation (108,137,138). Galectin-3 also increases vascularisation of prostate tumors and melanomas, while galectin-7 reduces angiogenesis of colon tumors (47,83,139). Galectins have been shown to take part in the tumor immune escape. Indeed, galectin-1 promotes immunoevasion of neuroblastoma, prostate, breast and pancreatic cancer cells (140-142). Galectin-3 also increases tumor immune escape of melanomas, colorectal and lung cancer cells (117,123,143). Most studies suggest that extracellular galectins are responsible for these functions. The involvement of intracellular galectins in these processes remains unknown.

4. CRD-independent functions for intracellular galectins?

Galectins are primarily known for their ability to bind to glycans containing lactose or N-aceyllactosamine via Van der Walls interactions between the carbohydrate and binding pocket. They have a relatively broad specificity depending on the type and the length of the carbohydrate and the mode of presentation of ligand to the CRD. It is thus logical to assume that inside the cells, they will also preferentially bind to intracellular glycoconjugates, which are abundantly found in the cytosol. There is compelling evidence, however, that galectins might have non-carbohydrate binding partners and functions. CRD-independent functions have been particularly well documented for intracellular galectins (144-146). For example, galectins do interact with Bcl-2 family members via a CRD-independent interaction (33,85,89,90). This galectin/Bcl-2 interaction is important since the balance of activity between pro- and anti-apoptotic signals of members of the Bcl-2 family regulates apoptosis. Other CRD-independent functions of galectins include RNA processing in the nucleus (57) and regulation of cell cycle progression (Wnt signaling?) (25,99,101,102,147). All these galectin functions are independent of their saccharidic binding activities and rather rely on protein-protein interactions. Some galectins, such as galectin-10, harbor very low affinity for galactosides and are believed to act mainly through other specificities, while their CRD binding activity remains debated (148,149). These CRD-independent functions represent a paradigm shift in our understanding of galectin function and the development of galectin-specific antagonists.

A new challenge: studying the redundancy of galectin functions. The existence of redundant or antagonistic functions between galectins is a major concern because these proteins can converge under normal or pathological conditions. The cross-talk between intracellular galectins remains completely unknown although cells often express more than one intracellular galectin. For example, MCF-7 breast cancer cells express galectin-3, -8 and -9 (150). MCF-10 and MDA-MB-468, two other human mammary epithelial cell lines, express both galectin-3 and -7, but not galectin-8 or -9 (151,152). Moreover, many galectins could be present within the same intracellular compartments. A case in point is the mitochondria, where both galectin-3 and -7 are found. Galectin-12 can also be present in mitochondria and not surprisingly, it seems to be involved in the control of cellular metabolism (38-40). Whether galectins have redundant or opposed functions in the mitochondria is an interesting question given the critical role of cellular metabolism in cancer. A better understanding of the functional redundancy among homologous proteins, which is frequently observed in eukaryotes, is also critical. Such redundancy often occurs in order to increase maintenance of important gene function and to limit losses following mutations/deletions of specific genes (functional compensation). Lessons learned from such studies could also bring important insight into many others fields, from understanding pathologies to general developmental biology.

Future directions. Because of their critical role in cancer, considerable efforts have been directed towards the development of carbohydrate-based inhibitors that would limit the binding of galectins to glycosylated residues on cell surface receptors. For example, GCS-100 is a galectin-3 antagonist with a modified citrus pectin carbohydrate that has been shown to inhibit tumor growth and metastasis in several preclinical models (153-155). Others, like OTX008, a galectin-1 antagonist, act as allosteric CRD-dependent inhibitors following binding to a site distant from the carbohydrate-binding site (156). Nevertheless, despite almost two decades of research, the development of effective galectin antagonists for the treatment of cancer has met with limited success. The emerging evidence that galectins have critical intracellular and CRD-independent functions calls for a refocusing of our efforts on development of new galectin-specific antagonists to modulate apoptosis. Our knowledge of the subcellular localization of galectins will also significantly improve target identification during the drug discovery process. It is thus imperative to better understand the role of intracellular galectins and to provide novel insight into how galectins collaboratively modulate cancer progression from within the cells.

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ANNEXE V

L'EXPRESSION DE LA GALECTINE-7 EST INDUITE PAR P53 MUTANT DANS LES CELLULES DU CANCER DU SEIN

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Expression of Galectin-7 Is Induced in Breast Cancer Cells by Mutant p53

Carole G. Campion, Marilyne Labrie, Geneviève Lavoie, Yves St-Pierre*

Institut national de la recherche scientifique, Institut Armand-Frappier, Laval, Québec, Canada

Abstract

Galectin-7 was initially described as a marker of epithelial differentiation expressed in the stratified epithelium of various tissues. Like other members of the galectin family, its expression level is often significantly altered in cancer cells. In breast cancer, its expression is significantly augmented in aggressive molecular subtypes, most notably in estrogen receptor-negative tumors and in cell lines with a basal-like phenotype. Studies using experimental mouse models have further shown high expression of galectin-7 was sufficient to increase the metastatic behavior of poorly metastatic breast cancer cells, rendering them more resistant to apoptosis. This expression pattern in breast cancer cells is unexpected because galectin-7 was originally identified as a *p53*-induced gene. To address this paradox, we have examined the molecular mechanisms regulating galectin-7 in breast cancer cells. Our results showed that transfection of breast cancer cells with expression vectors encoding mutant p53 was sufficient to induce galectin-7 at both mRNA and protein levels. Doxorubicin treatment of breast cancer cells harboring a mutant p53 also induced galectin-7. This induction was specific since knockdown of endogenous mutant p53 inhibited doxorubicin-induced galectin-7 expression. The p53-induced galectin-7 expression in breast cancer cells correlated with increased NF-κB activity and was inhibited by NF-κB inhibitors, indicating that the ability of mutant p53 to induce galectin-7 was dependent on NF-κB activity. The implication of NF-κB was further supported by data showing that NF-κB bound to the endogenous galectin-7 promoter and that TNFα-induced *galectin-7* expression was abolished by NF-κB inhibitors. Taken together, our data provide an explanation to the observed high galectin-7 expression levels in cancer cells and suggest that galectin-7 could be part of a common pathway used by mutant p53 to promote cancer progression.

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* E-mail: yves.st-pierre@iaf.inrs.ca

Introduction

Evidence suggesting that interactions between lectins and their ligands play a major role in different steps of cancer progression has gained the attention of several oncologists [1]. This is particularly true for galectins. Changes in their expression levels correlate with alterations in cancer cell growth, intercellular adhesion, and apoptosis [2–8]. A case in point is galectin-7. In normal tissues, galectin-7 is present in epithelial cells in various tissues [9–11]. Using tissue microarrays constructed from samples obtained from normal breast tissues and breast carcinomas, we previously reported that galectin-7 was expressed at abnormally high levels in tissues collected from patients with a poor prognosis [12]. These results were consistent with the genomic profiling data previously reported by Perou et al. [13], who provided a molecular portrait of 65 surgical specimens of human breast tumors from 42 individuals. Their data revealed that while *galectin-7* transcripts were expressed at low levels in normal breast tissues and mammary epithelial cell lines, they were highly expressed in estrogen receptor (ER)-negative breast cancer and in cell lines with a basal-like phenotype. This abnormally high expression level of galectin-7 is not restricted to breast cancer cells. It is also found in pancreatic cancer cell lines [14], and in esophageal, buccal, and hypopharyngeal squamous cell carcinoma [10,15–17]. Such high levels of galectin-7 in cancer cells are somewhat paradoxical because galectin-7 has generally been considered a pro-apoptotic protein under the control of p53. *Galectin-7* (also called *p53-induced gene 1*) was indeed identified as one of the 14 transcripts induced by ectopic p53 in colon carcinoma cells [18]. In normal epidermal keratinocytes, increase levels of galectin-7 also correlate with p53 stabilization [19]. Yet, galectin-7 is constitutively expressed in cells harboring mutant

p53 [18,19]. In the present work, we have examined this apparent contradiction by investigating the molecular mechanisms controlling galectin-7 expression in human breast cancer cells.

Material and Methods

Cell lines and reagents

Breast cancer cell lines were a generous gift from Dr. Peter Siegel (McGill University, Montreal, Qc, Canada) [20]. Immortalized human keratinocytes (HaCaT) were provided by Dr. Thierry Magnaldo (Génétique et physiopathologie des cancers épidermiques, Faculté de Médecine, Nice, France) [19]. MCF-7 cells were originally obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in complete Dulbecco's modified Eagle's medium supplemented with 8% (v/v) FCS, 2 mmol/L L-glutamine and 10 mmol/L HEPES buffer. One mmol/L sodium pyruvate was added for maintenance of MCF-7 cells and one mmol/L of non-essential amino acids for HaCaT cells. All cell culture products were purchased from Life Technologies (Burlington, ON, Canada). Doxorubicin, quercetin and parthenolide were purchased from Sigma Chemicals (St. Louis, MO). Recombinant human TNFα was from R&D Systems (Minneaopolis, MN). Caffeic acid phenethyl ester (CAPE) was from Tocris Bioscience (Ellisville, MO).

Vectors, transfection and luciferase assay

The plasmids encoding the luciferase reporter vector containing p53 (plasmid 219077) or NF-κB (plasmid 219083) were purchased from Stratagene (Mississauga, ON, Canada). The vectors encoding mutant p53 (R175H, plasmid 16436; R273H, plasmid 16439; V143A, plasmid 16435; R249S, plasmid 16438 and R248W, plasmid 16437 [21]) were obtained from Addgene (Cambridge, MA). The vector encoding the wild-type human *p53* gene was purchased from Origene (Burlington, MA). The expression vector encoding human c-Rel was provided by Dr. Nathalie Grandvaux (University of Montréal, St-Luc Hospital, Montreal, Canada). pSuper and pSuper-p53 siRNA vectors (siRNA CTRL and siRNAp53) were kindly provided by Dr. Reuven Agami (The Netherlands Cancer Institute, Amsterdam, Netherlands) [22]. The pCDNA3.1 vector was purchased from Invitrogen (Burlington, ON, Canada). For transfection, cells were plated at equal density 24 h before transfection. Cells were then transfected with the indicated vector(s) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After transfection, cells were incubated in complete medium at 37° C in 5% CO_2 for the indicated periods of time and subjected to a dual reporter assay. Luciferase activity was measured using the Luciferase Assay System protocol (Promega, Madison, WI, USA) and a luminometer (Lumat LB 9507, Berthold). β-galactosidase activity was measured using a colorimetric enzyme assay using the Luminescent β-Galactosidase Detection Kit II according to the manufacturer's instructions (Clontech Laboratories, Mountain View, CA). Luciferase expression levels were normalized to the levels of β-galactosidase expression.

RNA Isolation and RT-PCR

Total cellular RNA was isolated from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 μg of cellular RNA in a total reaction volume of 20 μL using the reverse transcriptase Omniscript (QIAGEN, Mississauga, ON, Canada). After reverse transcription, human *galectin-7* (gene ID 3963, sense primer: 5'- ACC AAC CCG GTC CCA G -3' and antisense primer: 5'- GCG GGC TAA CGC TTT ATT TGC -3'), human *galectin-3* (gene ID 3958, sense primer: 5'- ATG GCA GAC AAT TTT CCG CTC C -3' and antisense primer: 5'- ATG TCA CCA GAA ATT CCC AGT T -3'), human *TP53* (gene ID 7157, sense primer: 5'- CCA GCC AAA GAA GAA ACC A -3' and antisense primer: 5'- TAT GGC GGG AGG TAG ACT GA -3'), human *p21WAF1* (gene ID 1026, sense primer: 5'- CTG GAG ACT CTC AGG GTC GAA -3' and antisense primer: 5'- GGA TTA GGG CTT CCT CTT GGA -3'), human *C/EBPβ* (gene ID 1051, sense primer: 5'-GCG ACG AGT ACA AGA TCC -3' and antisense primer: 5'- AGC TGC TTG AAC AAG TTC C-3') and *GAPDH* (gene ID 2597, sense primer: 5'- CGG AGT CAA CGG ATT TGG TCG TAT-3' and antisense primer: 5'-CAG AAG TGG TGG TAC CTC TTC CGA -3'). cDNAs were amplified using the following conditions: 94° C for 3 min, followed by 35 cycles of the following: 94° C for 1 min, 60° C for 1 minute, and 72° C for 1 min, followed by a final extension step at 72° C for 10 min. PCR was performed in a thermal cycler (MJ Research, Watertown, MA). The amplified products were analyzed by electrophoresis using 1.5% agarose gels and SYBR Safe (Life Technologies) staining and UV illumination.

p53 knockdown

MCF-7 and MDA-MB-231 cells were plated in 6-well plates at $3x10^5$ cells/well before transfected with pSuper and pSuper-p53 siRNA vectors using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, 0.5 μg/ml of doxorubicin was added, and cells were incubated for an additional 24 h before RNA isolation and RT-PCR.

Western Blot analysis

Cells were solubilized in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo, Fisher Scientific, Ottawa, ON, Canada) containing a cocktail of protease inhibitors (Roche, Laval, QC, Canada). Equal amounts of proteins (25 μg) were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada). The membranes were first blocked with 5% milk in PBS/0.05% Tween 20 for 1 h and subsequently blotted overnight at 4° C with primary antibodies: rabbit anti-human p53 polyclonal antibody (1:1000; Santa-Cruz Biotechnology, Santa Cruz, CA) and mouse anti-β-actin monoclonal antibody (1:20000; Sigma). Secondary antibodies consisted of horseradish peroxydase conjugated anti-rabbit or anti-mouse (GE Healthcare, Mississauga, ON, Canada). The immunoblots were developed using ECL detection reagent (GE Healthcare).

ELISA Assay

Human galectin-7 proteins levels were quantified in whole cells using a commercial ELISA kit (R&D Systems) according to the manufacturer's protocol. Samples were analyzed using a Model 680 microplate reader (Bio-Rad).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the EZ-Chromatin-Immunoprecipitation Assay Kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Briefly, cells were fixed in 1% paraformaldehyde. Nuclei were isolated, sonicated, and pre-cleaned with protein G agarose/salmon sperm DNA. The pre-cleaned chromatin solution was either set aside as input DNA or incubated with anti-NF-κB/p50 (06-886), (Millipore), anti-NF-κB/c-rel (B-6), anti-p53 (DO-1), or anti-RNA polymerase II (05-623B) (Santa-Cruz Biotechnology) on a rotation platform at 4° C overnight. Input DNA and mouse IgG-pulled DNAs were used as controls for all the experiments. After reversal of the cross-linking, DNA was purified from the immune complex and amplified using PCR primers specific for the *galectin-7* promoter region: sense: 5'-GGC ATA GTC CTA GTG GAT CCC-3' and antisense: 5'-TGT AAT GGA ACA GCG GCC ACAA-3'. The samples were incubated for 3 min at 94° C, followed by 40 cycles as follows: 1 min at 94° C, 1 min at 62° C, and 1 min at 72° C, with a final extension at 72° C for 10 min. The amplified products were analyzed by electrophoresis using 1.5% agarose gels and SYBR Safe (Life Technologies) staining and UV illumination.

Confocal analysis

MCF-7 cells were cultured onto glass coverslips to semi-confluency. After 24 h, the cells were washed with cold PBS, fixed with 3% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% BSA in PBS (PBA) for 30 min. Cells were first incubated overnight at 4° C with a goat anti-human galectin-7 polyclonal antibody (1:100; R&D Systems) or a rabbit anti-human COX-IV antibody (1:500; New England Biolabs, Ipswich, MA) with PBA. After several washes, the cells were incubated with an Alexa Fluor 488-conjugated donkey anti-goat IgG (1:500; Life Technologies) or an Alexa Fluor 568 goat anti-rabbit IgG (1:500; New England Biolabs) for 1 h at room temperature. Samples were mounted using ProLong gold antifade Reagent with 4'-6-diamino-2-phenylindole (DAPI) (Life Technologies) on glass slides and visualized using a Zeiss LSM780 laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY).

Results

Wild-type p53 up-regulates galectin-7 expression in human breast cancer cells

Using MCF-7 cells transfected with a vector encoding wild-type p53 ($p53^{wt}$), we found that increased levels of wild-type p53 were able to induce galectin-7 expression at both mRNA and protein levels (Figure **1A and B**). Similar results were obtained using the MDA-MB-231 cell line (Figure **1B and C**). Treatment of MCF-7 cells, which harbor wild-type alleles of

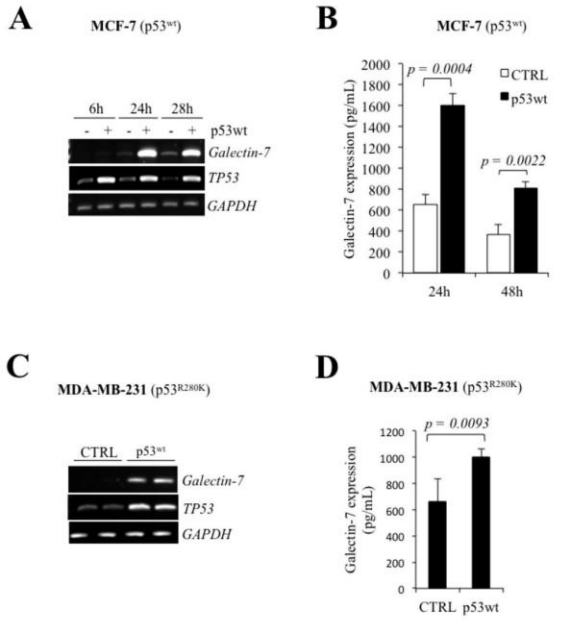

Figure 1. Wild-type p53 induces galectin-7 expression in breast cancer cell. Galectin-7 and *TP53* mRNA and protein levels in MCF-7 (**A** and **B**) and MDA-MB-231 (**C** and **D**) cells following transfection with an expression vector encoding wild type *p53*. Protein levels were measured by ELISA, as described the materiel and methods section. Control cells were generated using an empty pCDNA3.1 vector. *GAPDH* was used as loading control.

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TP53, with doxorubicin (Dox) also induced galectin-7 expression at both mRNA and protein levels (Figure **2A and C**). At the protein level, the expression of galectin-7 was restricted to the cytosol and into mitochondria where it colocalized with COX-IV, a well established mitochondrial marker (Figure **S1**). This pattern of expression is consistent with recent data reported [23]. Knockdown of $p53^{wt}$ further inhibited Dox-induced *galectin-7* expression in MCF-7 cells (Figure **2B**).

Mutant p53 can induce galectin-7 expression in human breast cancer cells

We and others have previously found that galectin-7 was expressed in mammary tissues of aggressive subtypes of breast cancer cells. Because of the high incidence of p53 mutations in human breast cancer, we thus tested whether mutant p53 can also induce galectin-7 expression. To test this, transfected breast cancer cells with vectors encoding the most common forms of mutant p53, including "hot spots" mutants such as $p53^{R175H}$ and $p53^{R248W}$ [24]. Our results showed that $p53^{R175H}$, $p53^{R273H}$ and $p53^{R248W}$ were all able to induce *galectin-7* in MCF-7 (Figure **3A**). No such increase in *galectin-7*

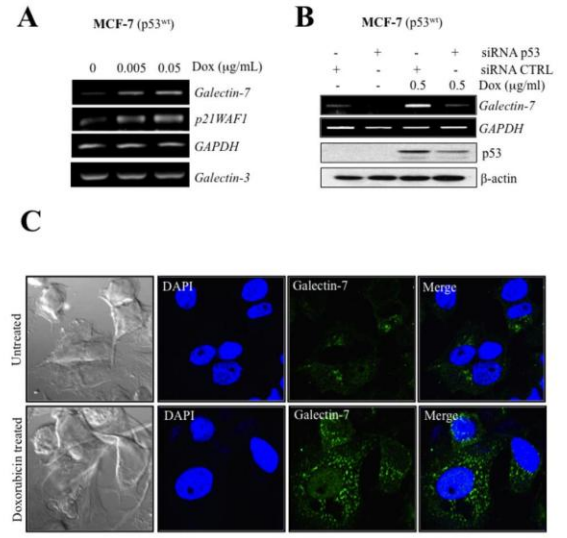

Figure 2. Doxorubicin induces galectin-7 expression in MCF-7^{p53wt}. (**A**) Increased expression of *galectin-7* and *p21WAF1* in MCF-7 cells treated with increasing doses of doxorubicin. No such increase was observed in *galectin-3* mRNA levels. *GAPDH* was used as loading control. (**B**) Inhibition of *galectin-7* mRNA levels and p53 protein levels by p53 siRNA in MCF-7 cells treated with 0.5 µg/ml of doxorubicin. A siRNA CTRL vector was used as control and GAPDH or β-actin was used as loading control. (**C**) Confocal images showing galectin-7 expression in doxorubicin-treated MCF-7 as compared to untreated control cells. Fixed cells were labeled with a goat anti-human galectin-7 polyclonal antibody and an Alexa Fluor 488-conjugated donkey anti-goat IgG (green fluorescence). Nuclei were stained with DAPI (blue).

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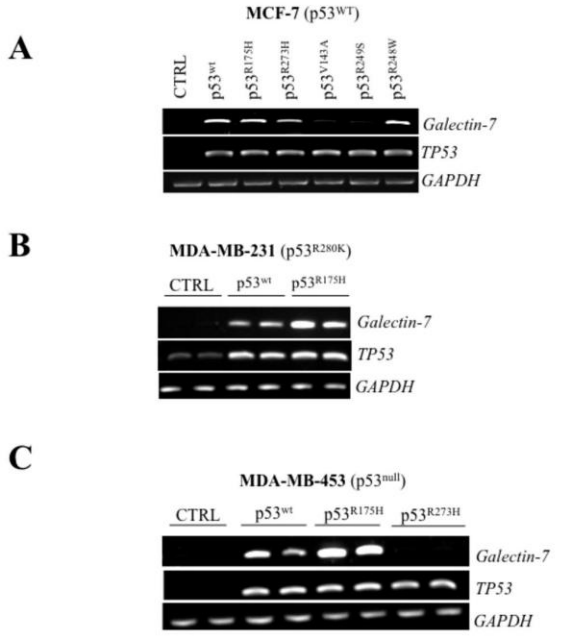

Figure 3. Mutant p53 induces *galectin-7* expression in breast cancer cell lines. Semi-quantitative RT-PCR analysis showing *galectin-7* and *TP53* mRNA levels following transfection with an expression vector encoding the wt p53 or a mutated form of p53 (R175H, R273H, V143A, R249S or R248W) in MCF-7 (**A**), MDA-MB-231 (**B**) and MDA-MB-453 (**C**). An empty pCDNA3.1 vector was used as a control (CTRL). GAPDH is used as loading control.

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was obtained using the control (empty) vector. The p53^{R175H} also induced *galectin-7* when expressed in MDA-MB-231, a human breast cancer cell line harboring a mutant *TP53 gene* (p53^{R280K}) and in the p53^{null} MDA-MB-453 cells (Figure **3B, C and S5**). Of note, mutant p53 were not all equally capable of inducing *galectin-7* in all cells types. In MCF-7 cells, for instance, ectopic expression of p53^{V143A} and p53^{R249S} were insufficient to induce *galectin-7*. Similar results were obtained in MDA-MB-453 transfected with a vector encoding p53^{R273H}, suggesting that the ability of mutant p53 to induce *galectin-7* was cell-specific. Our results showing that Dox-treated MDA-MB-231 cells expressed increased galectin-7 confirmed that endogenous p53^{R280K} was capable of inducing galectin-7 at both mRNA and protein levels (Figure **4A and B**). No such increase was observed when the p53^{null} MDA-MB-453 cells were treated with Dox, indicating that the effect of Dox was p53-specific. This conclusion is supported by our data showing that Dox-induced *galectin-7* expression by endogenous p53^{R280K} in MDA-MB-231 cells was inhibited by partial knockdown of p53^{R280K} (Figure **4C**).

The role of NF-κB in galectin-7 expression

An *in silico* analysis of the human *galectin-7* promoter revealed the presence of a putative p53 binding site in its 5' proximal region relative to the transcriptional start site (Figure **5A**). Several putative NF-κB binding sites were also present within this region, raising the possibility that *galectin-7* could be regulated by via a transcriptional crosstalk between NF-κB and p53. This hypothesis is supported by our data showing that both endogenous NF-κB and p53 were equally capable of binding to the human *galectin-7* promoter (Figure **6**). Further support to this hypothesis is provided by our data showing that MDA-MB-468 cells expressing galectin-7 constitutively have high levels of NF-κB activity (Figure **5B and C**). These cells do not have any detectable p53 activity since they have lost the wild-type allele of *TP53* and only express the transcriptionally inactive *TP53*^{R273H} allele. Similar results were obtained in the case of HaCaT cells, a transformed keratinocyte cell line known to express high levels of galectin-7 [23,25]. Like MDA-MB-468 cells, HaCaT cells do not have detectable p53 transcriptional activity and only express the transcriptionally inactive *TP53*$^{H179Y/R282W}$ alleles (Figure **5C, S4 and S5**). These

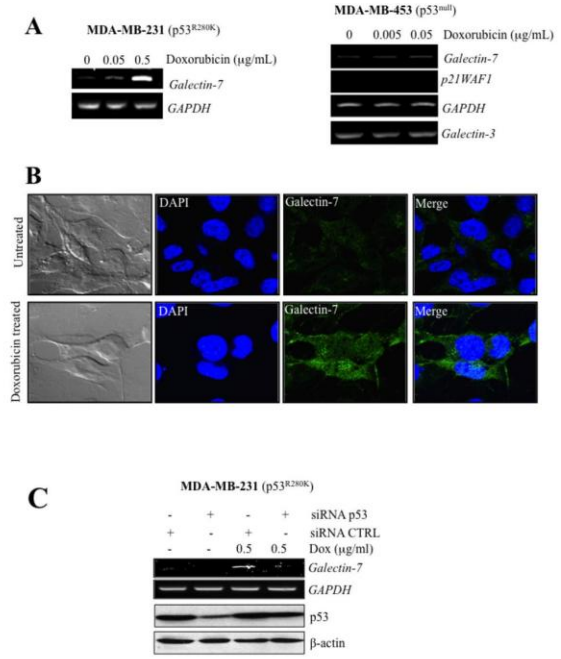

Figure 4. Doxorubicin induces *galectin-7* expression in MDA-MB-231^{p53R175H}. (**A**) Effect of increasing doses of doxorubicin on galectin-7 expression in MDA-MB-231, a mutated p53^{R280K} cell line and MDA-MB-453, a p53^{null} cell line. No increase in *galectin-3* mRNA levels was observed in MDA-MB-453. *GAPDH* was used as loading control. (**B**) MDA-MB-231 were treated or not with 0.5 μg/ml of doxorubicin before cells fixation and permeabilization. A goat anti-human galectin-7 polyclonal antibody was used in combination with an Alexa Fluor 488 donkey anti-goat IgG to detect endogenous galectin-7. MDA-MB-231 cells are shown with galectin-7 (green) and nucleus (blue). (**C**) Inhibition of *galectin-7* mRNA levels and p53 protein levels by p53 siRNA in MDA-MB-231 cells treated with 0.5 μg/ml of doxorubicin. A siRNA CTRL vector was used as control and GAPDH or β-actin was used as loading control.

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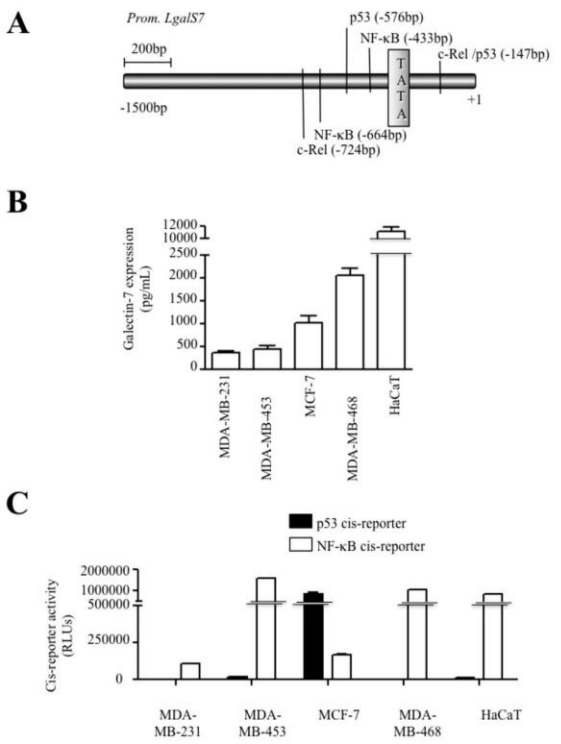

Figure 5. Correlation between galectin-7 expression and NF-κB activity. (**A**) Putative binding sites for NF-κB, c-Rel and p53 within the proximal promoter region of the human *galectin-7* gene. The location of the putative binding sites were obtained by analyzing the sequence of the galectin-7 promoter using the TFSearch program which searches highly correlated sequence fragments against TFMATRIX transcription factor binding site profile database in the 'TRANSFAC' databases by GBF-Braunschweig (http://www.cbrc.jp/research/db/TFSEARCH.html). (**B**) Galectin-7 protein levels measured by ELISA in MDA-MB-231, MDA-MB-453, MCF-7 and MDA-MB-468 breast cancer cell lines. Human epithelial keratinocytes (HaCaT) was used as a positive control. Similar results were obtained by Western blot analysis (**Figure S4**). (**D**) NF-κB and p53 activity as measured following transient transfection with luciferase reporter vectors in breast cancer cell lines with different p53 status and HaCaT cells. Transfection efficiency was normalized with co-transfection with a β-galactosidase reporter vector.

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results are thus consistent with a role for NF-κB in regulating galectin-7 expression. This conclusion is supported by our data showing that transfection of an expression vector encoding c-Rel or treatment of cells with TNFα a well-known inducer of NF-κB activity, both induced *galectin-7* expression in MCF-7, HaCaT, and MDA-MB-468 (Figure **7A, B and S2**). Our results with TNFα were in fact consistent with gene profiling data in a public profiling database (Geo Profiles, NCBI) showing a specific NF-κB-dependent increase of *galectin-7* in epidermal keratinocytes stimulated by TNFα (Figure **7C**). We also found that parthenolide, a specific inhibitor of NF-κB, suppressed *galectin-7* expression in HaCaT cells (Figure **7D**). Similar results were obtained following treatment of MDA-MB-468 cells with caffeic acid phenethyl ester (CAPE) and quercetin, two other inhibitors of NF-κB [26,27] (Figure **7E and F**). This inhibition of *galectin-7* expression in HaCaT and MDA-MB-468 cells following treatment with parthenolide and CAPE correlated with reduced NF-κB activity (Figure **S3**). Taken together, these results suggest that NF-κB plays a central role in the expression of galectin-7.

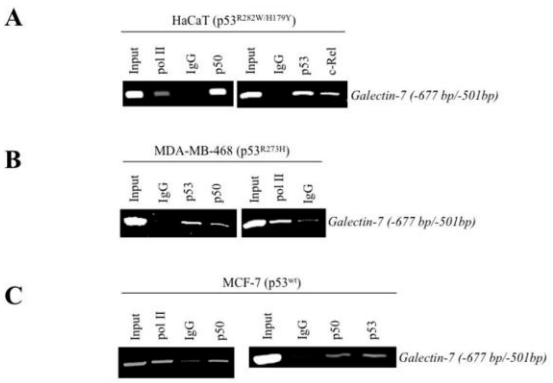

Figure 6. Binding of NF-κB and p53 on the endogenous galectin-7 promoter. Binding of NF-κB p50 and c-Rel isoform, p53 and RNA polymerase II (PolII) on the endogenous galectin-7 promoter was measured by ChIP assay using genomic DNA collected from HaCaT (**A**) and human breast cancer cell lines MDA-MB-468 (**B**) and MCF-7 (**C**). An isotypic control (IgG) was used as a negative control. Total DNA extract was used as a positive control (Input).

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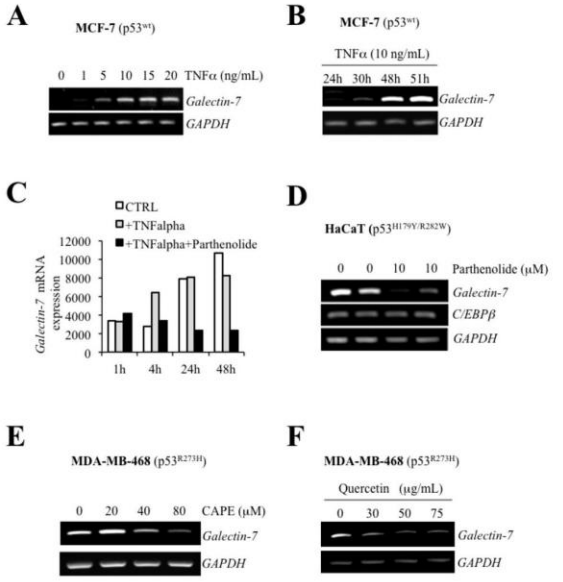

Figure 7. Galectin-7 expression is dependent of NF-κB signalling pathway. mRNA levels following treatment with increasing doses of TNFα (**A**) or at different time post-treatment with TNFα (**B**). (**C**) *In silico* analysis showing effect of parthenolide on TNFα-induced *galectin-7* expression in epidermal keratinocytes. Data were retrieved from a dataset (GDS : 1289) in the Gene Expression Omnibus (GEO) repository. (**D**) Effect of parthenolide on galectin-7 expression in HaCaT cells. mRNA levels of *galectin-7* was measured by semi-quantitative RT-PCR analysis 48h after treatment. No such effect was observed on C/EBPβ expression. GAPDH was used as loading control. (**E**) Dose-dependent inhibition of *galectin-7* mRNA levels in MDA-MB-468 cells treated with CAPE. GAPDH was used as loading control. (**F**) Dose-dependent inhibition of *galectin-7* mRNA levels in MDA-MB-468 cells treated with quercetin. GAPDH was used as loading control.

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Wild-type and mutant p53 requires NF-κB for the induction of galectin-7.

We next investigated whether NF-κB-dependent galectin-7 expression could be triggered by increased levels of wt or mutant p53. For this purpose, cells expressing wt and mutant p53 were either treated with Dox or transfected with wt or mutant p53. Our results showed that increased expression of wt or mutant p53 ($p53^{R175H}$) both increased NF-κB activity in MCF-7, MDA-MB-231 and MDA-MB-453 cells (Figure **8A–C and S4**). Similar results were obtained using the MDA-MB-$231^{p53R280K}$ cells treated with Dox (Figure **8D and S4**). Moreover, the induction of galectin-7 at the mRNA and protein levels by wt or mutant p53 was inhibited in a dose-dependent manner by CAPE (Figures **9** and **10**).

Discussion

The present study shows that both wild type and mutant p53 can induce galectin-7 in breast cancer cells. This conclusion is based on the following: 1) transfection with an expression vector encoding wt or mutant p53 induced galectin-7 in several breast cancer cell lines, including $p53^{null}$ cells; 2) treatment of breast cancer cells harboring wild-type or mutated allele of *TP53* with Dox induced galectin-7 expression in human cancer cell lines, and 3) depletion of endogenous p53 inhibited Dox-induced *galectin-7* expression. We further showed that NF-κB was also capable of inducing galectin-7. This conclusion is supported by our data showing that: 1) NF-κB binds to the endogenous galectin-7 promoter; 2) transfection of NF-κB subunits or treatment of cells with TNFα, a well-known inducer of NF-κB, both increased galectin-7; 3) inhibitors of NF-κB decreased constitutive or TNFα-induced *galectin-7* expression. Finally, our results showing that p53-induced galectin-7 expression correlated with increased NF-κB activity and was inhibited by NF-κB inhibitors suggest galectin-7 expression results from a crosstalk between p53 and NF-κB.

Over the last decade, a large number of observations have brought support to the idea that NF-κB plays an important role in cancer. In most cancer cells, NF-κB is constitutively active and is responsible, at least in part, for resistance to apoptosis. It is therefore not surprising that a crosstalk exists between NF-κB and p53. In fact, our results are consistent with a number of studies showing that: 1) mutant p53 expression correlates positively with NF-κB activity in cultured cancer cells [28]; 2) elevated expression of mutant p53 is associated with increased NF-κB activation in cancer tissues [29]; and 3) mutant p53

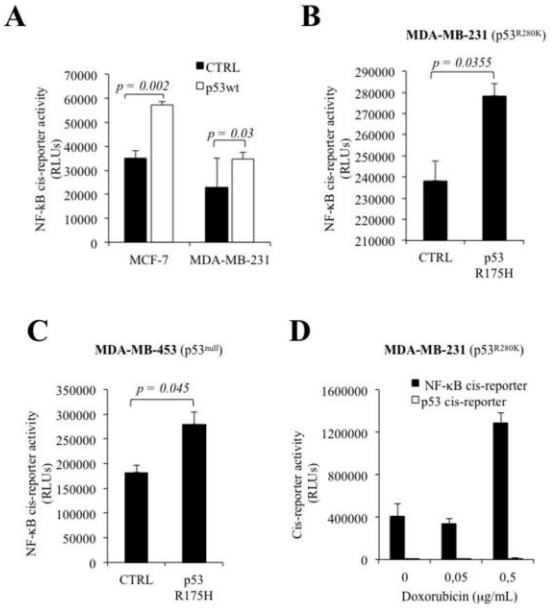

Figure 8. p53 induces galectin-7 expression via NF-κB. (**A**) Increased NF-κB reporter activity in MCF-7 and MDA-MB-231 cells 24h following transfection with an expression vector encoding wt p53. An empty pCDNA3.1 vector was used as a control (CTRL). Transfection efficiency was normalized by co-transfection with a β-galactosidase reporter vector. NF-κB reporter activity in MDA-MB-231 (**B**) and MDA-MB-453 (**C**) cells following transfection of the mutated $p53^{R175H}$. An empty pCDNA3.1 vector was used as a control (CTRL). Transfection efficiency was normalized by co-transfection with a β-galactosidase reporter vector. (**D**) Effect of increasing doses of doxorubicin on NF-κB reporter activity in MDA-MB-231, a mutated $p53^{R280K}$ cell line. Transfection efficiency was normalized by co-transfection with a β-galactosidase reporter vector.

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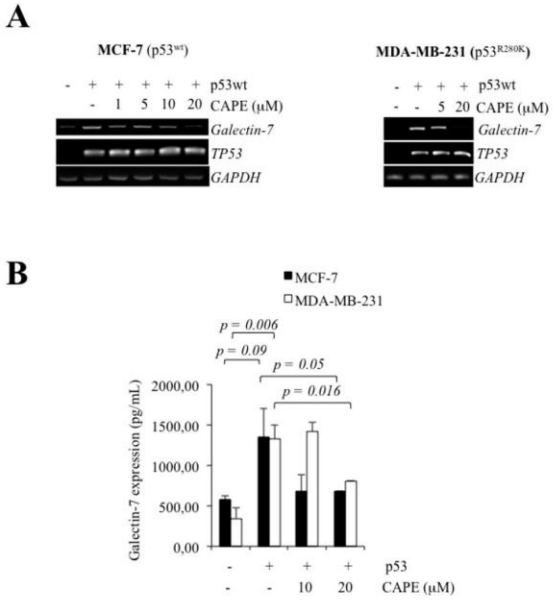

Figure 9. *Galectin-7* expression induced by wild-type p53 required NF-κB. Inhibition of galectin-7 mRNA (**A**) and protein levels (**B**) by increasing doses of CAPE in MCF-7 and MDA-MB-231 cells transfected with an expression vector encoding wt p53. GAPDH was used as loading control.

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augments NF-κB activity by affecting both the strength and duration of NF-κB activation [30]. Because high levels of NF-κB have been associated with resistance to apoptosis [31], and galectin-7 induces resistance to apoptosis in breast cancer cells [12], our results suggest that galectin-7 may contribute to NF-κB-mediated resistance to apoptosis. Whether the clinical efficiency of some NF-κB inhibitors alone or in combination with other therapies is linked to reduced galectin-7 is an interesting question that merits future investigations. It will also be interesting to determine whether specific isoforms are all equally capable of binding to the *galectin-7* promoter to induce its expression. Homo- and heterodimerization between NF-κB isoforms generates a diverse range of DNA binding proteins which vary according to the cell type, the stimulus, and the duration of the signals. Using several pharmacological inhibitors to block NF-κB activity thus ensures that we target all NF-κB isoforms. It is important to note, however, there is yet no evidence yet that NF-κB alone is sufficient to induce galectin-7. For example, galectin-7 is not detected in MDA-MB-453 (null p53) cells, which harbor a constitutively high level of NF-κB activity. Moreover, in contrast to other cell models, we could not induce galectin-7 upon treatment of MDA-MB-453 (p53-null) cells with TNFα.

Mutations in the *TP53* gene are among the most common genetic alterations found in breast cancer. The presence of p53 mutations correlates with a high histologic grade, lack of ER and/or PR expression, deregulated apoptosis, and poor prognosis [32]. Such correlation is consistent with the expression pattern of galectin-7, which is exclusively found in ER/PR negative breast cancer [12,13]. Whether galectin-7 is part of a common pathway used by mutant p53 to aid in oncogenesis is an interesting possibility.

The mechanism by which mutant p53 can induce galectin-7 in breast cancer cells via NF-κB is unclear. Given the frequent overlap between p53 and NF-κB binding sites in gene promoters, it is possible that mutant p53 could influence the transcriptional output of specific NF-κB target genes by binding to their promoters. Increased basal NF-κB activity could also result from the ability of mutant p53 to transactivate the *NF-κB2* gene encoding p100/p52 [28]. Perhaps, this NF-κB/p53 complex is required for opening of chromatin at NF-κB loci and for the persistence of NF-κB complex to its binding motifs [33].

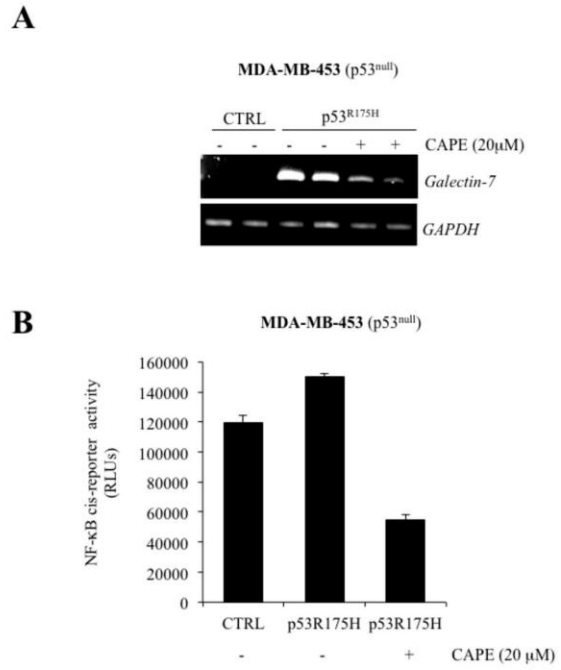

Figure 10. *Galectin-7* expression induced by $p53^{R175H}$. mutant is inhibited by CAPE. (**A**) Inhibition of *galectin-7* mRNA levels by CAPE in MDA-MB-453 cells transfected with the expression vector encoding $p53^{R175H}$ or with the (empty) pCDNA3.1 control vector (CTRL). GAPDH was used as loading control. (**B**) Effect of CAPE on NF-κB reporter activity in MDA-MB-453 cells transfected with the expression vector encoding $p53^{R175H}$ or with the (empty) pCDNA3.1 control vector (CTRL). Transfection efficiency was normalized by co-transfection with a β-galactosidase reporter vector.

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It is important to note, however, that a constitutive NF-κB activity is not sufficient to induce galectin-7. For example, the $p53^{null}$ MDA-MB-453 cells do harbor high levels of NF-κB activity but do not express detectable levels of galectin-7 (Figure **5B and C**), suggesting that a NF-κB/p53 complex is required to transactivate the *galectin-7* promoter or that additional epigenetic regulatory mechanisms are involved. Future investigations are needed to clarify this issue. However, our results reveal a regulatory circuit placing NF-κB under the control of p53 and suggest that p53 can dictate cell fate decisions on induction of pro- or anti-apoptotic genes depending on cell status. Cells with p53 levels insufficient or instable to promote transcriptional activities of NF-κB would be disfavored. Inversely, cells with high levels of mutant p53 could mimic NF-κB activation and readily induce expression of anti-apoptotic NF-κB target gene like *galectin-7*. Our results using the HaCaT cell line, a keratinocyte cell line with high levels of NF-κB activity and galectin-7 expression, are interesting in this regard. In these cells, *TP53* is mutated on both alleles, at codon 179 of exon 5 and codon 282 of exon 8 (Figure **S5**). These mutations are commonly found in human tumors and are believed to play a significant role in the immortalization of this line [34]. The mutation at position 179 is known to destabilize the p53 protein by altering its ability to interact with a zinc molecule, causing loss of DNA-binding specificity [33]. While the mutation at position 282 alters the global architecture of the protein, it does not affect its ability to bind to DNA [35]. These specific properties of mutant p53 possibly explain the high transcriptional activity of NF-κB in HaCaT cells and the observed binding of mutant p53 to the endogenous galectin-7 promoter. Similar results were obtained in MDA-MB-468 cells, which express a single *TP53* allele that encodes a missense mutation at codon 273 (R→H) (Figure **S5**). This mutation is one of the most common mutations in cancer cells. It disrupts the sequence-specific DNA-binding core of p53 [36] while maintaining the overall architecture of the DNA binding surface, explaining why binding of specific DNA is not completely abolished. Interestingly, we found that this mutant induced *galectin-7* expression in MCF-7 breast cancer cell line while binding to the endogenous galectin-7 promoter. Surprisingly, it was not able to induce *galectin-7* in the $p53^{null}$ MDA-MB-453 cells. The mutant R249S was also unable to induce *galectin-7* in all cell lines tested, suggesting that cell-specific corepressors/coactivators are involved. We believe, however, that it is too early to determine if a relationship can be established between a specific type of mutation and its effect on galectin-7 expression as we have tested a relatively small repertoire of mutations (Figure **S5**). Moreover, it is important to take into account that the effect of a specific mutation may depend on the cell type and/or the expression of the other wild-type or mutated allele. Future studies will have to be conducted to answer this question. We have nevertheless added a sentence in the discussion regarding this possibility.

In conclusion, we found that both wt and mutant p53 can induce galectin-7 in breast cancer cells. While our data help to explain why galectin-7 is constitutively expressed in cancer cells, they also raise questions about the role of galectin-7 when induced by wt versus mutant p53. Our data support the view that galectin-7 cannot be solely considered a pro-apoptotic protein. The biological role of galectin-7 is likely dependent on functional interactions with other p53-induced genes and/or NF-κB-induced genes, a possibility consistent with its elevated levels in breast cancer tissues where mutant p53 is commonly found. Future investigations will be needed to determine how galectin-7 biological function is modulated by the crosstalk between p53 and NF-κB.

Supporting Information

Figure S1. (TIF)

Figure S2. (TIF)

Figure S3. (TIF)

Figure S4. (TIF)

Figure S5. (TIF)

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Author Contributions

Conceived and designed the experiments: CGC YSP. Performed the experiments: CGC GL ML. Analyzed the data: CGC GL ML YSP. Contributed reagents/materials/analysis tools: CGC GL ML. Wrote the manuscript: CGC YSP. Helped to acquire funding: YSP.

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