

Sialylation regulates galectin-3/ligand interplay during mammary tumour progression - a case of targeted unclocking

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ABSTRACT Galectin-3 is involved both in facilitating detachment of cells from primary tumour sites and favouring cancer cell adhesion and survival to anoikis in the blood stream. The mechanisms behind these apparently contradictory roles of the lectin have not yet been resolved. In order to investigate possible interplays between galectin-3 and its ligands underlying their role in the metastatic process, we examined mucin-1 (MUC1) and epidermal growth factor receptor (EGFR), well-known galectin-3 ligands, as well as galectin-3-binding site expression in a series of spontaneous canine malignant mammary tumours (CMMT) and a metastatic CMMT cell line. Despite the fact that CMMT cells expressed MUC1 and EGFR homogeneously over their plasma membrane, intravascular tumour cells, positive for galectin-3, expressed MUC1 and EGFR in a more focal membrane localization. Moreover, MUC1 overexpression in primary CMMT was present in parallel with down-regulation of galectin-3. Furthermore, in the CMT-U27 cell line, galectin-3 knock-down led to increased MUC1 expression, while MUC1 knock-down led to down-regulation of the lectin. Finally, removal of sialic acid from both CMMT and CMT-U27 xenograft samples exposed galectin-3-ligands throughout the tumour tissue, whereas these ligands were only present in galectin-3-positive invading cells in untreated samples. Interestingly indeed, we show that in vessel-invading cells, there is interaction between galectin-3 and the T antigen *in vivo*. We therefore hypothesized that loss of galectin-3 and sialylation-related masking of its ligands, in conjunction with their overexpression in specific tumour cell subpopulations, are crucial in regulating adhesive/de-adhesive events in the progression and invasive capacity of metastatic cells.

KEY WORDS: *galectin-3, galectin-3 ligand, sialylation, metastasis, mammary tumour*

Introduction

The metastatic process involves a complex series of coordinated events leading to distant metastases - the main cause of tumour-related death (Chambers *et al.*, 2002). Crucial steps in this process include detachment of tumour cells from primary sites, by loss of homotypic (between cancer cells) and heterotypic cell-extracellular matrix (ECM) adhesion and survival of tumour emboli in the blood stream, which in contrast, requires homotypic adhesion between tumour cells (Orr and Wang, 2001). The prospect of

better understanding the adhesion/de-adhesion capacity of tumour cells is paramount to in turn understand tumour progression and

Abbreviations used in this paper: CMMT, canine malignant mammary tumours; CRD, carbohydrate recognition domain; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; Gal3/AP probe, galectin-3/alkaline phosphatase probe; ICAM-1, intercellular adhesion molecule 1; MAL, *Maackia Amurensis* Lectin; MUC1, Mucin-1; PLA, proximity ligation assay; RCPs, rolling circle products; RUNX, Runt-related gene family; shRNA, short hairpin RNA; SNA, *Sambucus Nigra* Agglutinin; T antigen, Thomsen-Friedenreich antigen.

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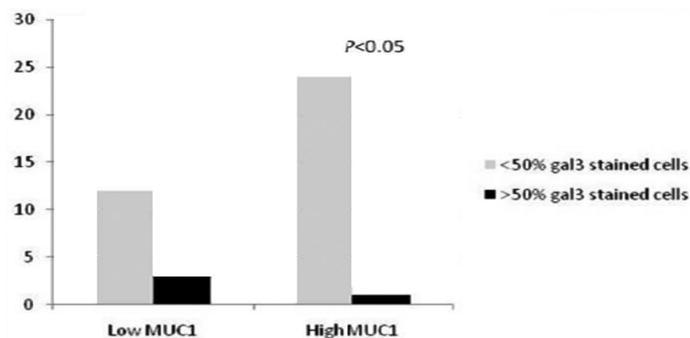


Fig. 1. Mucin1 (MUC1) is overexpressed in a series of canine malignant mammary tumours (CMMT) with galectin-3 down-regulation. MUC1 immunohistochemical staining was analyzed in a series of 40 CMMT, and tumours were compared according to the percentage of MUC1 and galectin-3-expressing tumour cells. Most CMMT presented MUC1 staining in more than 50% of tumour cells, while the majority of these tumours presented galectin-3 expression in less than 50% of tumour cells. There was a significant difference ($P < 0.05$) by Pearson chi-square test.

metastasis. Galectin-3 is a member of the family of carbohydrate binding proteins (β -galactoside-specific lectins) that has been implicated in multiple wide spanning roles such as, cell-cell and cell-ECM adhesion, angiogenesis promotion, cell proliferation and apoptosis resistance, the latter actions ultimately facilitating tumour progression and metastasis (Liu and Rabinovich, 2005). To date, galectin-3 up- or down-regulation is, for reasons still unknown, dependent not only on tumour type but also on specific localization of tumour cells (Liu and Rabinovich, 2005). Recently, we reported on the down-regulation of galectin-3 and galectin-3-binding-sites in canine malignant mammary tumours (CMMT). This observation indicated a possible role for a decrease of galectin-3/galectin 3-binding sites in the detachment and migration of tumour cells from primary tumours. In contrast, in vessel-invading cells galectin-3 and its binding-sites were up-regulated supporting a role for such

an increase in the metastatic process (de Oliveira et al., 2010).

Galectin-3 has multiple well-known ligands co-implicated in different steps of carcinogenesis (Liu and Rabinovich, 2005). Mucin-1 (MUC1) and epidermal growth factor receptor (EGFR) are among these and play a fundamental role in cancer progression and invasion (Kufe, 2009, Salomon et al., 1995). MUC1 is a large highly glycosylated membrane-associated protein expressed in the apical surface of normal epithelial cells (Kufe, 2009). Aberrant expression of MUC1 has been associated to poor prognosis in several types of cancer (Reis et al., 1998; Sipaul et al., 2011), including canine mammary cancer and human breast cancer (de Oliveira et al., 2009; Kufe, 2009). An abnormal under-glycosylated form of MUC1 is overexpressed in scattered distribution throughout the membrane of cancer cells (Kufe, 2009). In these cells, the mucin carries truncated glycan structures with terminal galactose or N-acetylgalactosamine often being sialylated. Such is the case of the cancer associated Thomsen-Friedenreich antigen (T antigen), Gal β 1-3GalNAc, i.e. core 1 disaccharide, recognized by galectin-3 (Yu et al., 2007). The T antigen is generated by the T-synthase, initiating the synthesis of core 1-derived O-glycans (Seko et al., 1996). The core 1 disaccharide is a substrate for a number of sialyltransferases, which synthesize different forms of the sialylated T antigen, including ST3GalI (Picco et al., 2010), ST6GalNAcI and ST6GalNAcII (Marcos et al., 2004). Recently, our group has also reported that sialylation is a prevalent type of glycosylation in CMMT (Pinho et al., 2009a). The presence of sialic acid can inhibit cell-cell interactions by masking carbohydrate ligands thus blocking receptor recognition (Powell et al., 1987). In cancer MUC1 promotes tumour cell detachment from primary sites by inhibiting cell-cell and cell-ECM adhesion, through its large size and through negative repulsive charges conferred by the presence of sialic acid (Kufe, 2009). However, in vessels, galectin-3 induces MUC1 clustering on the tumour cell surface by binding T antigen. This exposes adhesion molecules such as inter-cellular adhesion molecule 1 (ICAM-1) that facilitate interaction between

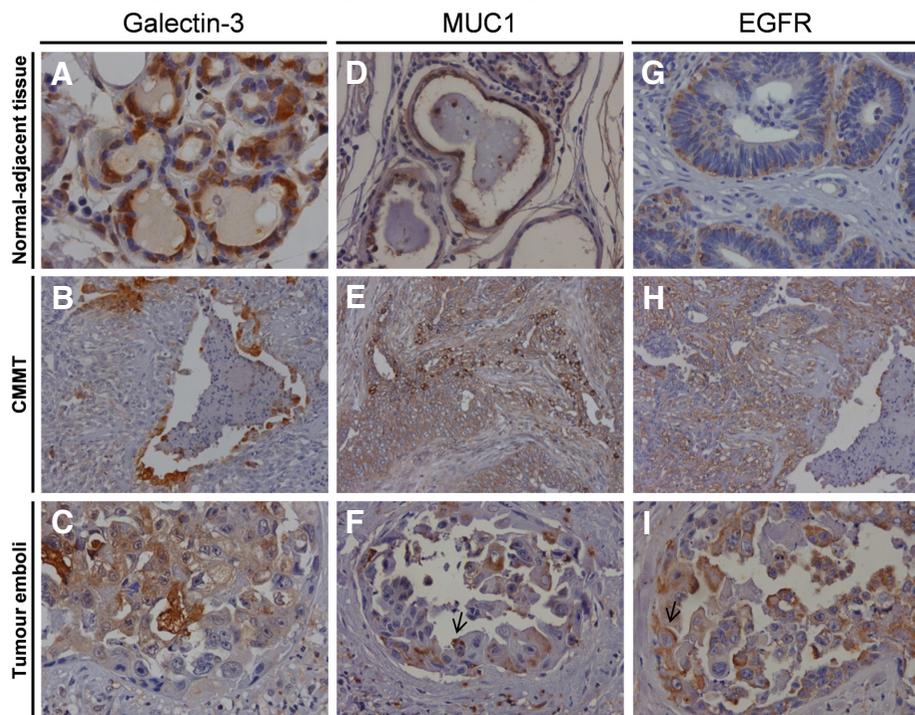


Fig. 2. MUC1 and epidermal growth factor receptor (EGFR) are differentially expressed in the membrane and cytoplasm in a series of CMMT. Photomicrographs depict galectin-3, MUC1 and EGFR immunostaining (brown colour) with haematoxylin counterstain in malignant CMT. Galectin-3 is expressed in normal-adjacent tissue (A) (x630 magnification) but its expression is decreased in malignancy (B) (x400 magnification) with the exception of among others, intravascular tumour cells (C) (x400 magnification). In normal-adjacent tissue MUC1 is expressed predominantly in the apical membrane (D) (x630 magnification); in CMMT MUC1 is expressed more focally in the membrane in cytoplasmic, vesicular and whole membrane patterns (E) (x400 magnification) and consistently in intravascular tumour cells (arrow) (F) (x630 magnification). In normal-adjacent tissue EGFR is expressed in the basolateral membrane (G) (x630 magnification); in CMMT, however, EGFR is expressed all over the membrane and cytoplasm (H) (x400 magnification). Tumour cell emboli express EGFR more focally at the membrane (arrow) (I) (x400 magnification).

tumour and endothelial cells (heterotypic interactions) (Zhao *et al.*, 2009). Furthermore, in vessel-invading cells, MUC1 clustering by galectin-3 enables E-cadherin-mediated homotypic aggregation hence promoting survival over anoikis (Zhao *et al.*, 2010).

EGFR is a member of the growth factor receptor family of tyrosine kinase receptor proteins, expressed in the basolateral membrane of the normal epithelial cells. EGFR expression reportedly is associated to poor prognosis in a range of epithelial cancers although this issue is controversial with respect to human breast cancer (Salomon *et al.*, 1995; Klijn *et al.*, 1992). EGFR signalling regulates several cellular functions that promote resistance to apoptosis and cell survival (Salomon *et al.*, 1995; Burgess *et al.*, 2003). In addition to its canonical ligand, EGFR can be activated by other proteins, including mucins, which contain EGF-like domains (Couto *et al.*, 1996). Besides a reported direct interaction between MUC1 and EGFR (Kufe, 2009), MUC1-EGFR bridging by galectin-3 has been demonstrated *in vitro* using breast cancer cell lines (Ramasamy *et al.*, 2007). Notably, the presence of both MUC1 and EGFR was recently shown in peripheral blood samples of dogs bearing CMMT (da Costa *et al.*, 2010).

In this study we aimed to determine the mechanisms underlying the role of galectin-3 in tumour cells adhesion/de-adhesion during the progression of CMMT. To test the relationship of the lectin with the levels and patterns of expression of its ligands MUC1 and EGFR, we used two experimental settings: 1) histochemistry, co-expression and Proximity ligation assay (PLA) interaction studies in a spontaneously occurring CMMT series; 2) Galectin-3 co-precipitation with MUC1 and EGFR and a short hairpin RNA (shRNA) strategy to knock-down galectin-3 and MUC1 in a CMMT metastatic cell line. In primary tumours, on the one hand, down-regulation of galectin-3 is associated with MUC1 overexpression (in turn significantly associated to vascular invasion and distant metastases) and the two are broadly not co-localized. Notably, an increase in MUC1 levels of expression is observed upon galectin-3 knock-down in the CMT-U27 cell line. On the other hand, galectin-3 is co-expressed at the cell membrane with EGFR and MUC1, by vessel-invading tumour cells, and interacts with the mucin *via* T antigen. Moreover, a decrease in galectin-3 levels of expression is observed upon MUC1 knock-down in the CMT-U27 cell line. We also analysed the effects of sialylation in modulating the availability of galectin-3-ligands during tumour progression in spontaneous CMMT and in an experimental nude mice model of CMMT. Removal of sialic acid greatly increased galectin-3 binding to primary tumours in both models. Our data demonstrate an important role of the levels and glycosylation status of galectin-3 ligands in regulating the lectin's functions in the complex multistage process of cancer metastasis.

Results

MUC1 expression in a CMMT series is up-regulated in metastatic cases, concomitant with a galectin-3 dual expression: generalized down-regulation in primary tumour vs. up regulation in tumour emboli and cells surrounding necrotic areas

In order to investigate a possible association between

galectin-3 and its ligand MUC1, the immunohistochemical expression of the mucin was studied in a series of 40 cases of spontaneous CMMT and compared to previous analyses of galectin-3 expression (de Oliveira *et al.*, 2010).

The majority of cases (25) showed MUC1 overexpression, with more than 50% of the cells being stained. Only fifteen cases expressed MUC1 in less than 50% of the cells. In contrast, there were only 4 CMMT cases with more than 50% cells stained for galectin-3. Therefore, we found a significant ($P < 0.05$) difference between galectin-3 and MUC1 expression in primary CMMT (Fig. 1). Concerning the pattern of expression, galectin-3 was found in normal-adjacent mammary epithelium (Fig. 2A) but was drastically down-regulated in the primary tumours (Fig. 2B). However an interesting, localized up-regulation of the lectin was consistently observed in well-defined areas, mainly in tumour emboli (Fig. 2C). Normal-adjacent mammary epithelium expressed MUC1 predominantly in the cytoplasm and at the apical membrane (Fig. 2D), whereas CMMT cells expressed MUC1 mainly in a cytoplasmic and vesicular pattern throughout the primary tumour cells (Fig. 2E). A total of 10 cases presented distant metastases. In these, MUC1

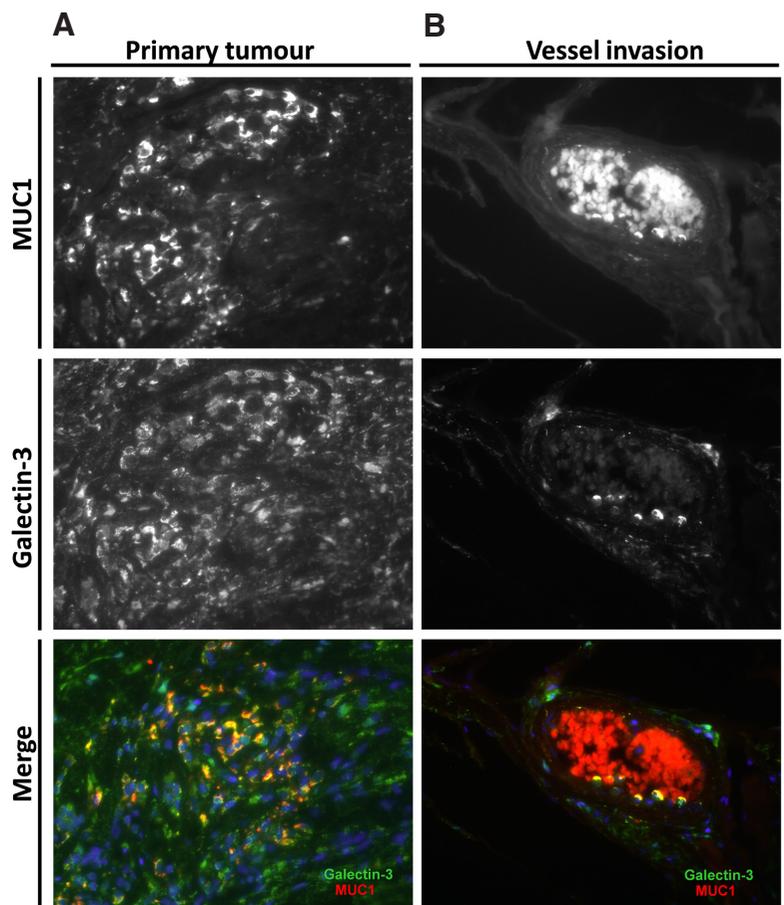


Fig. 3. Galectin-3 is co-expressed with MUC1 in cells at the vessel vicinity and in vessel-invading cells in CMMT. Co-localization was assessed by double-labelling immunofluorescence using anti-MUC1 and galectin-3 antibodies. (A) Co-localization between MUC1 (red colour) and galectin-3 (green colour) was frequently observed in cells with particularly aggressive characteristics focally. (B) Intravascular tumour cells strongly co-expressed (yellow colour) MUC1 and galectin-3 focally at the cell membrane. Photomicrograph magnifications 630x.

overexpression ($\geq 50\%$) was more common than in cases without distant metastases, ($p = 0.000$) (Table 1). Thirteen cases presented vascular invasion. MUC1 overexpression was also significantly associated to this feature ($p = 0.019$) (Table 1). Furthermore, MUC1 expression in vessel-invading cells was focally localized at the cell membrane (Fig. 2F). These results in a primary CMMT series indicate that MUC1 overexpression (and subcellular localization), which occurs in parallel with galectin-3 generalized down-regulation, plays an important role in metastasis.

EGFR expression is up-regulated in CMMT intravascular tumour cells and viable cells adjacent to necrotic areas paralleling that of galectin-3

Next, in order to compare EGFR expression to that of galectin-3 we performed immunohistochemical studies in the same CMMT series. Thirty-six CMMT cases were positive for EGFR expression. Of these, twenty-one cases expressed EGFR in less than 50% of the cells, whereas 15 showed EGFR expression in more than 50% of the cells. In CMMT, normal-adjacent mammary tissue expressed EGFR in the basolateral membrane (Fig. 2G). When present in malignant cells, EGFR expression was observed mainly in cytoplasmic and vesicular patterns (Fig. 2H). Furthermore, EGFR expression was observed consistently surrounding necrotic areas. In fact, tumours without necrosis, a classical hallmark of aggressiveness, had statistically significant lower expression levels of EGFR than tumours with necrosis ($p = 0.002$) (Table 2). Interestingly, intravascular tumour cells also showed enhanced EGFR expression focally at the cell membrane (Fig. 2I). Galectin-3 expression paralleled that observed for EGFR in the same cell subpopulations as previously shown (de Oliveira *et al.*, 2010). These results indicate a possible joint role of EGFR and galectin-3 in CMMT, in the survival process of tumour cells under stress conditions.

Galectin-3 is co-expressed with MUC1 and EGFR in intravascular tumour cells of CMMT and is present in both EGFR and MUC1 immunoprecipitates

Galectin-3 facilitates the interaction between MUC1 and EGFR in breast cancer cells *in vitro* (Ramasamy *et al.*, 2007). In order to assess co-expression between galectin-3 and its ligands *in vivo*, we performed double-labelling immunofluorescence studies in CMMT cases with vascular invasion. When present in the primary tumour, galectin-3 co-expressed with MUC1 mainly in cells of particularly aggressive characteristics (Fig. 3A). Interestingly, in intravascular tumour cells, galectin-3 co-expressed with MUC1 focally at the cell membrane (Fig. 3B). Likewise, co-expression between galectin-3 and EGFR was also observed in intravascular tumour cells (Fig. 4).

In order to further assess the interplay between galectin-3 and its ligands we took advantage of using two canine mammary tumour

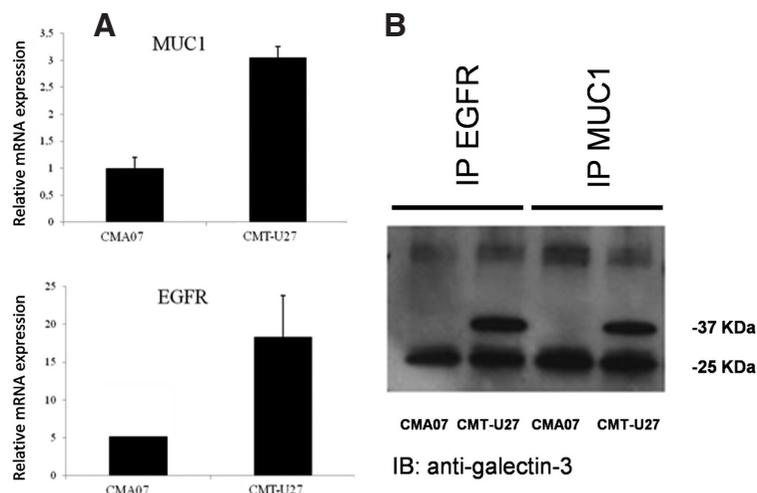


Fig. 5. CMT-U27 overexpresses MUC1 and EGFR and exhibits physical interactions between galectin-3 and its ligands when compared to CMA07 benign cell line. (A) Data from real-time PCR for MUC1 and EGFR using RNA from CMA07 and CMT-U27 cells shows that their levels are decreased in the benign when compared to the malignant cell line. cDNA contents were normalized on the basis of predetermined levels of 18S. The results represent the mean \pm SD of triplicate samples in one of three similar experiments. (B) Lysates from both cell lines were immunoprecipitated with anti-MUC1 and anti-EGFR. The precipitates were immunoblotted with the anti-galectin-3 antibody. Galectin-3 was present both in MUC1 and EGFR precipitates of the CMT-U27 cell line.

cell lines as a model: the CMA07 benign cell line and CMT-U27 metastatic cell line. Firstly, the levels of MUC1 and EGFR expression were compared between the two cell lines. CMA07 shows decreased expression of both MUC1 and EGFR when compared to CMT-U27 (Fig. 5A,B). Lysates from these cell lines were immunoprecipitated with anti-MUC1 and anti-EGFR antibodies. The obtained MUC1 and EGFR precipitates were subsequently immunoblotted with an anti-galectin-3 antibody. Galectin-3 was present both in MUC1 and EGFR precipitates (Fig. 5B). Altogether these results imply that, in CMMT, galectin-3 might interact with MUC1 and EGFR in certain dynamic states such as vessel-invasion and other stress-enduring locations (necrosis vicinity) and suggest a role for such interactions in the metastatic process of those tumours.

MUC1 and galectin-3 seem to function in a feedback regulatory loop in the CMT-U27 metastatic cell line

MUC1 has been observed to up-regulate galectin-3 expression by a glycosylation-dependent posttranscriptional mechanism in human breast cancer cells (Ramasamy *et al.*, 2007). In order to probe a similar role for MUC1 in CMMT, we performed MUC1 knock-down in the CMT-U27 cell line, which endogenously expresses both MUC1 and galectin-3, and evaluated its effects on galectin-3 expression. A decrease in galectin-3 mRNA expression was observed

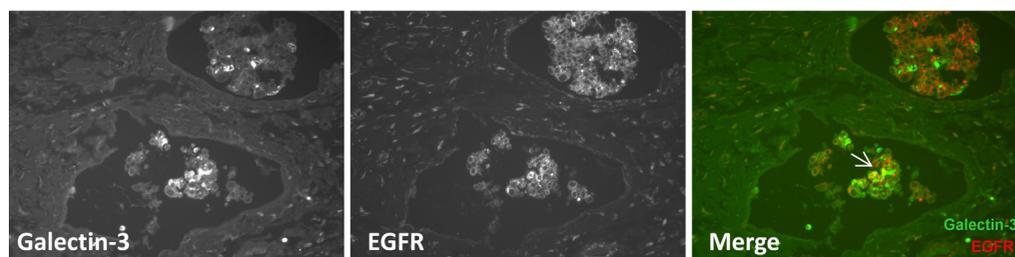


Fig. 4. Galectin-3 is co-expressed with EGFR in intravascular tumour cells of CMMT. Co-expression was assessed by double-labelling immunofluorescence using anti-EGFR and galectin-3 antibodies. Co-expression (yellow colour) between EGFR (red colour) and galectin-3 (green colour) was observed in vessel-invading tumour cells of CMMT (arrow). Photomicrograph magnifications 200x.

TABLE 1

RELATIONSHIP BETWEEN MUC1 EXPRESSION, DISTANT METASTASIS AND VASCULAR INVASION IN MALIGNANT CANINE MAMMARY TUMOURS

	Number (and %) of tumours showing MUC1 immunostaining in the stated percentage of cells					p value
	<25%	25-50%	50-75%	>75%		
Vascular invasion (n=37)						
No	24 (64.9%)	2 (8.3%)	9 (37.5%)	11 (45.9%)	2 (8.3%)	0.019
Yes	13 (35.1%)	0 (0.0%)	2 (15.3%)	7 (53.9%)	4 (30.8%)	
Distant metastases (n=39)						
No	29 (74.4%)	3 (10.3%)	11 (37.9%)	13 (44.8%)	2 (7.0%)	0.000
Yes	10 (25.6%)	0 (0.0%)	0 (0.0%)	5 (50.0%)	5 (50.0%)	

TABLE 2

RELATIONSHIP BETWEEN EGFR EXPRESSION AND NECROSIS IN MALIGNANT CANINE MAMMARY TUMOURS

	Number (and %) of tumours showing EGFR immunostaining in the stated percentage of cells						p value
	Negative	<25%	25-50%	50-75%	>75%		
Necrosis (n=38)							
No	9 (23.7%)	3 (33.3%)	1 (11.1%)	4 (44.5%)	1 (11.1%)	0 (0.0%)	0.002
Yes	29 (76.3%)	1 (3.4%)	1 (3.4%)	10 (34.5%)	14 (48.3%)	3 (10.3%)	

in CMT-U27 MUC1 knock-down cells when compared to control cells (Fig. 6A). There was a corresponding decrease in the protein level of galectin-3 in MUC1 knock-down cells compared to control cells (Fig. 6B). These results demonstrate that MUC1 is involved in galectin-3 regulation in CMMT.

The *in vitro* demonstration that MUC1 knock-down decreases galectin-3 expression, does not explain their respective expressions in the cell subpopulations *in vivo*. As a matter of fact, in most primary tumour cells, expression is increased for MUC1 and decreased for galectin-3 whereas in those cells constituting tumour emboli or

having aggressive characteristics they are both overexpressed. We thus hypothesized that in CMMT varying levels of galectin-3 expression could in turn play a regulatory effect on MUC1 expression. In order to verify this, we silenced galectin-3, by shRNA, in the CMT-U27 metastatic cell line. Upon galectin-3 knock-down there was a striking *circa* 5 fold increase in expression of MUC1 mRNA when compared to control cells infected with non-silencing shRNA (Fig. 6C). A corresponding increase in MUC1 protein expression was observed in galectin-3 knock-down cells when compared to control ones (Fig. 6B). Altogether, these results indicate that down-regulation of galectin-3 is involved in the up-regulation of MUC1 expression and suggest that MUC1 and galectin-3 are part of a feedback regulatory loop in CMMT.

Sialylation acts as a switch on/switch off mechanism modifying galectin-3 binding to its ligands during tumour progression in CMMT

Both galectin-3 and its binding-sites can be either down- (primary tumours) or up-regulated (intravascular tumour cells) according to specific settings in CMMT (de Oliveira *et al.*, 2010). Sialylation, particularly α 2,6-linkage, is a common posttranslational modification known to negatively affect galectin-3 recognition and binding to its β -galactoside ligands (de Melo *et al.*, 2007). Hence, we hypothesized that, in CMMT, galectin-3 binding to its ligands might be intermittently impaired by sialylation. In order to assess this, we verified the binding of a galectin-3/alkaline phosphatase (Gal3/AP) probe in 10 CMMT cases with or without neuraminidase treatment, prior to incubation. In the absence of treatment, the majority of tumour cells presented low galectin-3 binding site expression, with few exceptions among which the invasive front of CMMT (Fig. 7A). However, interestingly, following neuraminidase treatment of CMMT sections, binding of Gal3/AP probe to the neoplastic tissues was greatly increased and widespread (Fig. 7B,C).

Next, in order to further substantiate our hypothesis the reactivity of two plant lectins, *Sambucus Nigra* Agglutinin (SNA), which

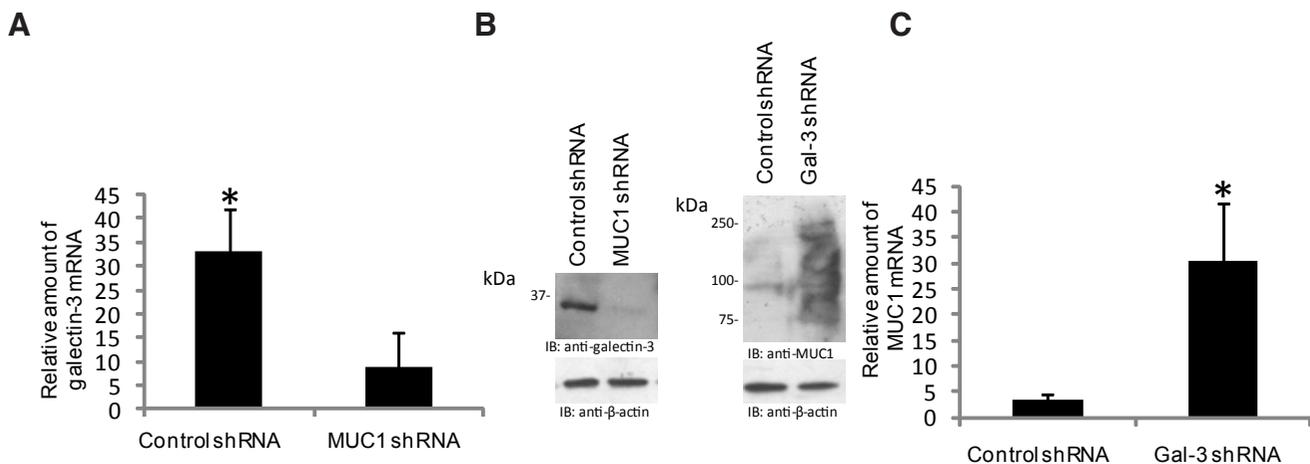


Fig. 6. Galectin-3 and MUC1 function in a regulatory loop in the metastatic CMT-U27 cell line. (A) Data from real-time PCR for galectin-3 using RNA from CMTU27 cells either uninhibited or inhibited for MUC1 using short hairpin RNA shows that levels of galectin-3 mRNA were decreased upon MUC1 knockdown. Cells infected with scrambled oligos were used as a control. Asterisks indicate significant differences ($p < 0.05$) by Student's *t*-test. cDNA contents were normalized on the basis of predetermined levels of 18S. The results represent the mean \pm SD of triplicate samples in one of three similar experiments. **(B)** Western blot study of the expression of MUC1 and galectin-3 upon knock-down. **(C)** Data from real-time PCR for MUC1 using RNA from CMTU27 cells either uninhibited or inhibited for galectin-3 using short hairpin RNA; levels of MUC1 mRNA were increased to around 10 fold upon galectin-3 knockdown. Cells infected with scrambled oligos were used as a control. Asterisks indicate significant differences ($p < 0.05$) by Student's *t*-test. cDNA contents were normalized on the basis of predetermined levels of 18S. The results represent the mean \pm SD of triplicate samples in one of three similar experiments.

recognizes α 2,6-linked sialic acid, and *Maackia Amurensis* Lectin (MAL), which recognizes α 2,3-linked sialic acid, was analyzed in the tumours, in parallel with that of Gal3/AP probe. Galectin-3-binding site expression was observed (Fig. 7D) in MAL-binding site positive areas (Fig. 7E) and was virtually independent of the neuraminidase treatment of the sections (Fig. 7F). In contrast, galectin-3-binding site expression was frequently absent (Fig. 7G) from SNA-binding site positive areas (Fig. 7H) prior to neuraminidase treatment, following which, increased Gal3/AP binding was observed (Figure 7I). These findings suggest that differential presence of α 2, 6-linked sialic acid in sedentary tumour cells and certain tumour subpopulations may account for differences observed in galectin-3-binding site expression and hence undermine galectin-3-mediated adhesion between tumour cells at these locations.

CMT-U27 xenografts in nude mice display a heterogeneous galectin-3-binding site expression pattern, resembling that of CMMT

The CMT-U27 highly metastatic cell line shows homogeneous expression of galectin-3-binding sites *in vitro* (de Oliveira *et al.*, 2010). Considering the heterogeneous pattern observed *in vivo* in CMMT, we aimed to investigate whether the homogeneous pattern observed in the CMT-U27 cell line would undergo microenvironment-

associated variations. We thus proceeded by injecting the cell line into the mammary fat pad of female nude mice. Interestingly, the xenografted tumours, presented a distinct pattern of Gal3/AP binding according to the location of the tumour cells. Galectin-3-binding site expression was low in the vast majority of sedentary tumour cells. On the other hand, at the invasive front of the primary tumours, this expression was high as previously seen in CMMT (Fig. 7J). In addition, binding of the Gal3/AP probe was again highly increased, following neuraminidase treatment of areas initially presenting lower levels of galectin-3-binding sites, resembling our observations in CMMT (Fig. 7K,L).

Accordingly, while MAL-binding site expression was low (Fig. 7M), high SNA-binding site expression was observed in sedentary cells (Fig. 7N). Neuraminidase treatment abrogated SNA binding to the tissues (Fig. 7O).

Altogether, these results suggest that the microenvironment may play a role in the sialylation-related availability of ligands for galectin-3-binding in CMMT.

T antigen and galectin-3 are co-expressed and interact during vessel invasion in CMMT

Several effects of galectin-3 interaction with MUC1, crucial in the metastatic process, are dependent on the lectin's binding to the

T antigen carried by the mucin (Zhao *et al.*, 2009; Zhao *et al.*, 2010). Hence, we set out to compare the expression levels of T antigen and its sialylated form in 13 cases with vessel invasion. Most cells of primary CMMT showed higher levels of sialylated T antigen when compared to the un-substituted form of the antigen which was expressed in low levels and was even often negative (Fig. 8A). However, surprisingly, intravascular tumour cells expressed mainly

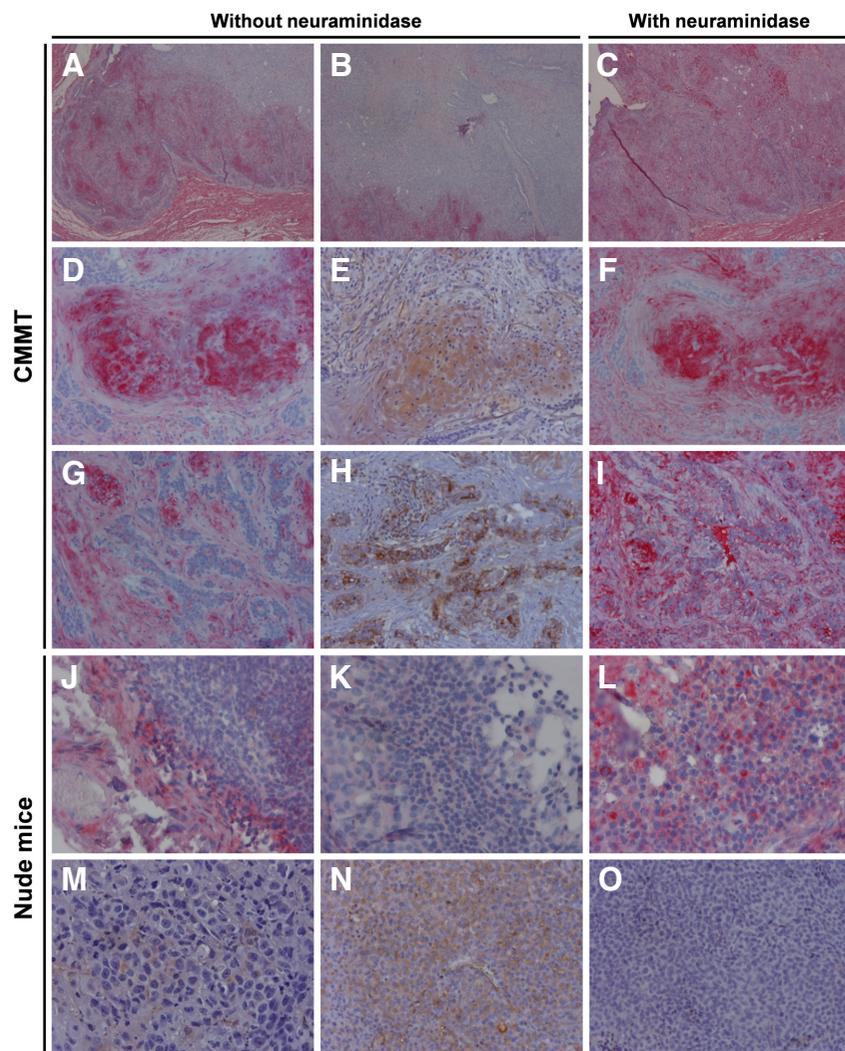


Fig. 7. Sialylation acts as a switch on/switch off mechanism modifying galectin-3 binding to its ligands during progression in CMMT. Incubation of the CMMT tissues with Gal3/AP was performed with or without prior neuraminidase treatment. High levels of galectin-3 binding sites were consistently observed in the invasive front of the primary tumours (A) (x200 magnification); however the majority of sedentary CMMT cells presented low levels of binding of Gal3/AP (B) (x200 magnification) which was increased following neuraminidase treatment in the CMMT sections (C) (x200 magnification). Galectin-3-binding site expression was observed (D) (x400 magnification) in positive MAL-binding sites areas (E) (x400 magnification) regardless of neuraminidase treatment (F) (x400 magnification). On the other hand, galectin-3-binding site expression was consistently decreased (G) (x400 magnification) in positive SNA-binding sites areas (H) (x400 magnification) but increased following neuraminidase treatment (I) (x400 magnification). Accordingly, xenografted tumours showed galectin-3-binding site expression also in the invasive front; (J) (x400 magnification), despite most areas of the tumours presented decreased expression of galectin-3-binding sites (K) (x630 magnification) which was again increased following neuraminidase treatment (L) (x630 magnification). Xenografted tumours present low levels of MAL-binding site expression (M) (x630 magnification) when compared to SNA-binding site expression (N) (x630 magnification), neuraminidase treatment abrogated SNA-binding to the xenografted tumours (O) (x630 magnification).

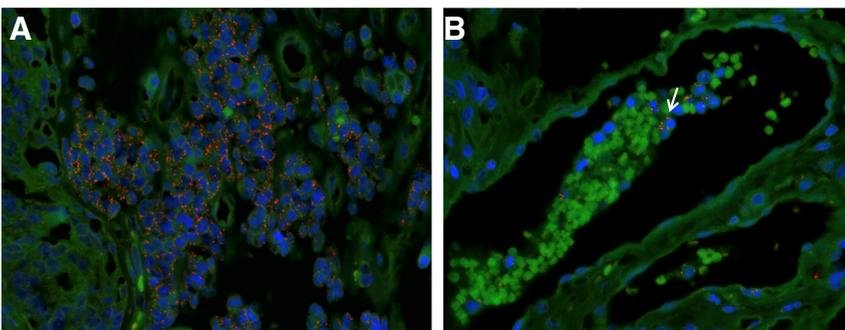
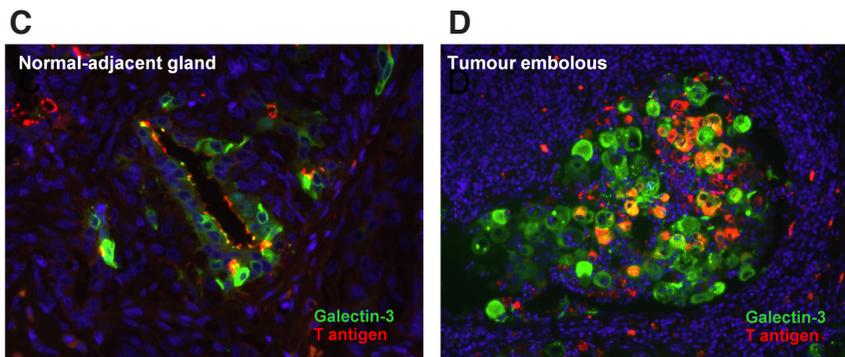
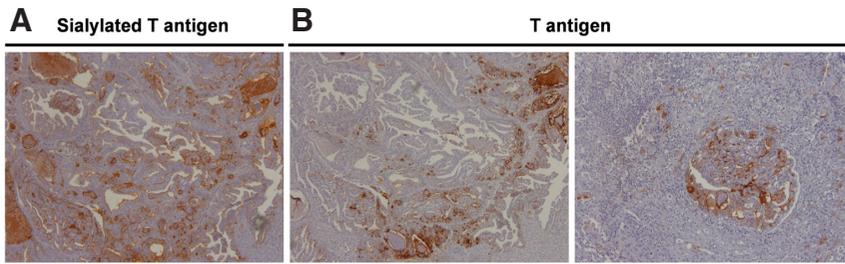


Fig. 8 (Left). Despite sedentary cell expression of increased levels of sialylated T antigen, intravascular tumour cells present co-localization between galectin-3 and the un-sialylated form of the antigen in CMMT. Photomicrographs depict T antigen and sialylated T antigen immunostaining (brown colour) with haematoxylin counterstain in CMMT. Most cells of CMMT, presented high levels of sialylated T antigen revealed by neuraminidase treatment (A) (x200 magnification) when compared to the T antigen (B) (x200 magnification). Surprisingly, intravascular tumour cells expressed the unsialylated form of the antigen (B) (x400 magnification). Co-localization was assessed by double-labelling immunofluorescence using anti-T antigen and galectin-3 antibodies (C) normal glands within primary tumours expressed T antigen only in the apical surface of the cells and despite galectin-3 was also expressed by these cells they did not co-localize (D) however, co-localization of galectin-3 with T antigen was interestingly observed in vessel-invading cells. Photomicrograph magnifications 400x.

Fig. 9 (Left). Intravascular tumour cells show interaction between galectin-3 and the un-sialylated form of the T antigen in CMMT *in vivo*. Interaction was assessed by proximity ligation assay using anti-T antigen and galectin-3 antibodies. (A) Although virtually no PLA signals were observed in sedentary cells of primary CMMT, they were abundantly observed in a few cells surrounding vessels showing interaction between T antigen and galectin-3. (B) Moreover, PLA signals were also consistently observed in intravascular tumour cells particularly at the cell membrane showing interaction between the T antigen and galectin-3 at these locations (arrow). Photomicrograph magnifications 400x.

the T antigen (Fig. 8B). Next, double-labelling studies between galectin-3 and this glycan were performed in the same cases. A few normal mammary glands adjacent to primary tumours which also expressed galectin-3 showed T antigen expression restricted to the apical surface of the cells. Nevertheless, co-expression of the two was never observed (Fig. 8C). However, in vessel-invading cells galectin-3 and the T antigen were co-expressed (Fig. 8D).

In order to prove the interactions between galectin-3 and the T antigen in circulating cells *in vivo* we used the proximity ligation assay, PLA, which is capable of detecting single events such as protein interactions. Assessment of galectin-3 interaction with the T antigen in the primary tumours, showed virtually no PLA signals. However in the vicinity of vessels (Fig. 9A) and in vessel-invading cells, signal presence was elevated (Fig. 9B). Our data shows the loss of T antigen's sialic acid capping in vessel invasion in CMMT. This allows an important interaction between galectin-3 and T antigen, and therefore emphasizes a combined role in metastasis.

Discussion

Metastasis is highly dependent on the ability of tumour cells to evade from primary sites, and to enter and survive in the circulation (Chambers *et al.*, 2002). It is well-known that structural modifications in glycans of glycoproteins can alter their interaction with endogenous lectins, modulating their role in cell adhesion and

signal transduction (Ohtsubo *et al.*, 2006). In an earlier study, we have established that the Gal β 1-3GalNAc-binding lectin, galectin-3, and its binding-sites are implicated in the metastatic process of mammary tumours (de Oliveira *et al.*, 2010). Specifically, we have demonstrated that i) overall galectin-3 expression is down-regulated in primary CMMT but up-regulated in its intravascular tumour emboli and that ii) both intravasated tumour cells and tumour endothelium overexpress galectin-3-binding sites while in the primary CMMT, on average, there is a decrease of their expression.

For the present study, our specific goal was to gain more insight in the mechanisms by which galectin-3 along with its ligands could be acting in tumour progression and invasion of CMMT.

Among several well-known galectin-3-ligands EGFR and MUC1 are two of the most closely associated to breast cancer (Klijn *et al.*, 1992; Kufe, 2009). In the present study, we observed a clear cellular sub-compartmentalization of MUC1 (apical) and EGFR (basal) membrane staining in normal-adjacent mammary glands. On the other hand, in CMMT neoplastic cells, MUC1 and EGFR were expressed in a predominantly vesicular and cytoplasmic pattern and scattered throughout the entire membrane. Nevertheless, in intravascular cells of CMMT they were often expressed focally at the cell membrane. Interestingly at these locations the proteins were co-expressed with galectin-3. In normal mammary tissues MUC1 is localized at the apical while EGFR is present at the basolateral side (Kufe, 2009; Klijn *et al.*, 1992) of cell membranes and therefore

there is virtually no interaction between them. Their interaction in a non-tumour context is a sign of temporary polarization disruption and hence appears to mediate cell survival programs (Kufe, 2009). Recent studies in the human and experimental animal models have indicated that in several types of carcinoma there is a permanent loss of polarization leading to constant interaction of transmembrane mucins and growth factor receptors (Kufe, 2009). Our data on galectin-3 ligands' expression in CMMT support this assumption. Moreover, in our study, EGFR expression was significantly associated to the presence of necrosis in CMMT. In accordance, in a previous work, EGFR expression was thoroughly studied by ligand binding in a series of canine normal mammary tissue, benign and malignant tumours and although no statistical differences were found between the levels of expression of the receptor between non-tumorous tissues and CMMT (Rutteman *et al.*, 1994), when analysing EGFR by autoradiography, a high level of activity in necrotic areas was found (Berns and Rutteman, unpublished data). Similarly, increased galectin-3 expression around necrotic (hypoxic) areas has also been reported in CMT (de Oliveira *et al.*, 2010) and in human cancer (Neder *et al.*, 2004). Adding that to the fact that vessel-invading tumour cells express both galectin-3 and EGFR, the hypothesis of a passive mechanism of invasion wherein tumour cells which intravasate, are mainly those exposed to stress conditions (e.g. hypoxia which also leads to activation of EGFR kinase and may enhance cell migration in cancer cells) (Bockhorn *et al.*, 2007; Wang and Schneider, 2010), seems very plausible in CMMT. Recently, interactions between MUC1 and EGFR were suggested to be facilitated by galectin-3, since it bridges them *in vitro* by recognizing and binding glycans on both proteins in breast cancer cells (Ramasamy *et al.*, 2007). This study corroborates the hypothesis that depolarized cancer cells are able to use MUC1-EGFR interactions as a sustained survival signalling loop (Kufe, 2009). Our data further suggest that galectin-3-mediated MUC1-EGFR interactions might play a role in the metastatic process *in vivo* as indicated by co-expression of the proteins with galectin-3 in vessel-invading cells, corroborated by the precipitation studies.

In accordance with the literature and data recently reported by our group (de Oliveira JT *et al.*, 2009), in the present work, high expression of MUC1 was significantly associated to vascular invasion and metastases occurrence in CMMT. Interestingly, MUC1 overexpression in primary CMMT was also significantly associated to down-regulation of galectin-3. Down-regulation of galectin-3 in several types of human and canine cancer appears to be related to tumour progression (Castronovo *et al.*, 1996; Ahmed *et al.*, 2007; de Oliveira *et al.*, 2010). Loss of this protein has been reported to impair cell-cell cohesion allowing increased tumour cell motility (Liu and Rabinovich, 2005). MUC1 mucin on its hand acts as an anti-adhesive molecule in cancer cells due to both its large size and sialic acid-conferred negative repulsive charges (Jonckheere, 2008). Our data *in vivo* show a concomitant up-regulation of galectin-3 and MUC1 in vessel invading cells. Other authors have reported in mice xenografts of breast cancer cell lines that increased presence of galectin-3 seems to favour cell-adhesion and consequently provide survival advantage to blood-borne tumour cells, by clustering MUC1 at the cell membrane, (Zhao *et al.*, 2010). The reasons why galectin-3 expression varies in cancer cells according to their location are still poorly understood.

In cancer, down-regulation of galectin-3 has been associated to

methylation of its promoter (Ahmed *et al.*, 2007) and altered expression of transcription factors involved in its regulation, such as the RUNX family (Vladimirova *et al.*, 2008). Irrespective of other possible existing mechanisms, in the present study we have demonstrated - to the best of our knowledge, for the first time - that a decrease of galectin-3 plays a role in MUC1 up-regulation, in mammary cancer, suggestive of an indirect contribution to de-adhesion and in accordance to our *in vivo* observations. So far, the only existing study showing a role for galectin-3 in MUC1's regulation was that of Merlin *et al.*, (2011) in which galectin-3 knock-down, in a pancreatic cancer cell line, led to a decrease in MUC1 expression in accordance with their *in vivo* data from human pancreatic carcinoma where both galectin-3 and MUC1 are overexpressed in primary tumours. Furthermore, our data showing that MUC1 knock-down leads to decreased levels of galectin-3 imply that MUC-1 in turn is able to up-regulate galectin-3. This is in accordance with a recent report showing that MUC1 stabilizes galectin-3 mRNA in breast cancer cells through an N-glycosylation dependent mechanism whereby extracellular MUC1 C-terminal domain (MUC1-C) suppresses the expression of a galectin-3-transcript de-stabilizing microRNA (Ramasamy *et al.*, 2007). The fact that galectin-3, on its hand, binds to the MUC1-C N-glycan involved in its own regulation suggests that changes in glycosylation could account for differences in the lectin's regulation by MUC1 according to different cellular contexts.

Sialylation a type of glycosylation prevalent in cancer, has long been suggested to be involved in cellular differentiation and migration through cyclical masking/unmasking of galectin-binding sites (Gillespie *et al.*, 1993). Another key finding of the current study was that galectin-3 ability to bind to its ligands was impaired by sialylation in most areas of primary CMMT. Vessel-invading and cells of invasive fronts in CMMT were notable exceptions to this impaired expression of galectin-3-binding sites. A report of Holíková *et al.*, (2002), demonstrated that $\alpha 2, 6$ - but not $\alpha 2, 3$ -linked N-acetyl-D-neuraminic acid is implicated in masking galectin-3 binding sites in squamous epithelial carcinomas. Hence, in parallel to the use of the Gal3/AP probe, before and after neuraminidase treatment, we assessed the reactivity profile of two plant lectins which recognize distinct types of sialylation. The binding pattern of $\alpha 2, 6$ -Neu5Ac-recognizing plant lectin (i.e. SNA) was inversely associated to Gal3/AP ability to bind to the CMMT tissues. In contrast to $\alpha 2, 6$ - expression of $\alpha 2, 3$ - linked sialic acid, assessed with MAL, was not associated to galectin-3 binding site negative areas. Several reports have shown that while only occasionally binding to $\alpha 2, 6$ -sialylated glycans, galectin-3 binds well to $\alpha 2, 3$ -sialylated glycans (Hirabayashi *et al.*, 2002; de Melo *et al.*, 2007). Thus, the findings of the present study suggest that, although probably not exclusively, $\alpha 2, 6$ sialylation can be an on/off switch for galectin-3 binding *in situ*.

The reasons why invading tumour cells consistently showed increased binding of galectin-3 remain to be determined. However, whatever reasons may exist they must be microenvironment-related as suggested by the fact that CMT-U27 cells acquire heterogeneous expression of galectin-3-binding sites upon being xenografted in nude mice, while these sites' expression is homogeneous during *in vitro* culture (de Oliveira *et al.*, 2010). This being so, at least two explanations are plausible and reflect existing pathophysiological mechanisms 1) cells expressing unmasked galectin-3 ligands have invasion advantage and 2) mainly applicable to vessel-invading cells and cells in vessel surroundings, sialidases of serum and immune cell origin can cleave the α -bound sialic acid from

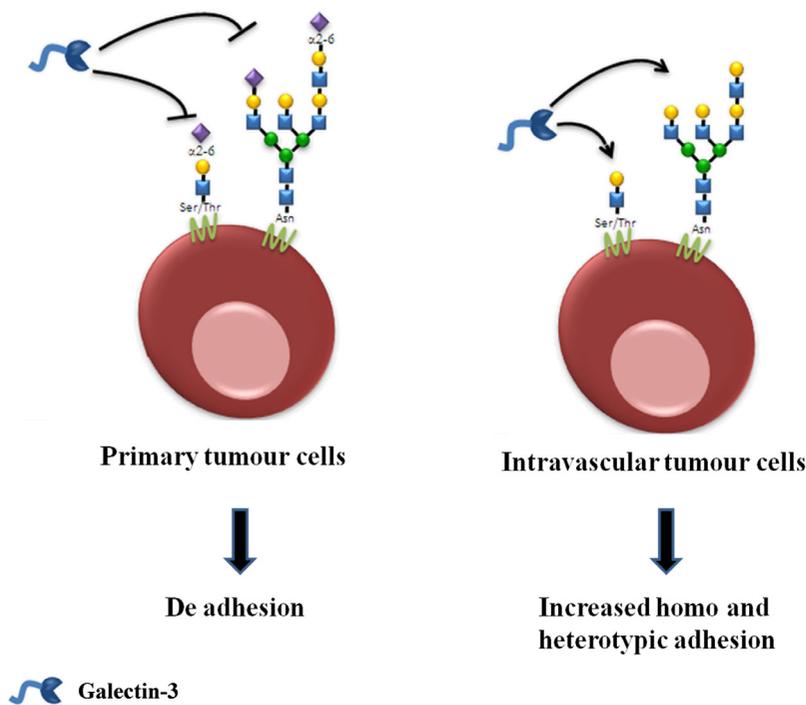


Fig. 10. Proposed model suggesting that microenvironment alterations may modify sialylation of galectin-3-ligands at the cell surface, masking terminal galactose saccharides and thereby cyclically altering galectin-3-mediated adhesion/de-adhesion in cancer cells.

galectin-3-ligands. The first explanation is based on the fact that several factors which could alone or in conjunction contribute to increased expression of unmasked galectin-3-ligands in invading cells have been related to migration and invasive capacity: (a) there is higher activity in invading cells of glycosyltransferases responsible for assembling ligands for this lectin, such as the metastasis-related Mgat5, with concomitant lower activity of competitive GnTIII, which leads to the production of N-glycans not recognized by galectin-3 (Nangia-Makker *et al.*, 2000; Pinho *et al.*, 2009b); (b) there is down-regulation of sialyltransferases which would otherwise mask galectin-3-ligands in these cells. Up or down-regulating of sialyltransferases is a well-known mechanism of motility tuning, turning galectin-3-binding positive cells into negative ones and *vice-versa*, allowing cells to migrate in a physiological context (Gillespie *et al.*, 1993). Furthermore, in normal lactating mammary glands sialyltransferase ST6Gal expression is increased (Dalziel *et al.*, 2001) while galectin-3 is down-regulated (Mengwasser *et al.*, 2002). The second explanation is supported by indications that there is increased sialidase activity in metastatic cells, and that sialidases are responsible for low levels of sialic acid in highly metastatic colon cells when compared to cells with low metastatic potential (Miyagi *et al.*, 2008). Also, increased tissue and serum sialidase levels have been observed in human breast cancer (Sónmez *et al.*, 1999). Moreover, macrophages, as well as other peri- and intravascular immune cells are able to secrete lysosomal sialidases and could therefore be co-responsible for decreased sialic acid capping of galectin-3-ligands in this context (Miyagi *et al.*, 2008).

Concerning the specific microenvironment of blood-borne

tumour cells, several studies have demonstrated that galectin-3 interacts with MUC1 by binding the T antigen (Yu *et al.*, 2007). Nevertheless its sialylated form, is one of the dominant glycoforms of MUC1 in breast cancer patients (Storr *et al.*, 2008). For this reason, we assessed and compared the expression of the T antigen and its sialylated form in CMMT. Our data showed an increased expression of sialylated T antigen in most sedentary tumour cells as compared to the un-substituted form of the antigen. Accordingly, sialylation of the T antigen has been reported to play an important role in promoting early stages of oncogenesis (Picco *et al.*, 2010). Interestingly, however, vessel-invading cells presented an increased T antigen expression. In corroboration of our data, T antigen enrichment has been recently proven to be very useful in assessing circulating tumour cells (Schindlbeck *et al.*, 2008). Moreover, interaction of the T antigen and galectin-3 was observed by PLA in vessel-invading cells but not in sedentary cells in CMMT. The galectin-3-MUC1 interaction via T antigen has been shown to induce MUC1 cell surface polarization and exposure of the cell surface adhesion molecules thereby enhancing tumour cell homotypic aggregation and preventing anoikis *in vitro* and *in vivo* experimental models (Yu *et al.*, 2007; Zhao *et al.*, 2010). However, until now, strong evidence in a spontaneous model of mammary carcinogenesis was lacking to support its relevance.

Our understanding of the initial steps of the metastatic process is still very limited and as such, the use of a spontaneously occurring animal model in which these events precipitate much faster than in humans is of great interest (Paoloni and Khanna, 2008). In conclusion, based on the results here presented and those of others, we suggest that in CMMT, down-regulation of galectin-3 in cancer cells can lead to MUC1 overexpression, while MUC1 decrease could lower galectin-3 expression, as part of a dynamic glycosylation-dependent regulatory loop. We hypothesize that differential glycosylation of the mucin, in specific tumour microenvironment scenarios, might account for the apparently contradictory role played by these proteins in each other's regulation, in CMMT. Further studies are needed in order to confirm this hypothesis. Moreover, our study provides new insight to the posttranslational sialic-acid dependent masking mechanisms that modulate the exposure of terminal galactose saccharides at the cell surface and thus cyclically alter galectin-3-mediated adhesion/de-adhesion capacity in different cancer cell subpopulations (Fig. 10). Future studies are warranted in order to completely uncover the role of microenvironment stimuli in differential/dynamic expression of galectin-3 and its ligands in CMMT.

Materials and Methods

Animal tissues

Malignant mammary tumour specimens were obtained from 40 female dogs at surgery or at post-mortem rapidly following euthanasia, in the years 2002 – 2006. Tumours stemmed from dogs of various pure and mixed breeds, and were randomly selected from the files of the veterinary pathology laboratory of the *Instituto de Ciências Biomédicas Abel Salazar* University of Porto. Tissues had been fixed in 10% neutral buffered formalin

and embedded in paraffin. Serial sections 3 µm thick were cut from each block. One section was stained with haematoxylin and eosin (HE) and the other sections were used for immunohistochemical studies. Follow up was performed in all operated animals for up to two years as described (de Matos *et al.*, 2006).

Histochemistry

Expression of MUC1, EGFR and galectin-3 was evaluated in the formalin-fixed, paraffin-embedded sections of spontaneously occurring CMMT, using standard immunohistochemistry protocols. Briefly, 4 µm thick paraffin sections were deparaffinised, then hydrated, and endogenous peroxidase was blocked with H₂O₂. After blocking with the appropriate normal sera, sections were incubated with 1:10 rat anti-galectin-3 monoclonal antibody (M3/38) and 1:150 rabbit polyclonal anti-galectin 3 (Abcam); 1:50 goat anti-MUC1 polyclonal antibody (Santa Cruz Biotechnology, directed against the C-terminal domain and hence recognizing the protein independently from its glycosylation), and 1:50 rabbit anti-EGFR polyclonal antibody (Santa Cruz Biotechnology) for 1h at room temperature and overnight respectively, followed by 1:200 donkey anti-rat (JacksonImmuno Research) and 1:200 donkey anti-goat (Santa Cruz Biotechnology) and donkey anti-rabbit peroxidase-conjugated secondary antibodies, respectively. For T antigen and sialylated T antigen assessment, slides were incubated overnight with 1:50 Mab 3C9 (kindly provided by Prof. Henrik Clausen from Copenhagen, Denmark), without or with previous neuraminidase treatment for 2 hours at 37°C respectively. Sialylation type profile was assessed using two biotinylated plant lectins, SNA (Vector), which recognizes α₂, 6- linked sialic acid and MAL (Vector), which recognizes α₂, 3- linked sialic acid, incubated for 1 hour at room temperature in a 1:250 dilution followed by incubation of avidin-biotin system (Vector) for 45 min at room temperature. Peroxidase activity was examined using diaminobenzidine tetrahydrochloride solution (DAB, Dako) as the substrate. Sections were counterstained with haematoxylin. Negative controls in which the primary antibody was replaced by BSA 5% in phosphate buffered saline (PBS) were performed in all series and sections of human gastric carcinomas were used as positive controls.

Galectin-3-binding sites were detected both in spontaneously occurring CMMT and malignant CMT-U27 xenografts using a Gal3/AP probe (de Melo *et al.*, 2007) in the same sections as previously described, without or with previous neuraminidase treatment (cleaving both α₂,6 and α₂,3 bound sialic acids). Neuraminidase treatment was carried out for 2h at 37°C at a final concentration of 0.1 U/mL (de Oliveira *et al.*, 2010). Alkaline phosphatase activity was observed using Fast Red (Dako). Sections were counterstained with haematoxylin. As a negative control, an assay using lactose to inhibit the carbohydrate recognition domain (CRD) of galectin-3 was performed.

Scoring of immunostaining and statistical analysis

Assessment of markers expression in CMMT was based on a semiquantitative analysis and tumours were grouped, according to the percentage of immunoreactive cells throughout the whole lesion, in: <10%, which was considered negative; <25%; 25-50%; 50-75%; >75%. Initially a descriptive study of the data was performed. Then association hypotheses were tested, using the Student t test for continuous variables and Fisher's exact test or Chi-square test for discrete variables. SPSS software (version 13.0) (SPSS, Chicago, IL, USA) was used for statistical analysis.

Double-labelling Immunofluorescence

For simultaneous visualization of galectin-3 with MUC1, T antigen and EGFR on the same tissue section, double-label immunofluorescence was performed. Representative tissue sections were chosen with high and low expression of each antigen and sections with the same percentage of positive cells for the antigens. Briefly, after blocking with 10% diluted normal sera for 20 min, sections were incubated with the first primary antibodies rabbit anti-galectin-3 1:150 in 5% bovine serum albumin (BSA) and anti-MUC1 1:50 overnight at 4°C, they were then washed in PBS and incubated with Alexa588-conjugated donkey anti-rat antibody (1:200) and biotinylated anti-goat secondary antibody the latter followed by Alexa594-conjugated

streptavidin incubation for 30 min. Sections were then washed and incubated with 10% donkey and swine serum for 20 min to which incubation with second primary antibodies anti-MUC1, anti-EGFR, 3C9 anti-T antigen and anti-galectin-3 respectively, followed overnight at 4°C. After washing, sections were incubated with biotin-conjugated secondary antibodies (Dako) for 30 min, to which Alexa594-conjugated streptavidin incubation for 30 min followed for MUC1, EGFR and T antigen and Alexa588-conjugated goat anti-rabbit antibody (1:200, Invitrogen) for galectin-3. All sections were then incubated with 1:100 PBS diluted 4'-6-Diamidino-2-phenylindole (DAPI) for 15 min. Finally slides were mounted in glycerol-based Vectashield medium (Vector, Burlingame, CA). Slides were analysed with a Leica DMIRE2 fluorescent microscope.

Cell lines and culture conditions

In this study we used a highly metastatic CMMT cell line, CMT-U27, kindly provided by Professor Eva Hellmen from Sweden. The cell line was cultured at 37°C in a humidified 5% CO₂ incubator and maintained in RPMI 1640 medium (with Glutamax and 25 mM Hepes) supplemented with 10% foetal bovine serum and gentamicin (50 µg/mL).

Experimental nude mice

Six-seven weeks old female N:NIH(s)ll-nu/nu mice were inoculated subcutaneously in the mammary fat pad with a suspension of 10⁶ - 10⁷ cells of CMT-U27 cell line. Mice were periodically observed, weighed and their tumours were measured once a week in three dimensions. Once the xenografted primary tumour attained the volume of 1 cm³ it was excised under general anaesthesia and all efforts were made to minimize suffering. The animals were humanely euthanized whenever their body weight started to decrease or any signs of poor body condition were shown. All studies with experimental animals were approved by the ethics committee of the University of Porto.

RNA interference

Establishment of galectin-3 knock-down cells was done using a retroviral expression system with short hairpin RNAs (shRNA) (Friedrichs *et al.*, 2007). Briefly, targeting and control vectors were transfected into PhoenixGP packaging cell line and transfected cells were selected using Puromycin (Sigma). For MUC1 knock-down Phoenix cells stably transfected with MUC1 shRNA were also used (kindly offered by Filipe Santos Silva, IPATIMUP, Portugal). Stable transfectants were seeded (1x10⁶ cells) and incubated for 24h at 32°C. The virus-containing media were then collected, filtrated through a 0.45 µm filter to remove remnant cells, and used to infect target CMT-U27. 6 and 12h post-infection, the media were replaced by selective puromycin-containing media to select infected cells.

RNA extraction and real-time PCR

Total RNA was isolated from the canine mammary tumour cell lines using TRI Reagent (Sigma) according to manufacturer's instructions. 1 µg of RNA were primed with random hexamers and reverse transcribed using Superscript II (Invitrogen) in a final volume of 20 µl. Two microliters of a 1:10 dilution of this mixture were amplified with SYBR Green (Applied Biosystems) and primers at a final concentration of 300nM each, in a fluorescence reader ABI Prism 7000, and duplicates were run for each RT product. The level of GAPDH RNA in each sample was measured and used for normalization of target genes abundance. Primer sequences are listed below.

GAL3:
5'CAGGCAGCTTTTCCATTCTGA3'/5'ACTGCAACAAATGGGCATCA3';
MUC1:
5'GCACTCGCCATCATCTATGTTTC3'/5'CCAGCTGCCACAGTTCCTTT3';
EGFR:
5'GGTCTGGAAGTTTTTCGGATGG3'/5'GAGGTTTTGCACAGGCTTCAA3'

Protein extraction, Immunoprecipitation and Western Blot

Cells were washed twice in PBS, centrifuged at 350 g, re-suspended in lysis buffer (12 mM Na₂HPO₄, 8 mM NaH₂PO₄, 0.2% SDS, 4 mM EDTA,

0.3 M NaCl, 2% NP-40, 0.1 M NaF, 50mM Sodium Deoxicolate) with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and Complete protease inhibitor cocktail (Roche) and incubated on ice for 15 min. The lysates were then centrifuged, the supernatants collected, and protein content was measured using the bicinchoninic acid method (Pierce), according to the manufacturer's instructions. 500 µg of protein extracts were precleared with 50 µl of protein G-sepharose beads for 1, 2 h. After centrifugation, the supernatant was incubated overnight with 1 µl of the polyclonal antibody against galectin-3 and 5 µl of the polyclonal antibodies against MUC1 and EGFR (both from Santa Cruz Biotechnology). After that, incubation with protein G-sepharose was performed, for 4 h. Next, the beads were washed three times with lysis buffer. The immune complexes were released by boiling 5 min at 95°C in Laemmli sampling buffer and the immunoprecipitates along with the total extracts were analysed by standard sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE, (pre-cast 4-20% tris-glycine gel), transferred to a nitrocellulose membrane (Amersham Biosciences) and blotted with the primary antibodies in 5% non-fat milk in 1x Tris Buffered Saline with 0.02% Tween-20 (Sigma). The primary antibodies were revealed using the appropriate peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence, ECL, detection kit (BioRad).

In situ PLA

In situ proximity ligation assay (PLA) (Söderberg *et al.*, 2006) was performed in sections where co-localization for galectin-3/T antigen combinations was observed by immunohistochemistry/double immunofluorescence. Deparaffinised tissue sections were pre-treated according to the immunohistochemistry/immunofluorescence protocol. Then, sections were incubated for 30min in PBS with 10% BSA, to block nonspecific staining. Excess blocking solution was removed and replaced by specific primary antibodies in PBS with 5% BSA at the optimal dilution. Sections were incubated overnight at 4°C. The PLA assays were performed using the DuoLink detection kit 613 (Olink AB, Uppsala, Sweden) according to the manufacturer's instructions. After washing, oligonucleotide-conjugated PLA secondary probes were added in 1:5 dilutions in PBS with 5% BSA and slides incubated in a pre-heated humid chamber for 1h45min at 37°C. Briefly, connector oligonucleotides were hybridized to probe pairs for 15min and circularized by ligation for 15min. The sections were incubated with the processive ϕ 29 DNA polymerase for 90min to produce rolling circle products (RCPs). RCPs were visualized with fluorescently labelled oligonucleotides and the sections counterstained with Hoechst 33342. Samples were examined under a Zeiss Imager.Z1 Axio fluorescence microscope equipped with DAPI, fluorescein isothiocyanate 1 (FITC) and Texas Red filters. PLA products are seen as bright fluorescent dots. Images were acquired using a Zeiss Axio cam MRm and the AxioVision Rel. 4.8 software. The resulting images were modified using ImageJ as follows: background with radius 4 was subtracted from the red channel of the RGB images and a maximum filter with radius 1 was applied. The result was intensity-scaled to suit printing demands.

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