

**MOLECULAR CHARACTERIZATION OF E-CADHERIN
GLYCOSYLATION IN CANCER**

SALOMÉ SOARES DE PINHO

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em Ciências Biomédicas submetida ao Instituto
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Esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na origem dos resultados apresentados neste trabalho, assim como a sua interpretação, discussão e redacção.

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ABSTRACT

Changes in glycosylation are a hallmark of cancer. A growing body of evidence supports crucial roles for glycans at various pathophysiological steps of tumour progression. However, the biological significance for the aberrant glycosylation in cancer constitutes a long-standing debate and understanding the roles of glycans sets the stage for treating cancer. It is therefore imperative to understand the physiological regulation of glycans in cancer.

Naturally occurring tumours in canine and human species share a wide variety of features. Canine mammary cancer has been considered a valuable spontaneous comparative model for study woman breast cancer in particular and also a precious tool to study cancer in general.

It has been suggested that aberrant glycosylation profoundly affects several cellular mechanisms including cell-cell adhesion. In line with this, E-cadherin is one of the most important cell-cell adhesion molecules having a central role in cancer. In addition, being a glycoprotein, E-cadherin can be post-translational modified by N-glycosylation. Nevertheless, till the beginning of the present work little was known about the role and the function of N-glycans structures on E-cadherin functionality, especially in what concerns E-cadherin mediated tumour progression.

We have found in canine mammary tumours that aberrant expression of Sialyl Lewis x (sLe^x) carbohydrate antigen was considered a marker of the malignant phenotype and may also has an important role in lymphatic metastization of cancer. Therefore we have concluded that sLe^x might be used as a prognostic tumour marker in canine mammary carcinomas. Furthermore we have proposed the existence of a molecular plasticity between sLe^x and E-cadherin expression during the process of tumour development and metastatic progression.

We have characterized for the first time the E-cadherin N-glycosylation profile in malignant and non-malignant tumour phenotypes. We have concluded that E-cadherin underwent an extensive modification of the N-glycans composition during the transformation to the malignant phenotype, that was characterized by an increase of β 1,6 GlcNAc branched structures, an increase in sialylation and an expression of few high mannose structures. In addition we have identified a new N-glycosylation site in E-cadherin from a mammary carcinoma cell line model, containing a potential of β 1,6 GlcNAc branched N-glycan form. In an attempt to further understand the critical functions of N-linked glycans on E-cadherin mediated tumour progression, we delved the regulatory mechanism between E-cadherin expression and the remodelling of its oligosaccharides structures by N-

acetylglucosaminyltransferase III and V (GnT-III and GnT-V). We have demonstrated that human E-cadherin regulates the *MGAT3* gene transcription resulting in increased GnT-III expression. In turn, GnT-III has glycosylated E-cadherin adding bisecting GlcNAc structures. Therefore a functional feedback loop between E-cadherin and GnT-III was proposed. Moreover, GnT-III knockdown resulted in a disruption of cell-cell adhesion with a membranar de-localization of E-cadherin leading to its internalization to the cytoplasm. Concomitantly with this, E-cadherin suffered a remodelling of its N-glycans structures with an increase of β 1,6 GlcNAc branched structures and a decrease of bisecting GlcNAc N-glycan structures. These results have clarified the existence of a bidirectional crosstalk between E-cadherin and GnT-III/GnT-V that was, for the first time demonstrated in a human sporadic diffuse gastric carcinoma model. These last results presented in this thesis opened new insights into the post-translational modifications on E-cadherin regulation in a tumour context that set the ground for future perspectives.

RESUMO

As alterações da glicosilação são uma característica marcante do cancro. Um crescendo de evidências tem vindo a atribuir um papel crucial aos carboidratos nas diversas etapas patofisiológicas da progressão tumoral. O significado biológico da glicosilação aberrante associada ao cancro é um tema em debate no qual a compreensão da função dos carboidratos se apresentará crucial para novas terapêuticas do cancro. É assim imperativo compreender a regulação fisiológica dos carboidratos no cancro.

Os tumores humanos e caninos partilham uma ampla variedade de características. Assim, os tumores de mama de canídeos são considerados um modelo valioso para o estudo comparativo do cancro de mama da mulher, em particular, e também uma ferramenta preciosa para o estudo do cancro em geral.

Tem sido sugerido que a glicosilação aberrante afecta profundamente vários mecanismos celulares, incluindo a adesão célula-célula. Neste contexto, a E-caderina é uma das mais importantes moléculas envolvidas na adesão intercelular tendo assim um papel central no cancro. Além disso, sendo uma glicoproteína, a E-caderina pode ser pós-translacionalmente modificada por N-glicosilação. Contudo, até ao início dos trabalhos que conduziram à presente dissertação pouco se sabia sobre o papel e a função da estrutura dos N-glicanos nas funções da E-caderina, nomeadamente na capacidade de mediação da progressão tumoral.

Em tumores mamários de canídeos, demonstramos que a expressão aberrante do carboidrato Sialyl Lewis x (sLe^x) foi considerada um marcador do fenótipo maligno tendo também um papel importante na metastização linfática das células tumorais. Desta forma concluímos que o sLe^x poderá ser utilizado como um marcador de prognóstico em carcinomas mamários de canídeos. Além disso, propusemos ainda a existência de uma plasticidade molecular entre a expressão do sLe^x e da E-caderina durante o processo de desenvolvimento tumoral e progressão metastática.

Caracterizamos, pela primeira vez, o perfil de N-glicosilação da E-caderina em fenótipos tumorais malignos e não-malignos. Concluímos que a E-caderina sofreu uma extensa modificação na composição dos seus N-glicanos durante a aquisição do fenótipo maligno, caracterizada por um aumento de estruturas β 1,6 GlcNAc branched, um aumento da sialilação e uma expressão de algumas estruturas high-mannose. Para além disso, identificamos ainda um novo sítio de N-glicosilação na E-caderina de um modelo celular de carcinoma mamário, o qual parece conter uma N-glicoforma do tipo β 1, 6 GlcNAc branched.

Com o objectivo de melhor compreender as funções críticas dos N-glicanos na mediação

da progressão tumoral pela E-caderina, decidimos explorar o mecanismo regulador entre a expressão da E-caderina e a remodelação das suas estruturas de oligossacáridos pelas enzimas N-acetilglucosaminiltransferases III e V (GnT-III e GnT-V). Demonstramos que a E-caderina humana regula a transcrição do gene *MGAT3* resultando assim no aumento da expressão da enzima GnT-III. Por sua vez, a GnT-III glicosilou a E-caderina adicionando estruturas bisecting GlcNAc. Desta forma, propusemos a existência de um “feedback loop” funcional entre a E-caderina e a GnT-III. Além disso, o silenciamento da GnT-III resultou numa ruptura da adesão célula-célula acompanhada por uma deslocalização membranar da E-caderina que conduziu à sua internalização para o citoplasma. Concomitantemente, a E-caderina sofreu uma remodelação dos seus N-glicanos caracterizada por um aumento de estruturas β 1,6 GlcNAc branched e uma diminuição de estruturas de N-glicanos bisecting GlcNAc. Estes resultados revelaram a existência de um “bidirectional crosstalk” entre a E-caderina e GnT-III/GnT-V que foi, pela primeira vez demonstrada num modelo humano de carcinoma gástrico difuso esporádico. Em suma, estes últimos resultados apresentados nesta tese abrem novas hipóteses sobre as modificações pós-tradução na regulação da E-caderina num contexto tumoral, definindo assim o terreno para perspectivas futuras.

RÉSUMÉ

Les changements dans la glycosylation sont souvent une des caractéristiques du cancer. Un nombre croissant de preuves appuie fondamental rôles des glycanes à différentes étapes physiopathologiques de la progression tumorale. Toutefois, la signification biologique par cette aberrante glycosylation dans le cancer, constitue un long débat et compréhension du rôle des glycanes, ouvre la voie pour le traitement du cancer. Il est donc impérative explorer la régulation physiologique de glycanes dans le cancer.

Les tumeurs formés naturellement dans l'espèce humaine et canine, et part une grande variété de fonctions. Le cancer mammaire canine a été considéré un précieux modèle spontané comparative pour l'étude en particulier du cancer du sein de femme et également un précieuse outil pour l'étude du cancer en général.

Il a été suggéré que l'aberrant glycosylation influe profondément sur plusieurs mécanismes cellulaires, y compris l'adhérence cellule-cellule. Au même niveau, E-cadhérine est une des plus importantes molécules de adhésion cellules-cellules, jouent un rôle central dans le cancer. Ajouté, être une glycoprotéine, E-cadhérine peut être post-translational modifié par le N-glycosylation. Néanmoins jusqu'au début du présent travail, très peu a été connu sur le rôle et la fonction des structures des N-glycanes sur la fonctionnalité de l'E-cadhérine, en particulier sur la progression tumorale.

Nous avons trouvé que l'expression aberrante du carbohydrate Sialyl Lewis x (sLe^x) a été considéré un marqueur du phénotype maligne et peut également jouer un rôle important dans la metastatization lymphatique des cellules cancéreuses. Par conséquent, nous avons conclu que sLe^x peut-être utilisé un marqueur pronostique de tumeurs dans les carcinomes mammaires canines.

En outre, nous avons proposé l'existence d'une plasticité moléculaire entre sLe^x et l'expression de l'E-cadhérine au cours du processus de développement de la tumeur et la progression métastatique.

Nous avons caractérisé pour la première fois le profil de la N-glycosylation de l'E-cadhérine dans les tumeurs phénotypes malignes et non malignes. Nous avons conclu que l'E-cadhérine a subi un extensif modification de la composition de N-glycanes au cours de la transformation au phénotype maligne, qui a été caractérisée par une augmentation des structures ramifiés de β 1,6 GlcNAc, une augmentation de sialylation et l'expression un peu élevé de structures mannoses.

En outre, nous avons identifié un nouveau N-glycosylation site dans l'E-cadhérine à partir d'une modèle de lignée cellulaire de carcinome mammaire, qui contient une potentiel forme de N-glycanes β 1,6 GlcNAc ramifié.

Dans une tentative de mieux explorer les fonctions essentielles de la N-glycanes sur l'E-cadhérine, nous avons exploré le mécanisme de régulation entre la expression de l'E-cadhérine et le réaménagement de ses oligosaccharides structures pour le N-acétylglucosaminyltrasférase III et V (GnT-III et GnT-V).

Nous avons démontré que l'E-cadhérine humaine règle la transcription du gène de *MGAT3* entraînant une augmentation d'expression de GnT-III. À son tour, GnT-III a glycosylée E-cadhérine ajoutant structures bisecting GlcNAc. Par conséquent une fonctionnelle boucle de rétroaction entre E-cadhérine et GnT-III a été proposée.

D'autre part, GnT-III knockdown entraîné dans une perturbation de l'adhérence cellule-cellule avec une délocalisation de la membrane de l'E-cadhérine qui conduit à son internalisation au cytoplasme. Parallèlement à cela E-cadhérine a subi un remodelage de ses structures N-glycanes avec une augmentation des structures ramifiés β 1,6 GlcNAc et une diminution des structures bisecting GlcNAc N-glycanes.

Ces résultats ont permis de clarifier l'existence d'un dialogue bidirectionnel entre l'E-cadhérine et GnT-III/GnT-V qui a été pour la première fois reproduit dans un modèle humaine de cancer gastrique sporadique diffuse.

Ces derniers résultats présentés dans cette thèse ouvert de nouvelles perspectives dans les modifications post-tranlationnels sur la E-cadhérine réglementation dans un contexte de tumeurs que fixe les perspectives d'avenir.

CONTENTS

Chapter 1	General Introduction	21
	Cellular Glycosylation	21
	N-glycosylation	23
	Glycoprotein Folding and Quality Control	26
	Glycosylation and Cancer	26
	Altered Glycosylation in Cancer: Sialylated glycans	27
	Altered Glycosylation in Cancer: branched N-linked glycans	29
	E-cadherin	33
	E-cadherin and cancer	35
	E-cadherin Post-Translational Modifications: <i>N-Glycosylation</i>	37
	Canine malignant mammary tumours	38
	References	41
	Rationale and Aims	53
Chapter 2	Aberrant Sialyl Lewis x expression and relationship with E-cadherin expression in canine mammary carcinoma model	57
	Paper I: <i>BMC Cancer</i> 2007	59
Chapter 3	Molecular plasticity of E-cadherin and Sialyl Lewis x expression	71
	Paper II: <i>PLoS ONE</i> 2009	73
Chapter 4	E-cadherin N-glycosylation profile in tumour phenotypes	81
	Paper III: <i>Biochem. Biophys. Res. Commun.</i> 2009	83
Chapter 5	Role of N-acetylglucosaminyltransferase III and V on E-cadherin Post-Translational Modifications	91
	Paper IV: <i>Human Mol. Genet</i> 2009	93
Chapter 6	General Discussion	107
	Biological role of Sialyl Lewis x antigen in canine mammary carcinoma model and its relationship with E-cadherin adhesion molecule.	108
	Molecular plasticity between Sialyl Lewis x and E-cadherin	110
	E-cadherin N-glycosylation profile in the acquisition of the malignant phenotype	111

E-cadherin Post-Translational Modifications: a potential mechanism in cancer	114
References	119
Summary and Concluding Remarks	125

Chapter 1

General Introduction
Rationale and Aims

GENERAL INTRODUCTION

Cellular Glycosylation

All cells in nature are covered with a dense and complex coat of glycans (Varki 2006). Glycans are one of the four fundamental components of cells (together with nucleic acids, proteins and lipids) and may also be the most abundant and diverse of nature's biopolymers (Marth and Grewal 2008; Ohtsubo and Marth 2006). Glycosylation is defined as the enzymatic process that produces glycosidic linkages of saccharides to other saccharides, proteins and lipids. The resulting *glycome* (biological repertoire of glycan structures), particularly mammalian *glycome* repertoire is estimated to be between hundreds and thousands of glycans structures containing a significant amount of biological information (Ohtsubo and Marth 2006). In fact, glycans have been found to be involved in a myriad of important biological functions such as molecular recognition, inter- and intracellular signaling, embryonic development, fertilization, immune defense, inflammation, cell adhesion and division processes, viral replication and parasitic infections (Varki 1993). Furthermore, glycan chains can significantly alter protein conformation and may consequently modulate the functional activity of a protein. Therefore, glycoprotein-glycans represent a valuable tool for fine-tuning and modulation of protein structure and function, thereby "intervening in the social life of cells" (Helenius and Aebi 2004; Varki 1993).

Glycosylation represents the most pronounced and complex form of protein post-translational modification. It is estimated that over 50% of all proteins are glycosylated (Apweiler et al. 1999). The structural diversity of oligosaccharides found in glycoproteins is enormous, since their biosynthesis is a non-template-driven process, with no proofreading machinery, and involves the coordinated action of an endogenous portfolio of cellular enzymes and substrates (Helenius and Aebi 2001; Petrescu et al. 2006). It is important to mention that the magnitude of the genomic commitment involved in the glycosylation process accounts for 1% to 2% of the translated genome of vertebrates (Ohtsubo and Marth 2006). This clearly reveals the impact and the importance of this process, such as glycosylation in eukaryotic biology. Some questions are now prone to arise: Why do cells need such an elaborate and costly system? Why are so many proteins in the eukaryotic cell glycosylated? It is the challenge of deciphering the purposes of glycobiology that now draws many researchers into the field of glicobiology and it also, in a certain way, one of the purposes of the present thesis.

Glycosylation is prominent in the lumen of the endoplasmic reticulum (ER) and in the Golgi apparatus, where the glycans become increasingly oligomeric and branched. The cellular repertoire of glycans (glycome) is produced predominantly in the secretory pathway of the cell and reflects the combinatorial expression of subsets of glycosyltransferases and glycosidases enzymes, of which there are more than 200 in the mammalian genome (Marth and Grewal 2008).

Glycosylation produces several main families of glycoconjugates (defined as a molecule in which one or more glycan units are covalently linked to a non-carbohydrate entity): the *N*-glycans; *O*-glycans; Glycosaminoglycans; Glycolipids (Glycosphingolipids); Glycosylphosphatidylinositol (GPI)-linked proteins (Figure 1).

Protein glycosylation encompasses *N*-glycans, *O*-glycans and Glycosaminoglycans (frequently termed proteoglycans). *N*-glycans are linked to asparagine residues of proteins, in a consensus sequence Asn-X-Ser/Thr (sometimes also Cys), where X is any amino acid excluding proline (Mirgorodskaya et al. 2000; Satomi et al. 2004; Vance et al. 1997). *O*-glycans are attached to a subset of serines or threonines residues of a protein. One of the most prevalent form is the so-called mucin-type *O*-glycosylation (Schachter 2000; Yan and Lennarz 2005). Although Glycosaminoglycans are also linked to serine and threonine, they are linear-repeating polymers and are often highly sulfated (Esko and Selleck 2002).

Lipid glycosylation in the secretory pathway is also a prevalent modification that includes Glycolipids, which consist of the lipid ceramid linked to one or more sugars. Sialic-acid-containing glycosphingolipids termed gangliosides (Maccioni et al. 2002). Finally, Glycosylphosphatidylinositol (GPI)-linked proteins, share a common membrane-bound glycolipid linkage structure that is attached to various proteins (Kinoshita et al. 1997).

It is also important to mention that some forms of glycosylation can occur outside of the secretory pathway, which is the example of *O*-linked GlcNAc (Figure 1). In fact various cytoplasmic and nuclear proteins contain *O*-linked N-acetylglucosamine (*O*-GlcNAc) (Hart 1997; Wells et al. 2001).

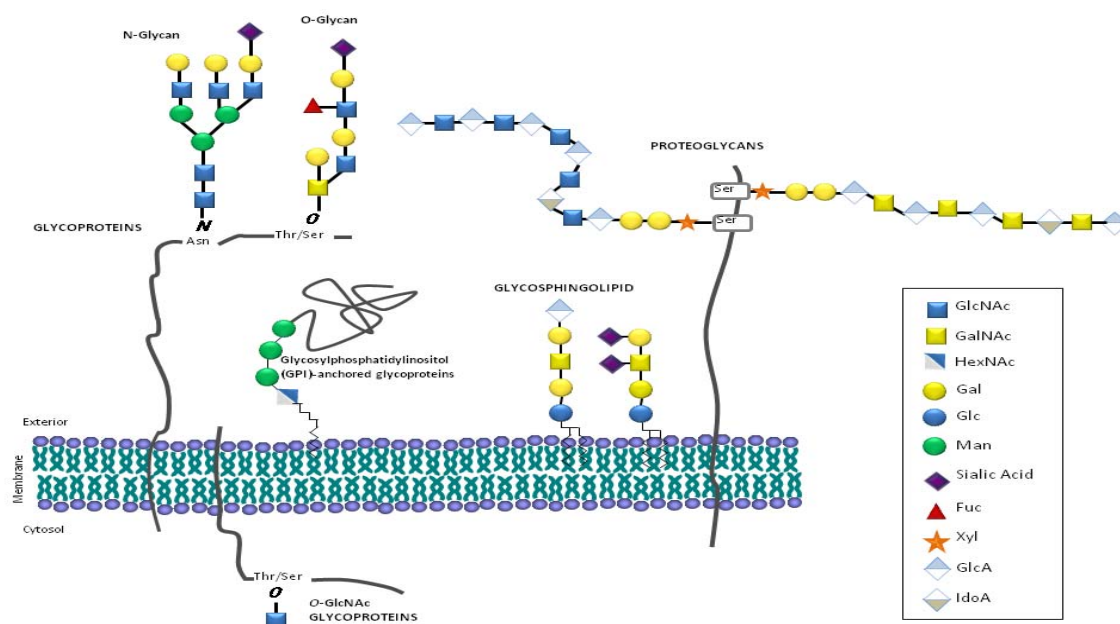


FIGURE 1. The most important families of glycoconjugates.

N-glycosylation

N-linked glycosylation has evolved into the most common protein post-translational modification in eukaryotic cells and it is a major subject addressed in the present work.

Apweiler et al, has predicted that more than half of all eukaryotic protein species are glycoproteins and about 90% of these are likely to carry N-linked glycans, with an average of 1,9 N-linked glycans per polypeptide chain (Apweiler et al. 1999). Moreover, recent statistical analysis reported that the count of N-glycosylated proteins reported so far is 883 compared with 188 for O-glycosylated proteins. Furthermore, of these 188 proteins, 104 contain both forms of glycosylation (Mitra et al. 2006). These data cover the N-glycosylation process with utmost importance in eukaryotic biology.

N-glycosylation is initiated by an *en bloc* transfer of a preassembled core oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) to the side chain nitrogen of the Asn residue in a tripeptide sequon (Asn-X-Ser/Thr) on a nascent protein in the lumen of the ER (Figure 2) (Kornfeld and Kornfeld 1985).

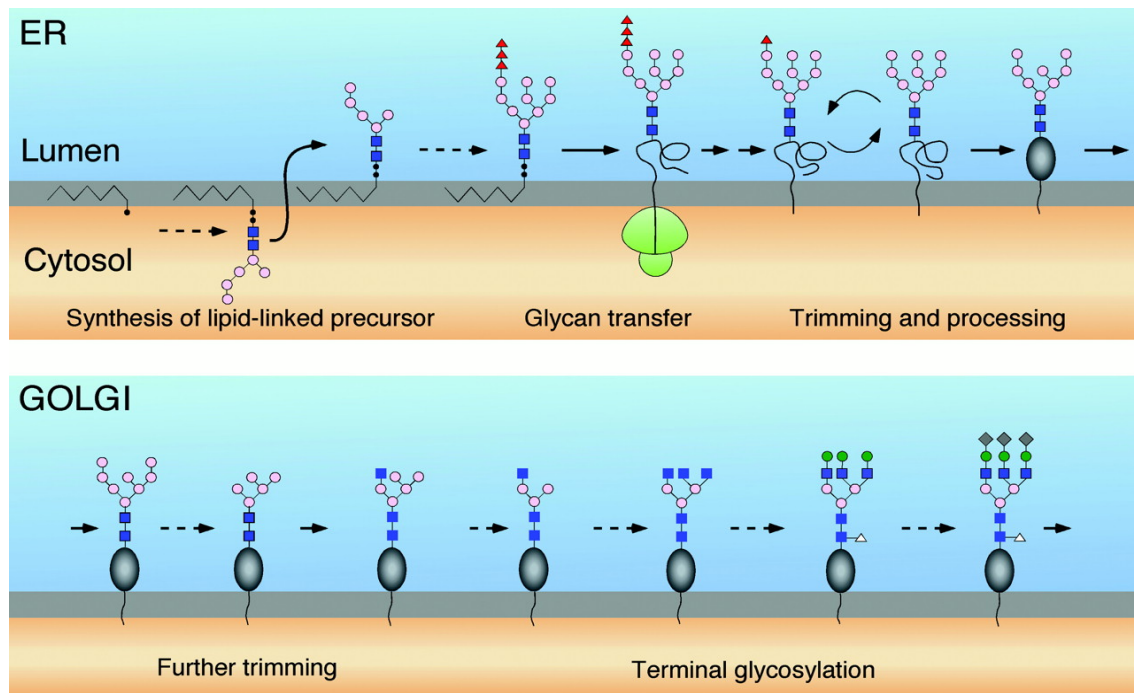


FIGURE 2. Biosynthesis of N-linked glycans. (Helenius and Aeibi 2001)

N-glycosylation can be viewed as four distinct phases (Figure 2), broadly associated with different compartments of the secretory pathway: 1) transfer of a preassembled $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide from a dolichol-linked donor to nascent glycoproteins in the lumen of the rough ER; 2) trimming by glycosidases in the rough ER and *cis*-Golgi; 3) substitution by N-acetylglucosaminyltransferases in the medial-Golgi; and 4) elongation in the *trans*-Golgi network to complete the glycan structures (Dennis et al. 1999).

All *N*-linked oligosaccharides, in the early secretory pathway, share a common core structure and therefore have a common role in promoting protein folding, quality control and certain sorting events. Later, when the glycoprotein travels from the ER to the Golgi, the glycan chains undergo enzymatic degradation and rebuilding (Figure 2) leading ultimately to a wide diversity of structures which differ extensively between species. Thus, the switch from structural uniformity in the ER to diversification in the Golgi coincides with a marked change in glycan function (Helenius and Aeibi 2001; Varki et al. 2009).

Inside the Golgi, a very well organized subsequent addition of sugars to different positions of the extending glycan is catalyzed by a panoply of glycosyltransferases, each adds a particular monosaccharides through a specific glycosidic bond in a step-wise manner (Hossler et al. 2007; Helenius and Aeibi 2004). The resulting *N*-glycan structures are generally classified in three principal categories: (1) High mannose, in which only mannose residues are attached to the core; (2) Complex, in which “antennae” are

attached to the core by *N*-acetylglucosaminyltransferases; and (3) Hybrid types, in which only mannose residues are attached to the Man α 1-6 arm of the core and one or two antennae are on the Man α 1-3 arm (Varki et al. 2009). An example of each *N*-glycan type is given in Figure 3.

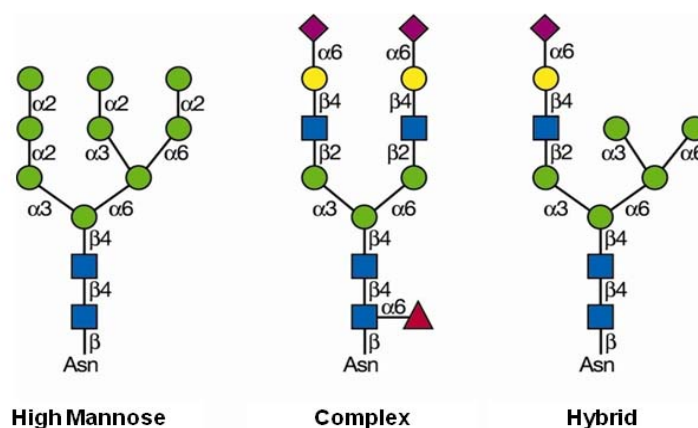


FIGURE 3. Types of *N*-linked glycans.

The processed high-mannose serves as a substrate for the diversification of *N*-glycans in the Golgi, resulting into a large repertoire of hybrid and complex *N*-glycans. As the *N*-glycan transits through the *medial*- and *trans*- Golgi, it becomes available to a wide variety of glycosyltransferases (Varki et al. 2009).

Therefore, varied expression levels of glycosyltransferases can affect the repertoire of *N*-glycan structures produced in a given cell type and in a given tissue. This diversity of *N*-glycan structures on each glycosylation site that occurs during the glycan processing in the Golgi, is referred to as microheterogeneity. It appears that factors other than protein sequence may influence *N*-glycan diversification. Such factors may include sugar nucleotide metabolism, transport rates in the ER and Golgi, and the localization of glycosyltransferases in the “assembly line” model of their action in the Golgi which can determine which enzymes encounter glycan substrate first. On the other hand, the variability in occupancy of the tripeptide sequon with the oligosaccharide is called macroheterogeneity (Hossler et al. 2007; Jones et al. 2005; Varki et al. 2009).

Overall, the number of *N*-glycans or multiplicity (*n*) is an encoded feature defined by the protein sequence that differs markedly between different surface glycoproteins. Whereas, the *N*-glycan structures are determining by the Golgi *N*-glycan processing pathway and metabolite supply to sugar nucleotide pools (Mendelsohn cancer res 2007).

Glycoprotein Folding and Quality Control

Protein folding occurs in the ER, which is known to have a primary quality control function by ensuring the fidelity and proper native conformation of proteins that traverse this compartment. The most important indirect effect of glycans on folding involves a unique chaperone system found in ER of nearly all eukaryotes, the so-called calnexin-calreticulin cycle (Helenius and Aebi 2001; Parodi 2000; Wang and Ng 2008). Glycans serve as an “admission ticket” to this cycle. Calnexin (membrane-bound) and calreticulin (soluble) are homologous ER lectins that bind transiently to virtually all newly synthesized glycoproteins (Hammond et al. 1994; Ou et al. 1993). Briefly, the calnexin-calreticulin system consists in a deglycosylation-reglycosylation cycle that ensures the proper folding and quality control of newly synthesized glycoproteins. Once correctly folded, the glycoprotein leave the ER to the Golgi complex where it will be further processed. Proteins that fail to reach their native conformation in the ER are selectively eliminated by ER-associated degradation (ERAD) (Klausner and Sitia 1990; Wang and Ng 2008). This fate is shared by misfolded and mutant proteins, by orphan subunits of oligomers, and by some heterologously expressed proteins. Thus, ERAD has a central clearance function in the cell.

Glycosylation and Cancer

Altered Glycosylation is a universal feature of cancer cells. Aberrant glycosylation expressed in glycoproteins in tumour cells has been implicated as an essential mechanism in defining stage and fate of tumour progression. In fact, in last decades a wide variety of studies have been suggested that aberrant glycosylation is a result of initial oncogenic transformation (being a result of cancer) as well as a key event in promoting tumour cell invasion and metastases (being a cause of cancer progression) (for review, (Hakomori 1996; Hakomori 2002). Nevertheless, the biological significance for this aberrant glycosylation in cancer is far to be fully understood.

One of most common type of aberrant glycosylation observed in cancer is an increase in the size and branching of *N*-linked glycans, due to increase enzymatic activity of *N*-acetylglucosaminyltransferase V (GnT-V) which catalyzes the synthesis of β 1,6 GlcNAc branching. Moreover, an increase in global sialylation has also been associated with cancer. Examples of other glycan epitopes commonly found on transformed cells include Sialyl Lewis x/a (sLe^{x/a}), Sialyl Tn (STn), Lewis y (Le^y) and polysialic acid (PSA) (Sell 1990; Taylor-Papadimitriou and Epenetos 1994). Another common feature of tumours is

the overproduction of certain glycoproteins which is the case of Mucin glycoprotein, characterized by dense clusters of *O*-linked glycans (Hollingsworth and Swanson 2004). However, little is known about the relevant glycoproteins or how these transformation-related changes in oligosaccharides biosynthesis may affect the malignant phenotype. Numerous clinicopathological studies have shown a clear correlation between aberrant glycosylation status of the primary tumours and the survival rates of patients (reviewed in (Hakomori 1996)). Herewith, some glycans on the surface of tumour cells can be measured in the bloodstream (serum) being some of them currently used to help diagnosis, track tumour recurrence or tumour burden or provide a surrogate measure for therapeutic response. Examples of these serological markers whose serum levels correlate with tumour burden and prognosis are: CA125 (tumour antigen associated with MUC16, in ovarian cancer); CA19-9 (associated with sLe^a in pancreatic cancer, gastric and colorectal carcinomas); CA15-3 (codified by MUC1, associated with breast cancer) and CA72-4 (which detects STn) in gastrointestinal cancers (reviewed in (Dube and Bertozzi 2005; Fuster and Esko 2005)).

Additionally, and based on the fact that cancer cell glycans differ from those found on their healthy counterparts, some interesting and promising studies have made an effort to generate vaccines, using glycans as novel therapeutic targets. A promising example, in Phase III clinical trials, is Theratope (Biomira) that is a STn-KLH conjugated vaccine. This vaccine tested in metastatic breast cancer patients is prepared by conjugating the STn glycan to a carrier protein such as Keyhole limpet Haemocyanin (KLH) in order to enhance antitumor immune response (Holmberg et al. 2003; Ibrahim and Murray 2003).

Altered Glycosylation in Cancer: Sialylated glycans

A family of important molecules related to aberrant glycosylation associated with malignant transformation is sialic acids. Sialic acids are typically found as terminal monosaccharides attached to cell surface glycoproteins and glycolipids. Usually, sialyl residues are linked via α 2,6 or α 2,3-linkage to galactosamine (Gal) or to N-acetylgalactosamine (GalNAc) via α 2,6-linkage. These sialylations are catalyzed by sialyltransferases (STs) which according to the carbohydrate linkages can be further classified as ST3Gal (α 2,3-ST); ST6Gal (α 2,6-ST) and ST6GalNAc (Takashima et al. 1999; Harduin-Lepers et al. 2005; Marcos et al. 2004). Sialic acids can also exist as α 2,8-linked homopolymers known as polysialic acid.

Sialylation plays roles in a variety of biological processes such as cell-cell communication, cell-matrix interaction, adhesion and protein targeting during the process of tumour development, differentiation and progression (Halliday et al. 2001; Kim et al. 1996).

The most widely reported alterations of sialylated-antigens associated with cancer, are those concerning Sialyl-Tn, Sialyl Lewis a and Sialyl Lewis x (Dabelsteen 1996).

Sialyl-Tn is a simple mucin-type carbohydrate antigen that is overexpressed in several epithelial cancers, such as human gastric (Baldus and Hanisch 2000; David et al. 1992; Victorzon et al. 1996), pancreatic (Kim et al. 2002), colorectal (Itzkowitz et al. 1990), ovarian (Kobayashi et al. 1992) and breast cancers (Leivonen et al. 2001; Yonezawa et al. 1992) and is usually associated with poor prognosis (Julien et al. 2006) and more aggressive cancer cell behaviour (Pinho et al. 2007).

Sialyl Lewis a (sLe^a) and Sialyl Lewis x (sLe^x) have been demonstrated to be highly expressed in many malignant cancers. They are involved in selectin-mediated adhesion of cancer cells to vascular endothelium, and these determinants are thought to be closely associated with hematogenous metastasis of cancers (Kannagi et al. 2004).

Sialyl Lewis a (sLe^a) has been demonstrated to be overexpressed predominantly in colon, pancreas and biliary tract cancer, playing therefore a role in hematogeneous metastases and consequently is associated with poor prognosis of those cancers (Ugorski and Laskowska 2002).

Sialyl Lewis x (sLe^x) is one of the sialylated carbohydrate antigen that is studied in the present work. The sLe^x (Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GInAc β 1-R) carbohydrate antigen is normally expressed on the surface of human leukocytes (neutrophils, monocytes and some T-lymphocytes) and is involved in the attachment of leukocytes to cytokine-activated endothelial cells through its interaction with the E-selectin cell adhesion molecule, leading to leukocyte extravasation and migration into the tissue, during inflammation (Munro et al. 1992). Furthermore, the expression of sLe^x antigen is also increased in several human carcinomas. The involvement of sLe^x antigen in tumour malignancy and progression occurs in a process that resembles those of inflammatory response. Thus and likewise inflammation, sLe^x, expressed at the surface of tumour cells, binds directly to E-selectin (expressed at the surface of cytokine-activated endothelial cells) which ultimately culminates with the extravasation of tumour cells to form new tumour foci. Therefore, sLe^x is closely associated with hematogeneous metastases of cancers, predominantly those from breast, ovarian, lung, bladder, gastric and colon. Additionally, the degree of sLe^x expression has been demonstrated to be correlated with shorter patient survival.

In this manner, sLe^x could be considered one of the key factors involved in metastatic spread of cancer.

The increase expression of sLe^x carbohydrate determinant in solid tumours is known to be due to two main mechanisms: “incomplete synthesis” of a pre-existing form of sLe^x, sialyl 6-sulfo Lewis x, found in normal tissues. The synthesis of this complex carbohydrate determinant (sialyl 6-sulfo Lewis x) tends to be impaired upon malignant transformation, leading therefore to the accumulation of sLe^x in cancer cells; and “neosynthesis” which results from the induction of some glycosyltransferases expression (sialyltransferases and fucosyltransferases) upon malignant transformation, that are involved in the *de novo* synthesis of sLe^x determinant (Dube and Bertozzi 2005; Kannagi 2004).

Tumour invasion and metastases is a multistep process that involves complex interactions between the tumour cells and the microenvironment (Laidler and Litynska 1997). The terminal step of this sequential process is colonization of the tumour cells at distant sites in the body. Tumour cells which show the ability to metastasize should therefore exhibit characteristic features differing not only from normal, but also from the tumour cells pre-existing within the primary lesion. Glycans are involved at every stage of this complex process of tumour progression (Fuster and Esko 2005). Enhanced sialylated glycans expression (an acidic sugar that imparts a negative charge to the glycan chain) might promote cell detachment from the tumour mass through charge repulsion, which physically inhibits cell-cell interactions (Alpaugh et al. 2002). In fact, few studies have reported that the presence of sialic acid weakened the strength of cell-cell adhesion and removal of cell surface sialic acid by sialidase increases the adhesiveness between cells (Acheson et al. 1991; Deman et al. 1974; Ligtenberg et al. 1992). However, the molecular mechanism between sialic acids and cell-cell adhesion in a tumour context is not yet completely understood and will be assessed in this thesis.

Altered Glycosylation in Cancer: branched *N*-linked glycans

β1,6 GlcNAc branched *N*-linked glycans

It is well recognized that another common change in glycosylation associated with cancer is an increase in the size and branching of *N*-linked glycans. This increased branching has been shown to be due to increased activity of *N*-acetylglucosaminyltransferase V (GnT-V or Mgat5), which catalyzes the synthesis of β1,6 GlcNAc branched *N*-glycans structures

(Dennis et al. 1987). In fact, enhanced β 1,6 GlcNAc branching of *N*-linked structures, caused by enhanced activity of GnT-V, is perhaps the most widely occurring glycosylation change inducing malignancy (Hakomori 2002; Pierce et al. 1997).

Actually, GnT-V, in the *medial/trans* Golgi, catalyzes the addition of a β 1,6-linked GlcNAc to the α 1,6-linked Mannose of the trimannosyl core of *N*-linked glycans to form tri- or tetraantennary branches (Figure 4) (Brockhausen et al. 1988a; Cummings et al. 1982; Brockhausen et al. 1988b). This branch provides the preferred substrate for the enzymatic subsequent synthesis of polylactosamine chains (van den Eijnden et al. 1988) and their terminal modifications including the Lewis antigens.

Several studies have been linked increased GnT-V activity with augmented cancer cell invasiveness and tumour progression (Fernandes et al. 1991; Ito et al. 2001; Yamamoto et al. 2000; Yao et al. 1998). GnT-V expression is transcriptionally regulated by oncogenes, such as *ras* (Yamashita et al. 1984), *src* (Buckhaults et al. 1997) and *her2/neu* (Chen et al. 1998), through the Ets pathway (Kang et al. 1996).

GnT-V activity is also highly associated with metastatic potential. This was clearly demonstrated by the observation that *MGAT5* knockout mice lacking the GnT-V activity show reduced tumour growth and have a 20-fold decrease in metastasis to the lungs (Granovsky et al. 2000). Furthermore, Partridge et al. reported that GnT-V-modified *N*-glycans with poly-N-acetyllactosamine, the favorite ligand for galectin-3, on surface receptors oppose their constitutive endocytosis and result in promoting intracellular signalling and consequently cell migration and tumour metastasis (Partridge et al. 2004). Moreover, a secreted type of GnT-V was also reported to be able to induce tumour angiogenesis without mediation of glycosylation, acting as an angiogenic cofactor of Fibroblast Growth Factor-2, giving, therefore, to GnT-V a bifunctional role in both tumour metastases and tumour angiogenesis (Saito et al. 2002). Additionally, it has been shown that the forced expression of GnT-V in epithelial cells results in a loss of contact inhibition, increased cell motility, and morphological transformation in culture (Demetriou et al. 1995). Furthermore, swainsonine, an inhibitor of *N*-glycan processing which blocks the pathway prior to the initiation of the β 1,6 branching, also inhibits organ colonization by MDAY-D2 cells and B16 melanoma cells (Dennis 1986; Humphries et al. 1986).

High expression of GnT-V and its *N*-glycan product, β 1,6 GlcNAc branched structure, was observed in some types of human cancers and correlate with disease progression.

The plant lectin leucoagglutinin (L-PHA) exhibits high specificity for β 1,6 branching on *N*-glycans and, therefore, has been used as a probe of the presence of an active GnT-V producing β 1,6 branched *N*-glycans. Many studies have demonstrated the association of increased L-PHA binding and GnT-V activity with increased tumour cell invasiveness.

Cancers of breast (Fernandes et al. 1991; Handerson et al. 2005; Siddiqui et al. 2005), colon (Seelentag et al. 1998), liver (Guo et al. 2001; Yao et al. 1998) and also melanomas (Przybylo et al. 2008) and gliomas (Yamamoto et al. 2000) consistently exhibited an increase of β 1,6 branched structures expression, as a result of increased GnT-V activity, that was correlated with poor patient outcome.

Alteration of the β 1,6 branched *N*-glycans on the cell surfaces has been implicated in the modulation of cell-cell adhesion and migration which were closely correlated to the tumour metastatic potential. In fact, Pierce and co-workers reported that GnT-V expression levels modulate N-cadherin-associated homotypic cell-cell adhesion. They showed that induction of GnT-V expression caused increased levels of N-linked β 1,6 branched structures with a concomitant decrease in the rates of homophilic cell-cell adhesion mediated by N-cadherin, as well as a stimulation of cell migration (Guo et al. 2003).

The GnT-V modification also affects other molecules that are important in modulating tumour adhesion, including the integrin family of adhesion receptors. Integrins represent a particularly important class of cell-surface adhesion receptors that mediate attachment to important Extracellular Matrix (ECM) protein ligands, such as fibronectin and laminin. Increased GnT-V expression results in increased β 1,6 branching on the β 1 subunit of tumour α 5 β 1 integrins, and this disrupt the ability of integrins to cluster on the tumour-cell membrane, which in turn increases tumour motility and invasiveness through the ECM (Guo et al. 2002). In spite of the existence of a strong association between GnT-V and its product, β 1,6 GlcNAc branched structures, in induction of malignancy and metastases, the exact molecular mechanism underlying this regulatory effect on tumour progression and metastasis, by GnT-V and β 1,6 branched structures, is not yet fully understood.

Bisecting GlcNAc structure of N-linked glycans

Contrary to the function of GnT-V, the N-acetylglucosaminyltransferase III (GnT-III) enzyme has been proposed as a functional antagonistic of GnT-V, thereby contributing to the suppression of cancer metastases.

GnT-III, a product of *MGAT3* gene, is a key glycosyltransferase in *N*-glycan biosynthetic pathway. GnT-III catalyzes the addition of GlcNAc via β 1,4 linkage to the β -mannose of the mannosyl core of *N*-glycans, and thereby alters not only the composition, but also the conformation of the *N*-glycan (Stanley 2002; Narasimhan 1982). This bisecting GlcNAc structure, catalyzed by GnT-III, preclude the action of other glycosyltransferases such as GnT-V, which is no longer able to act on the biantennary sugar chain and thus prevent the formation of β 1,6 branched structure (Brisson and Carver 1983; Gu et al. 1993; Schachter

1986) (Figure 4). The enzymatic competition between GnT-III and GnT-V, where GnT-III has been shown to exhibit a priority activity, contributes to the suppression of cancer metastasis (Zhao et al. 2006).

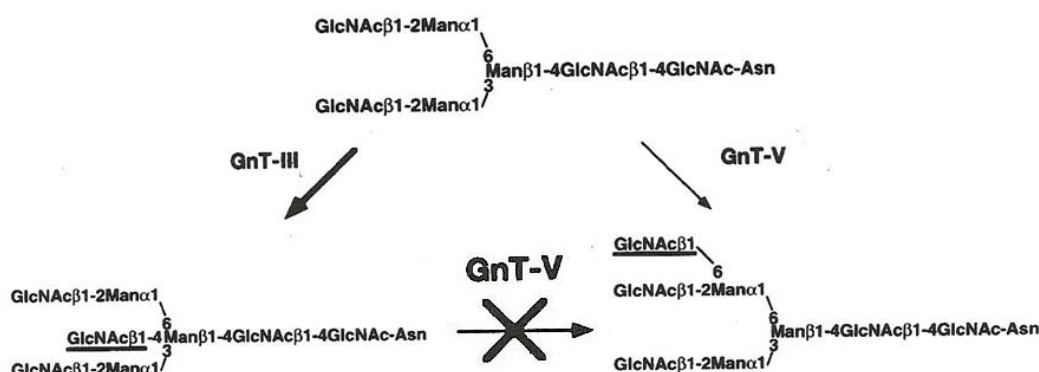


FIGURE 4. Reactions catalyzed by GnT-III and GnT-V. GnT-V fail to catalyze the transfer of GlcNAc to form β1,6 branch in the presence of bisecting GlcNAc. (Taniguchi et al. 1998)

In fact, overexpression of GnT-III in highly metastatic B16 melanoma cells reduced β1,6 GlcNAc branching in cell-surface N-glycans and increased bisected N-glycans, which ultimately result in a significant decrease of metastatic potential in experimental models of lung metastasis (Yoshimura et al. 1995). Additionally, and in order to examine whether the reduced metastatic potential of B16 melanoma cells expressing GnT-III was due to the altered biological function of E-cadherin-mediated cell-cell adhesion (since it acts as a suppressor of metastases), the same group (Yoshimura et al. 1996) have demonstrated that cells expressing GnT-III showed an enhanced cell-cell adhesion through the prolonged turnover of E-cadherin on the cell surface. These studies suggested that glycosylated E-cadherin by GnT-III seems to participate in the suppression of metastases of melanoma cells.

Furthermore, it was demonstrated that the addition of bisecting GlcNAc residues to E-cadherin down-regulates the tyrosine phosphorylation of β-catenin through EGFR. Therefore, β-catenin remains on the cell surface enhancing the homophilic interaction of E-cadherin which contributes to the suppression of cancer metastases (Kitada et al. 2001).

The role of GnT-III as a metastases suppressor was also demonstrated due to alteration of the function of integrins. Interestingly, the overexpression of GnT-III resulted in inhibition of $\alpha 5\beta 1$ -integrin-mediated cell spreading and migration. The affinity of the binding of $\alpha 5\beta 1$ -integrin to fibronectin was significantly reduced as a result of the introduction of a bisecting GlcNAc to the $\alpha 5$ subunit (Isaji et al. 2004). Thus, GnT-III has a role in suppression of tumour metastases through at least two mechanisms: enhancement of cell-cell adhesion and down-regulation of cell-ECM adhesion.

In summary, GnT-V displays a pro-metastatic effect, whereas GnT-III has an anti-metastatic effect. However, the molecular mechanism underlying these effects on cancer metastases and how they are regulated still remain to be fully understood.

E-cadherin

E-cadherin is a calcium-dependent cell-cell adhesion molecule, localized to the adherens junctions on the basolateral surface of the cell, playing pivotal roles in epithelial cell adhesion, tissue formation, and suppression of cancer.

The mature E-cadherin molecule, weighing approximately 120 kDa, contains a single transmembrane domain, a cytoplasmic domain (C-terminal) of about 150 aminoacids, and an ectodomain of about 550 aminoacids comprising five tandemly repeated domains (EC1 to EC5) (Figure 5). The extracellular N-terminal end (EC1) is essential to the process of homophilic calcium-dependent cell-cell adhesion. The presence of a tripeptide sequence Histidine-Alanine-Valine (HAV) within EC1 was described to be essential for the process of homophilic cell-cell adhesion (Blaschuk et al. 1990). The extracellular calcium is also essential for cadherin adhesive function, for protection against protease digestion and for ensures cadherin's proper folding (Hyafil et al. 1981; Yoshida and Takeichi 1982).

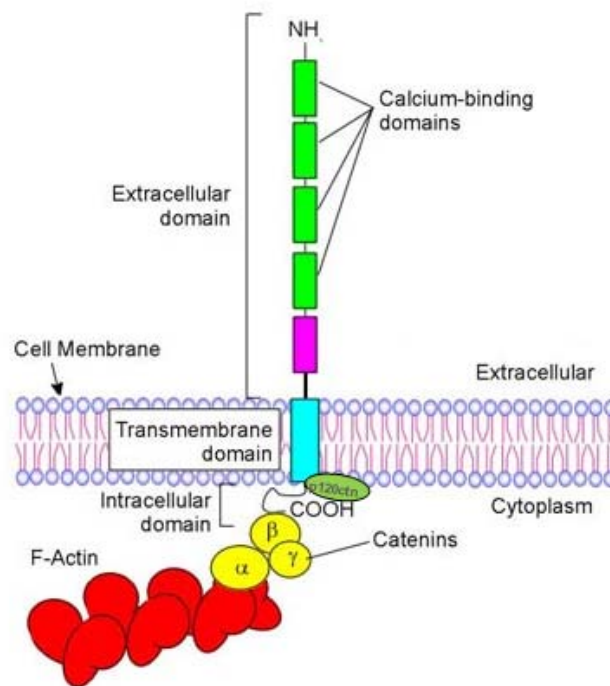


FIGURE 5. Structure of E-cadherin and its interaction with cytoskeleton.

Currently, it is quite established that the cadherins mediated adhesion occurs by first associating with cadherins on the same cell surface to form lateral or *cis* dimmers. These in turn adhere to dimmers on an adjacent cell to form *trans* adhesive bonds (Brieher et al. 1996; Chen et al. 2005; Leckband and Prakasam 2006). Binding between cadherin extracellular domains is weak, but strong cell-cell adhesion develops during lateral clustering of cadherins and through changes in the organization of the actin cytoskeleton (Chen et al. 2005; Nelson 2008).

The clustering into the junctional structure is also facilitated and mediated by interaction between E-cadherin cytoplasmic domain and catenins (Figure 5). β -catenin and plakoglobin interact directly with a core region of 30 aminoacids within the C terminus of the cadherin cytoplasmic domain, in the so-called catenin-binding domain (Jou et al. 1995). The N-terminal portion of both β -catenin and plakoglobin interacts with α -catenin, which links the cadherin to the cytoskeleton. Another catenin, p120-catenin, interacts with the highly conserved juxtamembrane domain of cadherins, and this binding between p120-catenin and E-cadherin is important to prevent the entrance of E-cadherin into degrading endocytic membrane pathways. Therefore, p120-catenin is perhaps the best characterized inhibitor of cadherin endocytosis by inhibiting clathrin-mediated endocytosis

(Miyashita and Ozawa 2007; Xiao et al. 2005), thereby stabilizing E-cadherin at the cell surface.

Until recently, α -catenin was believed to bind both β -catenin and actin, serving as the direct linkage molecule between the adhesion complex and the actin cytoskeleton. However a critical study recently showed that, although α -catenin associates with β -catenin and actin individually, it does not bind to them simultaneously (Yamada et al. 2005).

The authors confirmed the binding of α -catenin to actin filaments, but only for α -catenin homodimers; on the other hand, α -catenin could efficiently bind to E-cadherin/ β -catenin complex, but only in its monomeric form (Drees et al. 2005; Yamada et al. 2005). Very recently, it was reported that EPLIN is the “missing” link that connects the E-cadherin/ β -catenin/monomeric α -catenin to F-Actin. Abe and Takeichi demonstrated that EPLIN, which localizes to the apical cortical actin cytoskeleton in epithelial cells, binds to a C-terminal domain of monomeric α -catenin making therefore the linking to the apical actin belt (Abe and Takeichi 2008). Additionally, it was further reported that α -catenin helps transform an initial cell-cell contact consisting of just one bond into a nascent junction by mediating the formation of multiple bonds between cells without apparent input from the actin cytoskeleton (Bajpai et al. 2008).

Like other integral membrane proteins, newly-synthesized E-cadherin is packaged in the Golgi, transported to the cell surface and eventually internalized and degraded. Basal levels of E-cadherin internalization would be expected to support their metabolic turnover and perhaps contribute to local remodelling of cell-cell contacts. It is further feasible that cadherin endocytosis may be regulated by activating different uptake mechanisms, although they remain to be defined.

E-cadherin and cancer

The broad-ranging of effects of E-cadherin on physiological tissue organization make cadherins attractive targets during tumorigenesis, the disruption of which might contribute to the aberrant morphogenetic effects in cancer. Indeed, it is now clear that classical cadherin dysfunction is a major contributor to cancer development and progression (Birchmeier and Behrens 1994; Thiery 2002). Because the majority of solid tumours are carcinomas, the major target is the prototypical epithelial cadherin, E-cadherin.

The well recognized role of E-cadherin in tumour progression is based, briefly, in three major lines of evidence: 1) the pathological demonstration that advanced cancers often

have abnormal E-cadherin expression. Tumour progression correlates with loss of overall E-cadherin expression or loss of its normal localization at cell-cell contacts (Birchmeier and Behrens 1994; Yap 1998); 2) Evidence from both cellular and animal models that cadherin dysfunction promotes tumour progression to invasion and metastases (Vleminckx et al. 1991); 3) The identification of both somatic and germline E-cadherin mutations in a number of cancers (Berx et al. 1998).

It has been described in the literature that E-cadherin dysfunction associated with tumour progression can occur through several mechanisms including mutation of E-cadherin gene *CDH1*, epigenetic silencing (promoter hypermethylation) and transcriptional silencing through a variety of transcriptional factors that target the E-cadherin promoter. Somatic mutations have been most thoroughly characterized in woman lobular breast cancer, but have also been identified in other tumours (Berx et al. 1996). Characteristically, these mutations are accompanied by loss of heterozygosity (LOH) of the remaining E-cadherin allele, and correlate with tumour progression toward an invasive and metastatic phenotype. E-cadherin-inactivating mutations were first described in diffuse gastric cancer (Becker et al. 1993). In sporadic diffuse gastric cancer, somatic mutations preferentially cause skipping of exons 7 and 9, which corresponds to inframe deletions. In addition several truncation mutations have also been reported for the sporadic diffuse gastric carcinoma type (Becker et al. 1994). Germline mutations have also been reported in a number of families with diffuse gastric carcinoma. In fact Guilford et al., in 1998 has described for the first time *CDH1*-inactivating mutations in three Maori families with early-onset diffuse gastric cancer (Guilford et al. 1998). Since then, 68 different families carrying germline *CDH1* mutations have been identified worldwide (Carneiro et al. 2008), being also already described in Portuguese families (Oliveira et al. 2005). It has been calculated that approximately 30-40% of Hereditary Diffuse Gastric Cancer families harbour *CDH1* germline mutations as reviewed by Oliveira et al. (Oliveira et al. 2003; Oliveira et al. 2006).

However, mutation of the coding sequence, probably accounts for only a minority of cases of E-cadherin dysfunction in cancer. Promoter hypermethylation has also been identified as an important epigenetic event associated with the loss of E-cadherin gene expression during cancer progression. A large CpG island in the 5'proximal promoter region of the E-cadherin gene shows aberrant DNA methylation in at least eight different human carcinoma types and correlates with reduced E-cadherin protein expression (Machado et al. 2001; Yoshiura et al. 1995; van and Berx 2008).

Another common mechanism of E-cadherin down-regulation associated with tumour progression are the transcriptional repressors of E-cadherin (Thiery 2002). These include transcriptional repressors of the Snail/slugg family that are overexpressed in advanced carcinomas (reviewed in (Moreno-Bueno et al. 2008); and the transcriptional repressors SIP1 and ZEB1. Interestingly, strong expression of SIP1/ZEB2 is associated with loss of E-cadherin expression in gastric cancer of the intestinal type. In contrast, Snail is the transcriptional repressor that is up-regulated in diffuse gastric cancer sub-type (Rosivatz et al. 2002).

Overall, these several lines of evidence demonstrate that E-cadherin function is perturbed in many epithelial carcinomas, and this E-cadherin dysfunction promotes cancer progression to invasion and metastasis.

However, there are several different types of invasive cancers where E-cadherin dysfunction exist but whose dysfunction is not explained, at all, by any of the above mentioned mechanisms already described to underlie E-cadherin down-regulation. This question, that remains an open issue, is also one of the subjects of the present work. In order to address this interesting question, it can be hypothesized that other mechanisms operating at the post-translational level of E-cadherin may exist and therefore, justify by itself the E-cadherin dysfunction in those carcinomas without E-cadherin genomic alterations. Thus, the present work focuses on E-cadherin biology/functionality and its post-translational modification, *N*-glycosylation.

E-cadherin Post-Translational Modifications: *N*-Glycosylation

E-cadherin can be post-translational modified by phosphorylation, *O*-glycosylation and *N*-glycosylation. These post-translational modifications have been described to have an effect on E-cadherin functionality. Casein Kinase II, a serine-threonine kinase, phosphorylates the E-cadherin cytoplasmic domain increasing E-cadherin/ β -catenin interaction and ultimately interfering on the strength of cell-cell adhesion (Lickert et al. 2000). Moreover, cytoplasmic *O*-glycosylation (*O*-GlcNAc) of newly synthesized E-cadherin was described to block its cell surface transport, resulting in reduced intercellular adhesion (Zhu et al. 2001). Another study, has reported that E-cadherin was core fucosylated in highly metastatic lung cancer cells and this could impair E-cadherin mediated cell adhesion (Geng et al. 2004).

In addition, E-cadherin can be *N*-glycosylated. The extracellular domain of E-cadherin has three potential *N*-glycosylation sites at Asn residues 554, 618 and 633 (on the basis of

amino acid sequence). These three *N*-glycosylation sites are located, one in EC4 and two in EC5, and these sites are shared by human, canine and mouse E-cadherin. Human and canine can have an additional putative site, each in different parts of the extracellular region. Those *N*-glycans represent the most prominent modification of E-cadherin molecule, contributing up to 20% of its total mass.

In spite of this prominent modification of E-cadherin by *N*-glycosylation, little is known about the role and the function of those *N*-glycan structures on E-cadherin functionality, namely on E-cadherin mediated tumour progression.

Nevertheless, very recently it was described that, in fact, *N*-glycans seem to be essential for E-cadherin expression, folding and trafficking (Zhou et al. 2008). Furthermore, other recent studies have reported that *N*-glycans may affect the stability and the adhesive functions of E-cadherin (Liwosz et al. 2006; Zhao et al. 2008). In addition, the remodeling of E-cadherin *N*-glycans catalyzed by GnT-III and GnT-V was also demonstrated to have a potential role on E-cadherin mediated cell-cell adhesion, as described above.

In summary, it is known that almost 50% of the translated proteins are further glycosylated. As a result, proteins functions cannot be predicted simply by genome sequences. This indicates that much information is hidden in human and canine bodies, and cannot be revealed in terms of genome research. Therefore, it is clear that sugar chains contain a lot of information that extends beyond the genome and so it is essential to study glycans for understanding the structures and functions of proteins.

Hence, the role of post-translational modifications of E-cadherin by *N*-glycosylation in a tumour context is not fully defined so far, being therefore explored in this thesis.

Canine malignant mammary tumours

Mammary gland tumours represent the second most frequent neoplasia of canine species, after skin tumours. They are the most frequent tumours of female dogs accounting for 52% of the diagnosed tumours, although there appears to be some decline in its incidence due to the increase in the practice of ovariohysterectomy at an early age (Sorenmo 2003; Rutteman et al. 2001). The incidence of mammary tumours in the female dog is about three times higher than the incidence of woman breast cancer in the same geographical area (Owen 1979). This type of tumour is more prevalent in older female dogs of approximately 9 to 11 years old, sexually intact, or spayed later in life (Sorenmo 2003). Mammary tumours are rare in male dogs, as it happened in human breast cancer.

The exactly aetiology of canine mammary tumours is not fully defined. However, most attention has been paid to the steroids hormones and their influence on tumourigenesis. Studies have reported that the duration of exposure to ovarian hormones early in life determines the overall mammary cancer risk. Thus, the risk of developing mammary gland tumours increases from 0.5% to 8%, and to 26%, depending on whether the ovariohysterectomy is performed before the first, second, or any estrus thereafter, respectively (Schneider et al. 1969). Moreover, progestagens treatment also increases the risk of tumour development (Stovring et al. 1997). In addition to the hormonal influence on the risk for developing mammary gland tumours, there are also other factors, such as genetic predisposition and diet. It has been documented that certain breeds are at increased risk of developing mammary gland tumours accordingly to geographic location (reviewed in (Sorenmo 2003), which can also suggest a genetic component. In fact, Haga et al. has reported an overexpression of p53 gene product in canine mammary gland tumors (Haga et al. 2001). In addition, the oncogene *c-erbB2* has also been found to be overexpressed in the majority of canine malignant mammary gland tumours evaluated, and mutations in BRCA1 have also been documented in a few cases (Ahern et al. 1996; Ochiai et al. 2001). Germline mutations in BRCA1 and BRCA2 are known to be involved in breast cancer cases in women, and they may also be involved in the selected cases of mammary gland tumours in dogs (Martin and Weber 2000).

More recently, it was also described an association between E-cadherin expression and known factors of poor prognosis, which suggested that the loss of E-cadherin expression may have prognostic value in canine malignant mammary tumours (Brunetti et al. 2003; Matos et al. 2006).

Though canine mammary gland tumours may be benign or malignant, approximately 40% to 50% of these tumours are malignant. Accordingly to the World Health Organization International Histological Classification of Mammary Tumours of the dog and cat (Misdorp et al. 2001), most mammary gland tumours are of epithelial origin. Some, however, can have cellular mixed pattern consisting of both epithelial and myoepithelial tissue, with areas of cartilage and bone, and a few tumours are of purely mesenchymal origin.

All malignant canine mammary tumours have the potential to metastasize. In general canine malignant tumours metastasise via the lymphatics to the regional lymph nodes or hematogenously to the lungs that represent the most common site of distant metastases (Sorenmo 2003).

Taking into consideration all of these data from the literature, it seems clear that human and canine mammary tumours share a wide variety of epidemiological, biological and

clinical features and therefore canine mammary tumours has been considered a valuable model to study woman breast cancer. In fact, naturally occurring cancers in pet dogs and humans share many features, including histological appearance, tumour genetics, molecular targets, biological behaviour and response to conventional therapies (Munson and Moresco 2007; Paoloni and Khanna 2008). Studying dogs with cancer is likely to provide a valuable perspective that is distinct from that generated by study of human or rodent cancers alone or even by study *in vitro* models. Mammary cancer occurs among all taxonomic groups, and comparing the disease in animals with breast cancer in women could greatly improve our understanding of the molecular biology underlying the process of mammary tumourigenesis, and perhaps most importantly, the evaluation and development of novel cancer therapeutics that will benefit both human and his best friend the dog.

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RATIONALE AND AIMS

Glycosylation represents the most pronounced and most complex form of protein post-translational modification. Glycans have been found to participate in a myriad of cellular biological processes, contributing therefore to most interactions between the cell and its environment. It has long been evaluated that glycan expression changes with cellular condition and thereby changes in glycosylation are considered a hallmark of cancer state. A growing body of evidence supports crucial roles for glycans at various pathophysiological steps during tumour progression, such as tumour-cell dissociation and invasion and tumour-cell adhesion and metastases. However the critical functions of glycans in cancer biology are not fully defined, so far. It is therefore important to understand the physiological regulation of glycans in cancer science.

Naturally occurring cancers in canine and human species share a wide variety of features. Specifically mammary cancer is considered one of the major causes of death among women and female dogs, and comparing the disease in animals with breast cancer in women could greatly improve our understanding of the molecular biology underlying the process of mammary tumourigenesis. Therefore, canine mammary cancer has been considered a valuable spontaneous comparative model for study woman breast cancer in particular, and also a precious tool for study cancer in general.

Changes in interactions between adhesion molecules are thought to be crucial for tumour invasion and metastases. E-cadherin is a cell-cell adhesion molecule and is a well characterized tumour suppressor protein. It is now clear that E-cadherin dysfunction is a major contributor to cancer development/genesis and progression. Being a glycoprotein, E-cadherin can be post-translational modified by *N*-glycosylation. At the beginning of this project, little was known about the role of *N*-glycans on E-cadherin-mediated tumour progression.

The general aim of this work is to evaluate the role and the mechanism of aberrant glycosylation in cancer, emphasizing the role of the modulation of the *N*-glycans on E-cadherin functionality in a tumour context.

Specific aims

1. To analyse the expression of a specific carbohydrate determinant and its relationship with E-cadherin expression, in a model of canine malignant mammary tumour

Sialyl Lewis x (sLe^x) is a carbohydrate antigen that is considered not only a marker for cancer but also implicated functionally in the malignant behaviour of cancer cells. sLe^x, expressed *de novo* in cancer cells, is involved in selectin-mediated adhesion of cancer cells to vascular endothelium and this determinant is thought to be closely associated with hematogenous metastases of cancer. Consistently, E-cadherin down-regulation is also involved in malignant phenotype and tumour cell invasion and metastases. Therefore, we first aimed to evaluate the expression of sLe^x in a model of canine malignant mammary tumour in relation with clinicopathological features. We also explored the relationship between the expression of the two adhesion molecules, sLe^x and E-cadherin, both of them previously known to be involved in malignant progression. Chapter 2 presents this study.

2. To assess the existence of a molecular plasticity between E-cadherin and sLe^x expression throughout metastatic progression in two comparative mammary tumour models

The process of metastasis involves a series of steps and interactions between the tumour cells and the microenvironment. Key alterations in adhesion molecules are known to dictate progression from the invasive to malignant phenotype followed by colonization at a distant site.

In chapter 3, we delve into the results obtained from Aim 1, by exploring the existence of a coordinated mechanism between E-cadherin and sLe^x expression during tumour progression, using two comparative models of mammary tumourigenesis: canine malignant mammary tumours and woman inflammatory breast cancer model.

3. To characterize the N-glycosylation modifications of E-cadherin during transformation to the malignant phenotype

Modifications in protein glycosylation are often observed in disease states. In cancer, cells frequently display glycans with fundamentally different structures than those observed on the normal counterparts and on benign cells.

E-cadherin dysfunction is known to have a major role on cancer progression. Being a glycoprotein, E-cadherin can be *N*-glycosylated.

In chapter 4 we aimed to characterize the glycosylation profile of E-cadherin in two phenotypes, benign and malignant, and thus evaluate the importance of E-cadherin *N*-glycans in the process of tumour development and in the acquisition of the malignant phenotype.

4. To explore the regulatory mechanism between E-cadherin expression and the remodeling of its oligosaccharides structures by specific glycosyltransferases

E-cadherin dysfunction is a major cause of epithelial cell invasion. In spite of a wide variety of studies focusing on the epi/genetic regulation on the E-cadherin-switch-off during tumourigenesis, the Post-Translational Modifications of E-cadherin and its role in E-cadherin mediated tumour progression remain largely unknown.

N-acetylglucosaminyltransferase III and V (GnT-III and GnT-V) are two of the most important glycosyltransferases involved in *N*-glycans biosynthesis. These enzymes and its products have also been shown to have a role on cancer metastases, where GnT-III is considered a metastases suppressor whereas GnT-V has a role in induction of metastases.

Having concluded that in fact E-cadherin undergoes *N*-glycan modifications associated with malignancy (chapter 4) and taking into consideration recent studies reporting the importance of *N*-glycans on E-cadherin functions, in chapter 5 we aimed to elucidate the role of GnT-III and GnT-V glycosyltransferases and its products in E-cadherin expression, on a tumour context.

Chapter 2

Aberrant Sialyl Lewis x expression and relationship with E-cadherin expression in canine mammary carcinoma model

Paper I

Sialyl Lewis x expression in canine malignant mammary tumours: correlation with clinicopathological features and E-cadherin expression

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Research article

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Sialyl Lewis x expression in canine malignant mammary tumours: correlation with clinicopathological features and E-Cadherin expression

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Abstract

Background: Sialyl Lewis x (sLe^x) antigen is a carbohydrate antigen that is considered not only a marker for cancer but also implicated functionally in the malignant behaviour of cancer cells. Overexpression of sLe^x is associated with enhanced progression and metastases of many types of cancer including those of the mammary gland. Canine mammary tumours can invade and give rise to metastases via either lymphatic or blood vessels.

E-Cadherin is specifically involved in epithelial cell-to-cell adhesion. In cancer, E-Cadherin underexpression is one of the alterations that characterizes the invasive phenotype and is considered an invasion/tumour suppressor gene. Partial or complete loss of E-Cadherin expression correlates with poor prognosis in canine malignant mammary cancer.

The aim of this study was to analyse the sLe^x expression in canine malignant mammary tumours and to evaluate if the presence of sLe^x correlates with the expression of E-Cadherin and with clinicopathological features.

Methods: Fifty-three cases of canine mammary carcinomas were analysed immunohistochemically using monoclonal antibodies against sLe^x (IgM) and E-Cadherin (IgG). The clinicopathological data were then assessed to determine whether there was a correlation with sLe^x tumour expression. Double labelled immunofluorescence staining was performed to analyse the combined expression of sLe^x and E-Cadherin.

Results: sLe^x expression was consistently demonstrated in all cases of canine mammary carcinomas with different levels of expression. We found a significant relationship between the levels of sLe^x expression and the presence of lymph node metastases. We also demonstrated that when E-Cadherin expression was increased sLe^x was reduced and vice-versa. The combined analysis of both adhesion molecules revealed an inverse relationship.

Conclusion: In the present study we demonstrate the importance of sLe^x in the malignant phenotype of canine malignant mammary tumours. Our results support the use of sLe^x as a prognostic tumour marker in canine mammary carcinomas. Furthermore, we showed that sLe^x and E-Cadherin expression were inversely correlated. Future studies are warranted to clarify the molecular mechanism underlying the relation between sLe^x and E-Cadherin in canine mammary carcinoma cells which represents an important comparative model to woman breast cancer.

Background

Mammary tumours are the most common tumours in intact female dogs and approximately 40% to 50% of these tumours are malignant [1]. All malignant canine mammary tumours have the potential to metastasise. In general canine malignant tumours metastasise via the lymphatics to the regional lymph nodes or hematogenously to the lungs that represent the most common site of distant metastases. [1-3]

Malignant transformation is associated with abnormal glycosylation, resulting in expression of altered carbohydrate determinants, such as the Sialyl Lewis x (sLe^x) antigen. Altered cell surface glycosylation is a prominent feature of malignant tumour cells and define their invasive and/or metastatic properties in general [4-12].

Tumour metastasis is a multistep process requiring detachment of malignant cells from the primary tumour, invasion of blood or lymph vessels, interaction with endothelium, extravasation at distant sites and formation of new tumour foci [9,12,13]. It is generally accepted that every step of the metastatic cascade is dependent on specific adhesive interactions of cancer cells with other cells and components of the extracellular matrix. These interactions are mediated by different families of adhesion molecules including cadherins, integrins, members of the immunoglobulin superfamily, and selectins and their carbohydrate ligands – Sialyl Lewis a (sLe^a) and sLe^x [9,13,14].

sLe^x is a tetrasaccharide (NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4[Fuc α 1 $\rightarrow$ 3]GlcNAc β 1 $\rightarrow$ R) that is particularly relevant from a biological standpoint. It is involved in selectin-mediated adhesion of cancer cells to vascular endothelium and this determinant is thought to be closely associated with hematogenous metastases of cancer [12-17]

In humans, the expression of sLe^x is significantly increased in carcinoma cells [4,7,18]. Many clinical studies have shown an association between the expression of sLe^x on tumours and enhanced tumour progression and metastasis [7,19]. In woman breast carcinoma the presence of sLe^x was also correlated with poor prognosis [20,21]. In fact, the presence of sLe^x has been used as a prognostic tumour marker in various types of human cancer [7,19], e.g. lung [22], bladder [8], breast [20,21,23], prostate [24], colon [25] and gastric [26-28] carcinoma. Little is known about the expression of sLe^x in canine tumours. To the best of our knowledge only the study of Nakagawa et al describe the expression of sLe^x in canine and feline mammary gland tumours [29], but no significant correlation between the expression of sLe^x and prognosis has been described in canine or feline tumours.

sLe^x and E-cadherin are two adhesion molecules that seem to be involved in malignant progression with opposite roles [31]. Alpaugh et al have described a cooperative role between E-cadherin and sLe^x in the passive dissemination of tumour emboli and in the genesis of the lymphovascular embolus of woman's Inflammatory Breast Carcinoma [30-32].

Recently, Jeschke et al identified a negative correlation between Sialyl Lewis antigens and E-cadherin expression in woman breast cancer and their lymph node metastases [23]. This combined analysis of tumour antigens involved in adhesion of breast cancer cells has never been described in canine mammary tumours, which constitutes an important comparative model for woman breast cancer.

The purpose of the present study is to analyse, by immunohistochemical staining methods, the expression of the carbohydrate sLe^x in canine malignant mammary tumours and to evaluate the relationship between sLe^x expression and tumour clinicopathological features. The relation between the expression of the molecules sLe^x and E-Cadherin [33] in canine malignant mammary tumours was also investigated.

Methods

Tissue specimens

Fifty-three malignant mammary tumours and 102 local and regional lymph nodes were surgically removed from 35 female dogs aged from 3 to 16 (mean, 9.9 years), of various pure or mixed breeds. The specimens were fixed in 10% neutral buffered formalin. After dehydration and paraffin wax embedment, sections of 4 μ m were cut from each representative paraffin blocks for staining with haematoxylin and eosin (HE) and for sLe^x and E-cadherin immunohistochemistry (IHC).

Lymph nodes were classified according to the presence of cancer cells (positive or negative), using HE and cytokeratin IHC methods [33].

Histological examination of the tumours

Tumours, including benign proliferative lesions found in the vicinity of the tumours, were classified independently by two observers from HE-stained sections on the basis of the diagnostic criteria of the World Health Organization classification of tumours in domestic animals [34].

The presence of intra-tumoral necrosis was registered for each case and evaluation and classification of the mode of tumour growth was assessed as previously described [33].

Follow-up data

All animals were clinically evaluated every 3 months (by physical examination, thoracic radiography and abdomi-

nal ultrasound) for the presence of distant metastases during a follow-up period of 2 years after surgery. All dogs were followed until death or until the end of the observation period. In all dogs that died with suspected distant metastases, complete necropsies were performed and histological confirmation was obtained.

Immunohistochemistry

Canine mammary cancer tissues, which had been resected by the curative operation were routinely processed and used for immunostaining of sLe^x and E-cadherin.

Immunostaining was performed by the modified avidin-biotin-peroxidase complex (ABC) method [35]. Two distinct monoclonal antibodies (mAbs) were used: the mAb FH6 generated against sLe^x epitope [36] and the monoclonal mouse anti-human E-cadherin antibody (clone 4A2C7, Zymed, S.Francisco, California, USA) [33].

Tumour sections (4 µm thick) were deparaffinized in xylene, dehydrated through graded concentrations of ethanol and washed with distilled water. Sections were then treated with Citrate buffer (sodium citrate antigen retrieval solution; 10 mM citric acid, pH = 6) for 20 minutes in a microwave oven at 600 W. The slides were cooled for 10 minutes at room temperature and rinsed twice in Phosphate buffered saline (PBS) for 5 minutes. Endogenous peroxidase activity was blocked by treating the section with hydrogen peroxide 3% in methanol for 10 min. After washing the slides in PBS, non-specific staining was eliminated by incubating the sections with normal rabbit serum (Dako) diluted at 1:5 in PBS containing bovine serum albumin (BSA) 10%, in a humid chamber for 20 min at room temperature. Excess normal serum was removed and replaced by the anti-sLe^x mAb FH6 diluted at 1:5. After overnight incubation (≈18 hours) at 4°C, slides were washed with PBS and incubated for 30 min with a 1:200 dilution of biotin-labelled rabbit anti-mouse secondary antibody (Dako). Sections were then washed with PBS and incubated for 30 min with avidin-biotin complex (Dako) diluted at 1:100. This was followed by staining the sections for 5 to 7 minutes with 0.05% 3,3 diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M Tris/hydroxymethylaminomethane buffer, pH 7.6, containing 0.1% hydrogen peroxide. Finally, sections were lightly counterstained with haematoxylin, dehydrated, and mounted.

Dilution of primary antibody, biotin-labelled secondary antibody, and avidin-biotin complex were made with PBS containing 5% BSA.

All series included, as positive control sections of a human mixed gastric carcinoma previously shown to display prominent expression of sLe^x.

Negative controls were performed by substitution of the primary antibody with immunoglobulins of the same subclass and concentration as the monoclonal antibody.

Scoring of immunostaining and statistical analysis

The degree of mAb FH6 reactivity with individual tissue sections was scored by percentage of stained carcinoma cells in the section by three authors (S.S.P., C.A.R., F.G.) without knowing patients outcome and clinicopathological features of the case. In the event of disagreement, slides were reviewed by the observers, and a consensus was obtained.

Expression of sLe^x and E-cadherin [33] in canine mammary carcinomas were classified in the following manner: negative, no immunoreactivity or immunostaining in very rare cells; less than 25% of cancer cells stained; 25–50%, well defined areas with positive cells; 50–75% of cancer cells stained; and more than 75% stained cells. Scoring of immunoreactivity was evaluated irrespectively of localization of positive cells and intensity of the staining.

For statistical analysis the expression of sLe^x was regrouped in two percentual categories (<25% and ≥25%) in order to increase the number of cases in each category and in this manner improve the statistical power of the tests.

The statistical relationship among variables was analysed using tables of frequencies and their significance tested by the Fisher's exact test [37]. P values less than 0.05 were considered a significant association.

Double-labelling Immunofluorescence

For simultaneous visualization of sLe^x and E-cadherin on the same tissue section, double-label immunofluorescence was performed. We chose the following representative tissue sections: sections with <25% sLe^x and >75% E-Cadherin expression; sections with >75% sLe^x and <25% E-Cadherin expression and sections with the same percentage of positive cells for both mAbs.

Paraffin sections were dewaxed, rehydrated and then treated with Extran (Merck, Frankfurt, Germany) 0.05% in distilled water for 10 min in a microwave oven at 750 W. After cooled for 20 min at room temperature, slides were rinsed twice in Phosphate-buffered saline (PBS) and then incubated for 20 min in a humid chamber with rabbit non-immune serum at a dilution 1:5 in PBS containing bovine serum albumin (BSA) 10%. Sections were incubated with the first primary mAb, anti-human E-Cadherin (clone 36, BD Biosciences Pharmingen, diluted 1:100 in PBS), overnight at 4°C. After washing twice for 5 min in PBS, sections were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (code F261; Dako, Glos-

trup, Denmark; diluted 1:100 in PBS). Sections were washed two times for 5 min in PBS and blocked with non-immune goat serum diluted 1:5 in PBS containing BSA 10%. Sections were then incubated with mAb FH6 (mouse IgM) diluted at 1:5, overnight at 4°C. Sections were washed two times for 5 min with PBS and incubated with Texas red-conjugated goat anti-mouse IgM (Jackson ImmunoResearch) diluted 1:50 in PBS. Sections were washed as before and nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI, Sigma) for 15 min in the dark, diluted at 1:100 in PBS. Sections were washed two times for 5 min in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, USA).

Dilutions of primary antibodies, secondary antibodies, and DAPI were made with PBS containing 5% BSA.

Microscopy and image processing

Immunostained slides were examined under a fluorescence microscope (Leica DMIRE2) equipped with appropriate filters. Separate images for DAPI, Texas-Red and FITC staining were captured digitally at $\times 200$ and $\times 400$ magnification. The red (for Texas-Red), blue (for DAPI), and green (for FITC) components were merged and composite images were imported into Adobe Photoshop 7.0®.

Results

Clinical and pathological features of mammary carcinoma patients

All dogs that were included in this study were female. The mean age at surgery was 9.9 years old (range: 3–16 years old). Histological diagnosis of canine mammary carcinomas consisted of 2 *in situ* carcinomas, 13 complex carcinomas, 13 tubulopapillary carcinomas, 9 solid carcinomas, 1 spindle cell carcinoma, 3 mucinous carcinomas, 10 carcinosarcomas and 2 carcinomas in benign tumour.

The mode of tumour growth was assessed in all of 53 tumours. Invasive tumours were more prevalent (28 cases, 52.8%) followed by expansive tumours (17 cases, 32.1%) and tumours with vessel invasion (8 cases, 15.1%).

Lymph node metastases were evaluated in 50 tumours (in 3 cases the regional lymph nodes were not submitted). The majority of the cases did not show lymph node metastases (39, 78.0%) whereas eleven patients (22.0%) revealed metastases in regional lymph nodes. The majority of the cases that showed lymph node metastases were classified as carcinosarcomas (6 out of 11). The others were classified as complex carcinoma, tubulopapillary carcinoma and solid carcinoma. From the eleven cases that revealed nodal metastases, 4 patients revealed in addition, distant metastases. Seven patients with lymph node metastases died (in 4 animals was performed eutha-

nasia because of the tumour and 3 animals died of other causes).

The presence of intra-tumoral necrosis was observed in thirty tumours (56.6%), whereas 23 (43.4%) tumours did not show this feature.

Of the 35 dogs that were included in this study, 33 completed the follow-up period and ten dogs (30.3%) developed distant metastases. From the 17 dogs that died at the end of the follow-up period, 52.9% died because of the mammary carcinoma and 47.0% died from other causes.

Expression of Sialyl Lewis x in canine mammary carcinomas

All of the carcinomas studied by immunohistochemistry showed expression of sLe^x antigen (Figure 1). Thirty two (60.4%) were found to express sLe^x in less than 25% of the cells, whereas 21 (39.6%) showed sLe^x expression in more than 25% of the cells. This distribution of sLe^x expression include the early stage cases of canine mammary carcinomas such as *in situ* carcinoma and carcinoma in benign tumour (Table 1). The staining was observed in the cytoplasm and/or in the cell membrane of epithelial cells. In tumour areas with cellular squamous differentiation, sLe^x was always expressed (Figure 2). In all cases, the adjacent normal mammary gland tissue and the adjacent benign proliferative lesions were evaluated and they did not showed expression of sLe^x. It was analysed a total of 43 adjacent benign proliferative lesions (6 simple adenomas, 8 epitheliomas and the others were hyperplasias in general). In proliferative lesions sLe^x was expressed only in mammary gland secretion.

Relationship between Sialyl Lewis x expression and clinicopathological features

Table 1 summarizes the expression of sLe^x according to clinicopathological features.

No significant relationship was found between the expression of sLe^x antigen and the different histological types of canine mammary carcinoma according to the World Health Organization classification of tumours in domestic animals [34].

Similarly, no significant relationship was observed between sLe^x expression and the growth pattern of mammary carcinomas.

A total of 63.6% of tumours with lymph node metastases showed significantly higher sLe^x expression ($\geq 25\%$), whereas most (74.4%) of the tumours without lymph node metastasis showed underexpression ($< 25\%$) of sLe^x ($p = 0.034$).

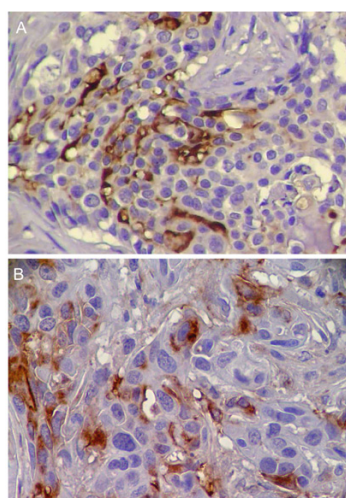


Figure 1
Immunohistochemical study of the expression of Sialyl Lewis x in canine malignant mammary tumours.
 A. Solid carcinoma; >75% of sLe^x expression; ×400 B. Carcinosarcoma; 25–50% of cells stained; ×400.

Although this association did not reach a statistical significance, we observed that tumours without necrosis showed less sLe^x expression than tumours with necrosis ($p = 0.07$).

No significant relationship was found between the expression of sLe^x antigen and distant metastases.

Relationship between Sialyl Lewis x and E-cadherin expression

The analysis of expression of sLe^x and E-cadherin disclosed an inverse correlation among the two molecules, higher sLe^x expression was accompanied with lower E-cadherin expression and vice-versa (Figure 3). When mammary carcinomas show underexpression of E-cadherin (<25%), all of them simultaneously revealed a higher sLe^x expression (≥25%). On the other hand when tumour samples showed overexpression of E-cadherin (>75%) the majority of them (93.1%) simultaneously revealed less than 50% of sLe^x expression. In summary, we found a significant relationship ($p = 0.013$) between sLe^x and E-cadherin expression in canine malignant mammary tumours (Table 2).

Table 1: Relationship between Sialyl Lewis x expression and clinicopathological features in canine malignant mammary tumours.

Clinical Features	Number of cases(%)	Sialyl Lewis x expression		P value
		<25%	≥25%	
Histological type (n = 53)				NS(0,075)
In situ carcinoma	2 (3.8%)	1 (50.0%)	1 (50.0%)	
Complex carcinoma	13 (24.5%)	12 (92.3%)	1 (7.7%)	
Tubulopapillary carcinoma	13 (24.5%)	7 (53.8%)	6 (46.2%)	
Solid carcinoma	9 (17.0%)	5 (55.6%)	4 (44.4%)	
Spindle cell carcinoma	1 (1.9%)	1 (100.0%)	0 (0.0%)	
Mucinous carcinoma	3 (5.7%)	1 (33.3%)	2 (66.7%)	
Carcinosarcoma	10 (18.9%)	4 (40.0%)	6 (60.0%)	
Carcinoma in benign tumour	2 (3.8%)	1 (50.0%)	1 (50.0%)	
Mode of Growth (n = 53)				NS(0,27)
Expansive	17 (32.1%)	13 (76.5%)	4 (23.5%)	
Invasive	28 (52.8%)	15 (53.6%)	13 (46.4%)	
Vessel invasion	8 (15.1%)	4 (50.0%)	4 (50.0%)	
Lymph node metastases (n = 50)*				0.034
No	39 (78.0%)	29 (74.4%)	10 (25.6%)	
Yes	11 (22.0%)	4 (36.4%)	7 (63.6%)	
Necrosis (n = 53)				NS(0,078)
Absent	23 (43.4%)	17 (73.9%)	6 (26.1%)	
Present	30 (56.6%)	15 (50.0%)	15 (50.0%)	
Distant Metastasis (n = 33)				NS(0,24)
No	23 (69.7%)	17 (73.9%)	6 (26.1%)	
Yes	10 (30.3%)	5 (50.0%)	5 (50.0%)	

* No lymph nodes were submitted with 3 tumours

NS – Not significant ($P > 0.05$)

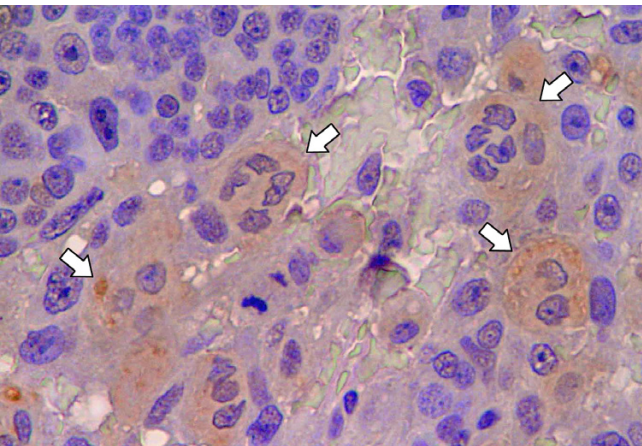


Figure 2
Immunohistochemical expression of Sialyl Lewis x in squamous metaplasia. All cells that exhibit squamous metaplasia (arrows) are positive for Sialyl Lewis x. $\times 400$

Simultaneous expression of sLe^x and E-cadherin was analysed using double-label immunofluorescence method (Figure 4), demonstrating the absence of overlapping between the two molecules (Figure 4). Based on these results, it seems that, when cells express sLe^x they do not express E-cadherin, and on the other hand when they are positive for E-cadherin they are negative for sLe^x.

Discussion

Malignant transformation of tumour cells is associated with abnormal glycosylation, resulting in expression of altered carbohydrate determinants including the expression of sLe^x and sLe^a antigens [4,9,12]. These carbohydrate antigens have been shown to be useful tumour markers in carcinomas of different organs [4,9,12]. The biosynthesis of these oligosaccharides are usually increased and altered during the acquisition of the malignant phenotype and tumour progression [5,12]. Some studies refer that aberrant glycosylation is a result of initial oncogenic transformation, as well as a key event in induction of invasion and metastases [10,12].

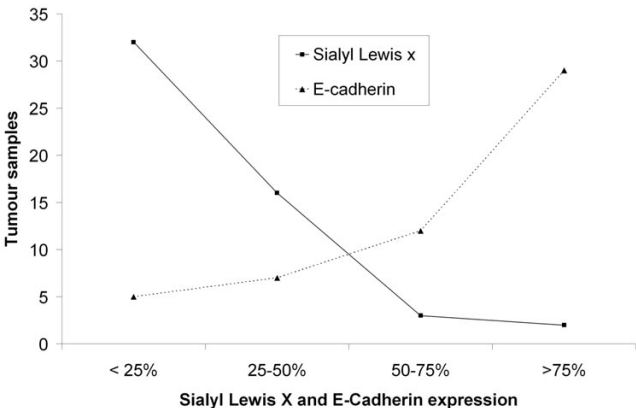


Figure 3
Relation between Sialyl Lewis x and E-cadherin expression. The figure illustrates the negative correlation between sLe^x and E-Cadherin expression in canine malignant mammary tumours. When expression of E-cadherin increase, the expression of sLe^x decrease and vice-versa.

Most human carcinomas show changes within cell surface carbohydrates compared to their normal counterparts [4,12].

sLe^x, present on the surface of tumour cells has been found to serve as ligands for endothelial E-selectin and was shown to play a major role in the process of adhesion of cancer cells to the endothelium [38]. sLe^x not only is a marker for cancer but also is functionally implicated in the malignant behaviour of cancer cells [4,12-14]. E-selectin, one of the selectin family member is expressed on the vascular endothelial cells and adheres to a carbohydrate ligand, sLe^x [15,16]. Binding of sLe^x, present on the cell membrane of neutrophil granulocytes, to E-selectin, expressed on activated endothelial cells, was shown to initiate neutrophil extravasation and migration into tissues [15]. It has been hypothesized that the E-selectin/sLe^x interaction can mediate the sequence of adhesion of tumour cells to endothelium and their subsequent extravasation in a process that mimics the above process

Table 2: Relationship between Sialyl Lewis x and E-cadherin expression in canine malignant mammary tumours.

E-Cadherin expression	Number of cases (%)	Sialyl Lewis x expression		P value
		<25%	≥25%	
<25%	5 (9.4%)	0 (0.0%)	5 (100.0%)	0.013
25-50%	7 (13.2%)	6 (85.7%)	1 (14.3%)	
50-75%	12 (22.6%)	9 (75.0%)	3 (25.0%)	
>75%	29 (54.7%)	17 (58.6%)	12 (41.4%)	

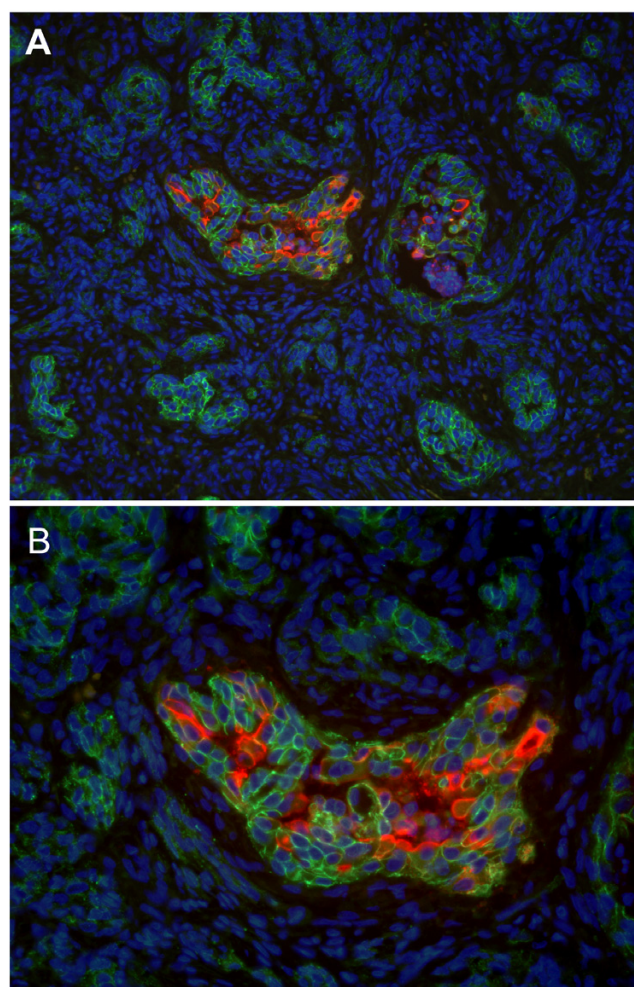


Figure 4
Double-label immunofluorescence of canine malignant mammary tumour (Complex carcinoma). A – Cells that are positive for Sialyl Lewis x (red) are negative for E-cadherin (green) and vice-versa. $\times 200$ B – Absence of co-expression of sLe^x and E-cadherin in the same cells. $\times 400$

of inflammation [15,17]. The sLe^x antigen is expressed by various human carcinomas such as: gastric carcinoma [26], colorectal carcinoma [25], lung cancer [22], prostate carcinoma [24], bladder carcinoma [8], oral [39], head&neck squamous cell carcinoma [40] and breast cancer [11,20,23]. The amounts of sLe^x expression are closely associated with progression and with poor prognosis in those cancers.

In canine cancers, Nakagawa et al has described the expression of sLe^x in canine and feline mammary gland tumours. However no association with clinicopathological features and prognosis was observed [29,41].

The present study demonstrates that in malignant canine mammary tumours the malignant transformation of the mammary gland is accompanied by expression of sLe^x. These results are in agreement with previous observations showing that in squamous metaplasia, used as criteria of malignancy in canine mammary cancer [42-44], there is a strong expression of sLe^x antigen. These observations support the use of sLe^x as a prognostic tumour marker in canine mammary carcinomas.

In this study, we found no significant relationship between sLe^x expression and the histological type of the tumours, classified according to World Health Organization classification. This result is in agreement with the results described by Nakagawa et al in canine and feline mammary gland tumours [29]. Similarly, in woman breast cancer, Nakagoe et al did not find an association between sLe^x expression and histological type of the carcinoma [20].

Previous studies have shown that, in woman breast cancer, tumour necrosis is associated with poorer survival [45-47] and higher recurrence rates [46]. In canine mammary tumours, the presence of large areas of necrosis within the tumour mass could be used as criteria for a diagnosis of malignancy [44]. In spite of no association between sLe^x expression and tumour necrosis we observed that some tumours with necrosis showed high amounts of sLe^x expression.

It is clear that the expression of sLe^x in tumours is a marker of the invasive and/or metastatic properties of the tumours [4,11]. In addition, it has been shown that sLe^x promotes binding of tumour cells at an invasion focus to endothelial cells through E-Selectin [11]. Many clinical studies show a clear association between the expression of sLe^x and tumours with enhanced progression and metastases [19]. In woman breast cancer, it was reported that the expression of sLe^x antigen in tumour cells was associated with poorer prognosis [20]. Matsuura et al demonstrated that the level of sLe^x was elevated in the sera of patients with metastatic breast cancers [21]. Still in woman breast cancer, Jeschke et al reports that overexpression of sLe^x was associated with poorer prognosis and malignant relapse [23].

To date, the expression of sLe^x in canine malignant mammary tumours, was not correlated neither with clinicopathological features, neither with prognoses [29].

In the present study we found a significant correlation between sLe^x expression and lymph node metastases. To the best of our knowledge, this is the first study to show such a relationship in canine malignant mammary tumours. These observations may suggest that sLe^x expres-

sion may also play a role in the process of local lymphatic invasion and metastization [38]. On the other hand, we did not observe an association between sLe^x expression and the development of distant metastases. This observation may suggest that in canine mammary tumours sLe^x does not contribute to haematogenous metastasis. However we could not rule out that the absence of association of sLe^x and haematogenous metastasis stems from the small number of cases studied for this clinicopathological feature.

These combined results from the relation between sLe^x expression and the clinicopathological features allow us to conclude that the sLe^x carbohydrate structure contributes for the malignant phenotype and tumour progression of canine malignant mammary tumours.

Tumour cell dissemination and development of metastases is a multistep process involving complex interaction between cancer cells, extracellular matrix, the vascular system, the immune system and the target organs [48]. Adhesion molecules are contributory factors toward metastatic activity. Adhesion can be divided into reduced adhesion of tumour cell, tumour cell interaction and increased adhesion of floating tumour cells to vascular endothelial cells [48]. Recently we and other authors have described that the loss of E-Cadherin expression may have prognostic value in canine malignant mammary tumours [33,49]. In the present study, our results also showed that increasing expression of sLe^x correlates with lymph node metastases in canine mammary carcinomas. To address a possible cooperative role between these two molecules involved in cell adhesion, sLe^x and E-cadherin, we have compared their expression in canine malignant mammary tumours. Our results showed an inverse relationship between sLe^x and E-cadherin expression. Cases expressing sLe^x showed decrease E-Cadherin expression and vice-versa. Similarly, we could observe in doubled labelled immunofluorescence images, that cells that expressed sLe^x were negative for E-cadherin and vice-versa. This observation is the first indication of a significant relationship between sLe^x and E-cadherin expression in canine malignant mammary tumours. Based on our results it seems that the expression of sLe^x and/or E-cadherin on the same tumour cell could be regulated by an internal mechanism of the cell. In a previous study in a model of woman Inflammatory Breast Carcinoma (IBC), Alpaugh et al have described a cooperative role of E-cadherin overexpression and sLe^x underexpression in the genesis of the lymphovascular embolus of IBC [30,31]. These results showed that both types of adhesion molecules might play opposing roles within the same tumour cell model. Furthermore, Jeschke et al have also shown, in breast cancer, a negative correlation between the expression of Sialyl Lewis anti-

gens and E-cadherin as the risk of breast cancer metastasis progresses [23].

Our findings, in canine mammary carcinomas, supports the generally accepted dogma that metastatic propensity is an active biologic process that includes the decrease of tumour cells adhesion at the primary tumour site (E-cadherin down-regulation) which is thought to be accompanied by higher mobility and invasiveness [50] contributing to the process of local lymphatic invasion and metastization (sLe^x overexpression).

Conclusion

Our results demonstrate the importance of sLe^x in malignant phenotype of canine mammary carcinoma and support the use of sLe^x as a prognostic tumour marker in canine malignant mammary gland tumours.

The significant relation between sLe^x and lymph node metastases allow us to conclude that sLe^x can be used as prognostic factor in canine malignant mammary tumours.

There is an inverse relationship between the expression of the adhesion molecules sLe^x and E-cadherin, in canine mammary carcinomas.

The present results warrants further investigation addressing the clarification of the molecular mechanism determining this inverse correlation between sLe^x and E-cadherin as well as the investigation of the mechanisms by which sLe^x interferes with homotypic cellular adhesion mediated by E-cadherin in canine malignant mammary tumours, as a comparative model to woman breast cancer.

Abbreviations

sLe^x = Sialyl Lewis x; sLe^a = Sialyl Lewis a; HE = Haematoxylin and eosin; IHC = Immunohistochemistry; ABC = Avidin-biotin-peroxidase-complex; mAbs = Monoclonal antibodies; PBS = Phosphate buffered saline; BSA = Bovine serum albumin; DAB = 3,3diaminobenzidinetetrahydrochloride; DAPI = 4'-6-Diamidino-2-phenylindole; NS = Non significant; IBC = Inflammatory Breast Carcinoma

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SSP and AJFM performed the study. SSP wrote the manuscript. CL participated in the immunohistochemistry method. NTM participated in the composition of the images. SSP and JC carried out the statistical analysis. CAR and FG participated in the design and coordination of the

studies and contributed strongly to the revision of the manuscript. All the authors read and approved the final manuscript.

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Chapter 3

Molecular plasticity of E-cadherin and Sialyl Lewis x expression

Paper II

Molecular plasticity of E-cadherin and Sialyl Lewis x expression, in two comparative models of mammary tumorigenesis

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Molecular Plasticity of E-Cadherin and Sialyl Lewis X Expression, in Two Comparative Models of Mammary Tumorigenesis

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Abstract

Background: The process of metastasis involves a series of steps and interactions between the tumor embolus and the microenvironment. Key alterations in adhesion molecules are known to dictate progression from the invasive to malignant phenotype followed by colonization at a distant site. The invasive phenotype results from the loss of expression of the E-cadherin adhesion molecule, whereas the malignant phenotype is associated with an increased expression of the carbohydrate ligand-binding epitopes, (e.g. Sialyl Lewis ^{x/a}) that bind endothelial E-selectin of the lymphatics and vasculature.

Methodology: Our study analyzed the expression of two adhesion molecules, E-cadherin and Sialyl Lewis x (sLe^x), in both a canine mammary carcinoma and human inflammatory breast cancer (IBC) model, using double labelled immunofluorescence staining.

Results: Our results demonstrate that canine mammary carcinoma and human IBC exhibit an inversely correlated cellular expression of E-cadherin and sLe^x within the same tumor embolus.

Conclusions: Our results in these two comparative models (canine and human) suggest the existence of a biologically coordinated mechanism of E-cadherin and sLe^x expression (i.e. molecular plasticity) essential for tumor establishment and metastatic progression.

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Introduction

Metastasis is classically defined as the lymphatic or haematogenous (blood-born) dissemination of cancer cells from the primary tumor (origin) to lymph nodes and/or to distant sites in the body [1]. The process involves a complex series of steps and interactions between the tumor cells and the microenvironment [2,3]. The terminal step of this sequential process is colonization of the tumor cells at distant sites in the body. The tendency of aggressive cancers to metastasize is the cause of mortality from the disease [4]. Determining the molecular mechanisms of metastasis is of the utmost importance in an attempt to manage and treat cancer.

Although all cells of the tumor embolus arise from the same parent cell, the embolus is composed of a heterogeneous population of cells. Even in the most malignant primary tumor not all cells have the capability of successfully colonizing at a distant site [5]. This diversity, as well as the molecular and cellular mechanisms underlying the metastatic potential of the tumor

embolus result from inherent (clonal selection) or acquired (adaptive) traits or both of the tumor cells composing it [6,7]. Only a portion of those heterogeneous cells progress to the malignant phenotype and even a smaller portion can successfully colonize, and form deadly metastases [6]. The ability of those tumor cells to conform to new environments is due to a molecular plasticity. Among the events involved in molecular plasticity, loss and gain of key adhesion molecules appears to be a significant factor [8,9]. Key alterations in adhesion molecules are known to dictate progression from the invasive to malignant phenotype followed by colonization at a distant site [10].

E-cadherin and Sialyl Lewis x (sLe^x) are two adhesion molecules that govern malignant progression. E-cadherin is an adhesion molecule that plays a key role in homotypic cell-cell adhesion, being classically considered a potent invasion/tumor suppressor gene [9,11]. Furthermore, sLe^x antigen is a carbohydrate structure that is involved in selectin-mediated adhesion of cancer cells to vascular endothelium and this determinant is thought to be closely

associated with haematogenous metastases of cancer [12,13]. sLe^x not only is a marker for cancer but also is functionally implicated in the malignant behaviour of cancer cells [14,15]. Overall, the loss and gain of expression of these molecules are not aberrant variations, but adaptive mechanisms of the cancer embolus, which can defeat our present treatment modalities.

Naturally occurring cancers in canines and humans share many features, including histological appearance, tumor genetics, molecular targets, biological behaviour and response to conventional therapies [16]. Mammary carcinomas, specifically, occur among all taxonomic groups, and comparing the disease in canine models with breast carcinoma in women could greatly improve our understanding of the molecular biology underlying the process of mammary tumorigenesis and progression [17].

In this report we demonstrate that canine mammary carcinoma and the highly metastatic inflammatory breast cancer (IBC) in women, exhibit an inversely correlated expression of E-cadherin and sLe^x in cells of the same tumor embolus. Both mammary carcinoma models support a coordinated expression between the two adhesion molecules, suggesting a dynamic transition state or molecular plasticity promoting tumor survival and dissemination during the metastatic progression.

Materials and Methods

Ethics Statement

All experiments were performed in compliance with the Memorial Sloan-Kettering Cancer Center Animal Care and Use Program (Protocol Number 06-04-006).

MARY-X Xenograft and in vitro spheroids

MARY-X was established from a patient with inflammatory breast cancer (IBC) [18]. *In vivo*, MARY-X recapitulates the human IBC phenotype of extensive lymphovascular invasion of the tumor cell emboli. The *in vitro* MARY-X spheroids are prepared by mincing the extricated tumor followed by an overnight incubation under standard tissue culture conditions. The IBC cells are released as sheets and small spheroids initially. Over a period of time, roughly 12–14 hours, the IBC cells as *in vivo*, begin to overexpress E-cadherin and form tight, compact spheroids in suspension. The compact, multicellular in cell suspension spheroids are further purified by partitioning through various size (100 μ m, 70 μ m and 40 μ m pore size; BD Biosciences) cell strainers to produce homogenous preparations of spheroid sizes of 40 μ m–70 μ m, 70 μ m–100 μ m and 100+ μ m in diameter. Spheroids were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C in an air-5% CO₂ atmosphere at constant humidity.

Canine mammary carcinoma cell line, CMT-U27

The canine mammary carcinoma cell line CMT-U27 was obtained from the Uppsala University, Sweden. CMT-U27 cell line was derived from a primary tumor (infiltrating ductal carcinoma) [19] and when inoculated in the fat mammary pad of female *nude* mice metastasizes to the lymph nodes, lungs, liver and heart. CMT-U27 was cultured in RPMI 1640 with Glutamax medium (Gibco, Invitrogen), supplemented with 10% fetal bovine serum (Gibco, Invitrogen) and 50 μ g/ml gentamicin (Gibco, Invitrogen). Culture was maintained at 37°C in a humidified 5% CO₂ atmosphere.

Tissue specimens

Ten tissue samples of primary canine mammary carcinomas of different histological types (3 Complex carcinomas; 3 Tubulopapillary carcinomas; 2 Carcinosarcoma; 2 Solid carcinomas) and 3

correspondent lung metastases were used in this study. Accordingly with Peña L et al., canine inflammatory mammary carcinoma (IMC) once diagnosed is involved with other histological types of canine mammary carcinoma including solid carcinomas, tubulopapillary carcinomas and sarcomatous type [20]. The same is also true for feline IMC where the histology revealed types of tubulopapillary mammary carcinomas [21]. In addition, ten *in vivo* (primary tumor and metastases in SCID mice) and ten *in vitro* specimens were harvested from the MARY-X model. The specimens were fixed in 10% neutral buffered formalin. After dehydration and paraffin wax embedment, 4 μ m thick sections were cut from each representative paraffin block for staining with haematoxylin and eosin and for double-staining immunofluorescence.

Double-Labeling Immunofluorescence

For simultaneous visualization of sLe^x and E-cadherin on the same tissue section of the two comparative models, MARY-X and canine mammary gland carcinomas, double-label immunofluorescence technique was performed.

Briefly, paraffin sections were dewaxed, rehydrated and then treated with Extran (Merck, Frankfurt, Germany) 0.05% in distilled water for 10 min in a microwave oven at 750 W. After being left to cool for 20 min at room temperature, slides were rinsed twice in Phosphate-buffered saline (PBS), and then incubated for 20 min in a humid chamber with rabbit non-immune serum at a dilution 1:5 in PBS containing 10% bovine serum albumin (BSA). Sections were incubated with the first primary monoclonal antibody (mAb), anti-human E-Cadherin (clone 36, BD Biosciences Pharmingen, diluted 1:100 in PBS), overnight at 4°C. After washing twice for 5 min in PBS, sections were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (code F261; Dako, Glostrup, Denmark; diluted 1:100 in PBS). Sections were washed twice for 5 min in PBS and blocked with non-immune goat serum diluted 1:5 in PBS containing BSA 10%. After incubation with mAb KM93 (mouse IgM) diluted at 1:40, overnight at 4°C, sections were washed twice for 5 min with PBS and then incubated with Texas red-conjugated goat anti-mouse IgM (Jackson ImmunoResearch) diluted 1:50 in PBS. Sections were washed as before and nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI, Sigma) diluted at 1:100 in PBS, for 15 min in the dark. Finally, sections were washed twice for 5 min in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, USA).

Dilutions of primary antibodies, secondary antibodies, and DAPI were made with PBS containing 5% BSA.

Immunostained tissue sections were examined under a fluorescence microscope (Leica DMIRE2) equipped with appropriate filters. Separate images for DAPI, Texas-Red and FITC staining were captured digitally at X200 and X400 magnification. The red (for Texas-Red), blue (for DAPI), and green (for FITC) components were merged and combined images were imported into Adobe Photoshop®.

Immunoprecipitation of E-cadherin from CMT-U27

Cells from CMT-U27 cell line were washed with cold phosphate-buffered saline (PBS) and then lysed with a lysis buffer (1% (v/v) Triton X-100; 1% (v/v) NP-40 in PBS, containing protease inhibitor cocktail (Roche), 100 mM Na₃VO₄, 100 mM PMSF) for 15 min on ice. After scraping cell monolayers with a rubber policeman the suspension was centrifuged at 14000 rpm for 10 min at 4°C. Protein concentrations were determined by using a BCA protein assay kit (Pierce).

Total protein (750 µg) of canine cell line was precleared with 50 µl of protein G-sepharose beads (Sigma Aldrich) at 4°C under agitation for 1–2 h to remove non-specific adsorption to the sepharose beads.

After centrifugation at 14000 rpm for 5 min at 4°C, supernatant was incubated overnight at 4°C under agitation with 5 µg of monoclonal antibody against E-cadherin (monoclonal mouse anti human E-cadherin, clone 36, BD Biosciences Pharmingen). Then, incubation with protein G-sepharose at 4°C for 2 h with agitation was performed. Next, the beads were washed three times with an immunoprecipitation buffer (10% (v/v) lysis buffer, 5 mg/ml Na₄P₂O₇, 1% (v/v) NaF 100X in PBS). The immune complexes were released by boiling 5 min at 95°C in Laemmli sampling buffer prior to analyses by Western.

For sLe^x carbohydrate detection of E-cadherin immunoprecipitated, nitrocellulose membrane was blocked and then incubated with monoclonal antibody that specifically recognizes sLe^x epitope (KM93) with a dilution of 1 µg/ml in PBS with 0.01% Tween 20 (PBS T) with 5% milk.

The membrane was washed three times with PBS T and incubated with HRP-conjugated anti-mouse IgM secondary antibody (Santa Cruz Biotechnology, 1:3000 diluted in PBS T with 5% milk) during 1 h at room temperature. After washing three times with PBS T, membrane was developed by an ECL according to the manufacturer's recommended protocol.

Results

Inversely correlated cellular expression of E-cadherin and sLe^x in the MARY-X spheroid

MARY-X, like IBC, grows exclusively within the murine lymphatics and blood vessels, which is the signature phenotype of this highly aggressive, malignant breast carcinoma [18]. Both the MARY-X *in vitro* spheroid (Figure 1A) and *in vivo* tumor embolus [data not shown] form on the basis of an intact, over-expressed E-cadherin/ α , β catenin axis [22]. Interestingly, MARY-X displays a unique relationship of two key adhesion molecules; over-expression of E-cadherin and under-expression of sLe^x confirmed by western blot analysis [23,24]. This relationship is contrary to what would be expected in a highly malignant carcinoma. To determine if expression levels were due to a homogenous population of cellular expression (i.e. each cell produced excessive amounts of E-cadherin and a limited amount of sLe^x) double-

labeling immunofluorescence of the two adhesion molecules was performed. The majority of cells of the MARY-X *in vitro* spheroid displayed membrane-positioned E-cadherin (green) expression only (Figure 1B). A minority population co-expresses both molecules with a cytoplasmic-positioned sLe^x (red) expression (Figure 1C and 1D). An even smaller population of cells, of the MARY-X *in vitro* spheroid display membrane-positioned sLe^x (red) expression only (Figure 1D). Co-expression of these two adhesion molecules is rarely seen. Hence the MARY-X spheroid displays an inverse relationship of expression of E-cadherin and sLe^x. Previous studies indicated a cooperative relationship between E-cadherin and sLe^x [23]. However, our study shows that the expression levels are not due to a homogenous population of cellular expression but rather a heterogeneous population of cellular expression. A similar inversely correlated cellular expression between these two key adhesion molecules has also been reported in a malignant canine mammary carcinoma model and was the basis of this study [25].

Inversely correlated cellular expression of E-cadherin and sLe^x throughout the metastatic progression of MARY-X and canine mammary carcinoma

Since adhesion molecules dictate metastatic progression of carcinomas, double labeling immunofluorescence of E-cadherin and sLe^x was performed on the primary tumor and corresponding metastases of both the MARY-X and canine mammary carcinoma models to determine the presence of a correlated cellular expression throughout metastatic progression. The majority of the cell population of the primary tumor of both the MARY-X and canine models display a strong membrane-positioned E-cadherin (green) expressing cells with no expression of sLe^x (Figure 2A & 2B). To a lesser extent, nests of sLe^x (red) expressing cells could be seen throughout both models (Figure 2A & 2B). In the MARY-X model, as seen *in vitro*, sLe^x is cytoplasmic-positioned and co-expressed with membrane-positioned E-cadherin (Figure 2A; middle panel). However, a small number of cells display membrane-positioned sLe^x expression only (Figure 2A; middle panel). The canine primary tumor is very comparable to MARY-X except that the nests of sLe^x expressing cells predominantly display membrane-positioned sLe^x only, i.e. no co-expression of the two molecules (Figure 2B; middle panel). The corresponding metastasis of MARY-X and the canine mammary carcinoma also show a dominant population of cells expressing

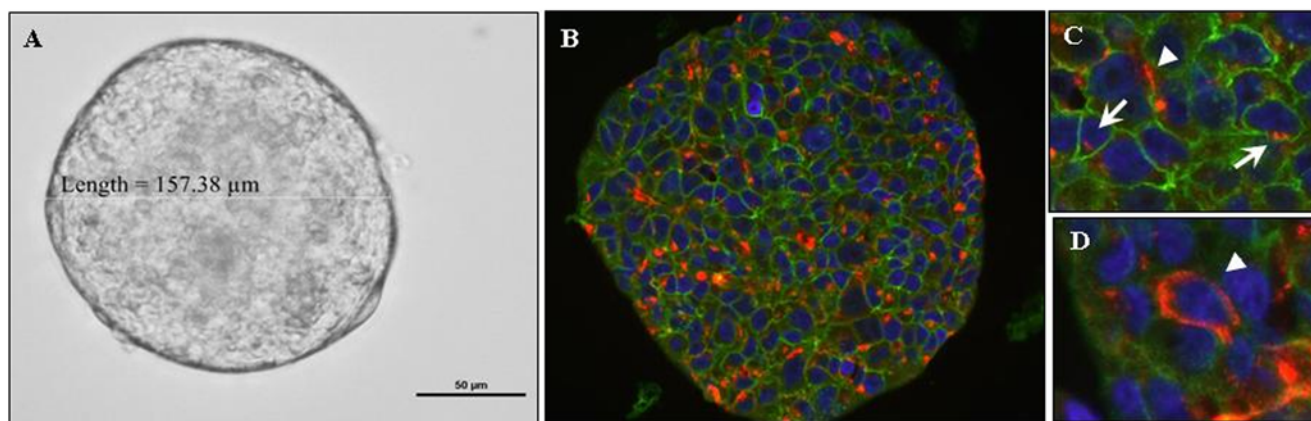


Figure 1. Expression of E-cadherin and sLe^x in the MARY-X spheroid. (A) MARY-X *in vitro* spheroid forms a compact, clump of cells due to over-expression of a key adhesion molecule, E-cadherin (light microscopy; 40× magnification). (B) Illustrates a heterogeneous population of cellular expression with a dominant population of cells expressing membrane-positioned E-cadherin (green) and a minority population of cells expressing sLe^x (red). (C & D) sLe^x was found to be either cytoplasmic- (long arrows) or membrane-positioned (blunt arrows). doi:10.1371/journal.pone.0006636.g001

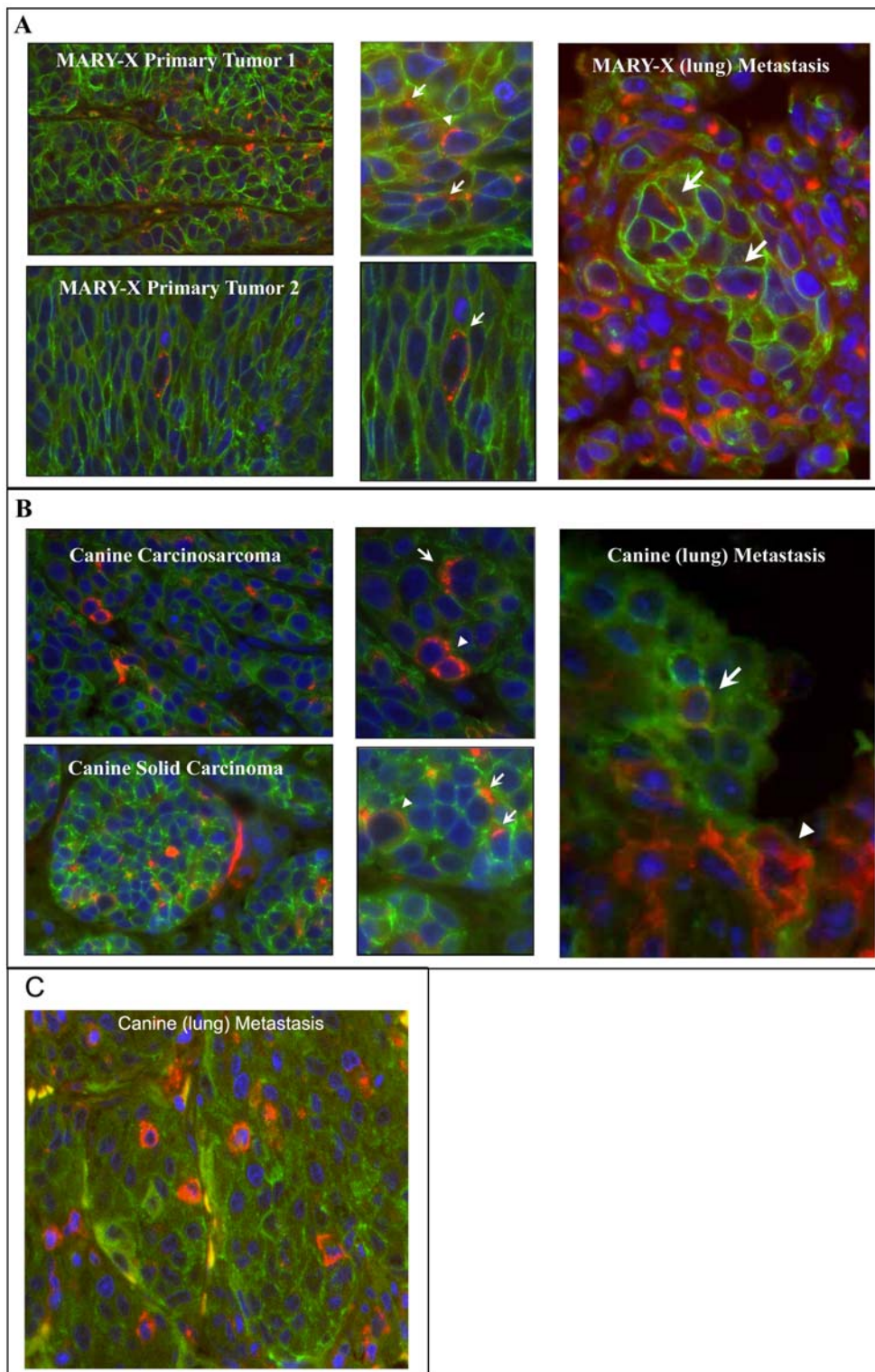


Figure 2. Expression of E-cadherin and sLe^x throughout the metastatic progression of MARY-X and canine mammary carcinoma. (A, B) Shows a heterogeneous population of cellular expression in both (A) MARY-X primary tumors and (B) canine primary tumors (carcinosarcoma and solid carcinoma) displaying a dominant population of cells with membrane-positioned E-cadherin (green) and smaller nests sLe^x (red) expressing cells (cytoplasmic or membrane-positioned). (A, B; middle panels) Magnified image shows the sLe^x found either cytoplasmic- (long arrows) or membrane-positioned (blunt arrows). Mary-X lung metastasis (MARY-X metastasis) displays a dominant population of cells expressing only E-cadherin (green), and a minority population of cells co-expressing cytoplasmic-positioned sLe^x (red) (long arrows). (B, C) Canine lung metastasis (Canine metastasis) displays a dominant population of cells expressing only E-cadherin (green), and minority populations of cells either co-expressing cytoplasmic-positioned sLe^x (red) (long arrows) or expressing only membrane-positioned sLe^x (red) (blunt arrows).
doi:10.1371/journal.pone.0006636.g002

only membrane-positioned E-cadherin (green) (Figure 2A & 2B; right panel and Figure 2C). However, in the few sLe^x expressing cells of the MARY-X metastasis co-expression of membrane-positioned E-cadherin with cytoplasmic-positioned sLe^x was seen (Figure 2A; right panel). The canine metastasis showed predominantly membrane-positioned sLe^x expressing only cells (Figure 2B; right panel and Figure 2C). In the primary tumor and corresponding metastases of both models, co-expression is a rare event. Therefore both models maintain a heterogeneous population of cellular expression through out the metastatic progression displaying an inversely correlated cellular expression of these two key adhesion molecules.

Sialyl-Lewis X does not mask E-cadherin expression

An E-cadherin immunoprecipitation (IP) was performed in order to rule out the possibility that sLe^x immunofluorescence (red) masked E-cadherin immunofluorescence (green) in cells that displayed sLe^x expression only (i.e. E-cadherin is a carrier of sLe^x). This approach was only performed on the canine model due to the greater number of cells that express only sLe^x as compared to the MARY-X model. The evaluation of the expression of sLe^x using a specific monoclonal antibody showed no detection of this carbohydrate antigen on the E-cadherin immunoprecipitate from canine mammary carcinoma cell line, CMT-U27 (Figure 3). This finding excludes the possibility that the sLe^x carbohydrate is attached to E-cadherin.

Discussion

Tumor cell formation and development of metastasis is a multistep process involving complex interactions between cancer cells, extracellular matrix, lymphovascular system, immune system and target organs [4]. The loss (e.g. E-cadherin) and gain (e.g. carbohydrate ligand-binding epitopes) of key adhesion molecules are contributing factors in the metastatic process [11,12]. This study investigated two interesting exceptions to the above rule, namely the inflammatory breast cancer model, MARY-X and a canine mammary carcinoma model. Both models are highly metastatic, yet display neither a loss in E-cadherin expression nor overall gain in the carbohydrate ligand-binding epitope of endothelial cells, sLe^x [25,26]. Rather, these two exceptions suggest a more “adaptable” metastatic progression as opposed to a “one way” progression from the invasive (loss of E-cadherin expression) to malignant phenotype (gain in sLe^x expression). The heterogeneous population of cellular expression suggests a dynamic “back and forth” transition state between phenotypes that might promote survival of the tumor embolus during metastatic progression.

Our study shows a correlated inverse relationship of E-cadherin and sLe^x expression in the genesis, dissemination and subsequent metastases in the MARY-X and canine mammary carcinoma models. These preliminary findings suggest that loss and gain in expression of adhesion molecules is not an aberrant variation but rather an adaptive cellular and molecular mechanism of the

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IP: E-Cadherin

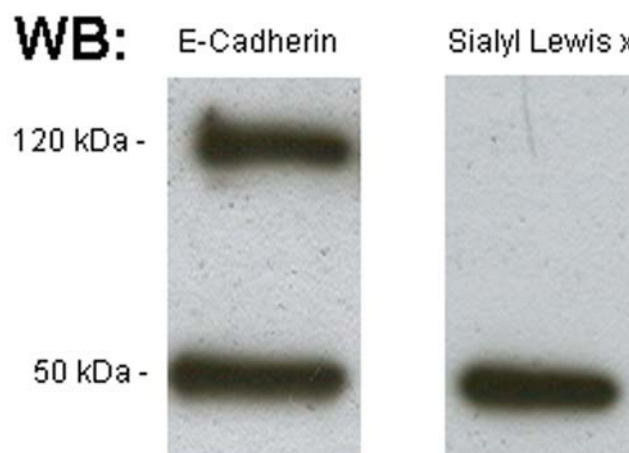


Figure 3. E-cadherin immunoprecipitation of CMT-U27 cell line. The left lane shows the E-cadherin immunoprecipitate. The right lane shows the absence of sLe^x in the E-cadherin immunoprecipitate. The 50 kDa bands in both lanes are the immunoglobulin heavy chain. doi:10.1371/journal.pone.0006636.g003

cancer that contribute to its metastatic ability. Our findings are the beginning to a better understanding of these adaptive mechanisms. More specifically, since molecular variation contributes to cancer progression and survival then the present findings set the ground for targeting these mechanisms resulting in new therapeutic strategies of metastatic disease. Further work is in progress regarding the clarification of the molecular mechanism underlying gene expression of these two key molecules through out the metastatic progression in both comparative models.

In conclusion, our study suggests the existence of a biologically coordinated mechanism of E-cadherin and sLe^x expression (i.e. molecular plasticity) essential for tumor establishment and metastatic progression.

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Author Contributions

Conceived and designed the experiments: SSP CAR FG MLA. Performed the experiments: SSP MLA. Analyzed the data: SSP CAR FG MLA. Contributed reagents/materials/analysis tools: SSP. Wrote the paper: SSP MLA. Contributed strongly to the revision of the manuscript: CAR FG.

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Chapter 4

E-cadherin N-glycosylation profile in tumour phenotypes

Paper III

Role of E-cadherin N-glycosylation profile in a mammary tumor model

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Role of E-cadherin *N*-glycosylation profile in a mammary tumor model

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ABSTRACT

Modifications in cell surface glycosylation affecting cell adhesion are common characteristics of transformed cells. This study characterizes the *N*-glycosylation profile of E-cadherin in models of canine mammary gland adenoma and carcinoma evaluating the importance of these glycosylation modifications in the malignant phenotype.

Our results show that the pattern of E-cadherin *N*-glycosylation in mammary carcinoma is characterized by highly branched *N*-glycans, increase in sialylation and an expression of few high mannose structures. Detailed mass spectrometry analysis demonstrated a new *N*-glycosylation site containing a potential complex type *N*-glycan in E-cadherin from a mammary carcinoma cell line.

Our study demonstrates the importance of E-cadherin *N*-glycans in the process of tumor development and in the transformation to the malignant phenotype.

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E-cadherin is a transmembrane glycoprotein that suffers various post-translational modifications, such as *N*-glycosylation [1,2]. These modifications have impact in the function of the molecule. *N*-glycosylation is common in E-cadherin, contributing up to 20% of its total mass [2,3].

Modifications in protein glycosylation are often observed in disease states. In cancer, cells frequently display glycans with fundamentally different structures than those observed on the normal counterparts and on benign cells [4–7].

N-linked glycosylation of E-cadherin has been implicated in the modulation of cadherin dependent tumor cell–tumor cell adhesion in association with tumor progression [8,9]. Previously, the structural modifications of *N*-glycans of E-cadherin by *N*-acetylglucosaminyltransferase III (GnT-III) were shown to cause increased cell–cell adhesion [10,11], whereas structural modification of *N*-glycans on E-cadherin with increased branched structures by GnT-V, resulted in decreased cell–cell adhesion, contributing to increased cellular motility and invasiveness [12]. These studies demonstrated that *N*-glycans may modulate E-cadherin mediated cell–cell adhesion contributing to tumor cell invasion and metastasization [2,13,14].

The present study, evaluates E-cadherin *N*-glycosylation in the process of mammary tumor development and acquisition of the malignant phenotype.

Material and methods

Cell lines. The canine mammary carcinoma cell line CMT-U27 was derived from a primary infiltrating ductal carcinoma and when inoculated in the fat mammary pad of female *nude* mice metastasizes to the lymph nodes, lungs and liver [15].

The canine mammary adenoma cell line CMA-07 was established and characterized in our laboratory. The CMA-07 cell line was originated from a primary complex adenoma and does not metastasize when inoculated in nude mice (unpublished data).

The human colon carcinoma cell line T84 and MDCK (ATCC) were used as positive control for E-cadherin expression.

Both canine cell lines were cultured in RPMI 1640 with Gluta-max medium, supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin. T84 and MDCK cell lines were cultured in DMEM/F-12 supplemented with 10% FBS and antibiotics (penicillin-10 U/ml, streptomycin-10 µg/ml). Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

Western-blotting. Cells from the four cell lines were washed with cold phosphate-buffered saline (PBS) and lysis with a lysis buffer. Protein concentrations were determined by using a BCA protein assay kit (Pierce).

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For analyzing, E-cadherin expression in adenoma and carcinoma cells, 30 µg of total cell lysate (TCL) of each cell line was electrophoresed on a 7.5% SDS–PAGE and transferred onto a nitrocellulose membrane. As a positive control, we used 30 µg of TCL from T84 and MDCK cell lines.

After blocking with PBS with 0.01% Tween-20 (PBS T) containing 5% milk, membranes were incubated with primary antibody (monoclonal mouse anti human E-cadherin, clone 4A2C7, Zymed; clone 36, BD Biosciences). Next, membranes were washed with PBS T solution, followed by incubation with HRP-conjugated anti-mouse IgG secondary antibody and the protein band was developed with ECL reagent.

Immunoprecipitation of E-cadherin and blotting. Equal amounts of total protein of each canine cell line (750 µg) were precleared with 50 µl of protein G-sepharose beads for 1, 2 h. After centrifugation, the supernatant was incubated overnight with 5 µg of monoclonal antibody (mAb) against E-cadherin. After that, incubation with protein G-sepharose for 2 h was performed. Next, the beads were washed three times with an immunoprecipitation buffer. The immune complexes were released by boiling 5 min at 95 °C in Laemmli sampling buffer and the immunoprecipitates were subjected to 7.5% SDS–PAGE and the separated proteins were transferred to a nitrocellulose membrane. The membranes were probed with 10 µg/ml of biotinylated lectins for lectin-blot (Table 1) or incubated with mAbs that specifically recognizes Sialyl Lewis x (FH6), Lewis x (SH1) and Lewis a (CA3F4), previously shown to display a positive expression in carcinoma and adenoma cell lines by immunofluorescence.

PNGase F and EndoH digestions. Total cell lysate from CMT-U27 was digested with 1 U of PNGaseF and EndoH (New England Biolabs) for 1 h at 37 °C, loaded onto a 7.5% SDS–PAGE, and examined by Western-blotting. For controls, samples were incubated without the enzymes.

Protein identification analysis. Immunoprecipitated sample from 1.5 mg of total protein from carcinoma cell line were obtained using anti-E-cadherin antibody and then submitted to 7.5% SDS–PAGE. The gel was silver stained. Gel pieces were digested with trypsin. Peptide extraction was performed by a 60% ACN/0.1% trifluoroacetic acid solution. Protein digests were desalted and concentrated using ZipTips (Millipore). Samples were crystallized onto a stainless steel 192-well MALDI plate and analyzed using the 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Peptide mass fingerprint data was collected in MS reflector mode in the range of 700–4000 *m/z* and was calibrated internally using trypsin autolysis peaks. Several of the highest intensity non-trypsin peaks were selected for MS/MS analysis. The spectra were analyzed using GPS Explorer (Version 3.6; Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local copy of the Mascot (Matrix Science) search engine software (Version 2.1.04). The obtained spectra were searched against a locally stored copy of the nr NCBI database with the following potential modifications: pro-

pionamide (Cys), oxidation (Met, His and Trp) and phosphorylation (Ser, Thr and Tyr). To be considered a match a confidence interval (CI) calculated by Applied Biosystems GPS Explorer of at least 99% was required.

Characterization of possible *N*-glycosylation sites of E-cadherin was performed using the Glycomod software [16,17] available at <http://www.expasy.ch/tools/glycomod/>. The analysis was performed by comparing the experimental mass spectra data of the tryptic peaks derived from the sample with the respective, non-glycosylated, identified protein (E-cadherin, gi|73957458).

Histochemistry. A total of six cases of canine mammary carcinomas and six cases of canine mammary adenomas of different histological subtypes were used in order to characterize the overall cellular glycosylation profile in tissue samples and evaluate differences between them.

Paraffin sections were used. Endogenous peroxidase activity was quenched with hydrogen peroxidase 3% in methanol for 10 min [18]. For L-PHA lectin, heat-induced antigen retrieval was done by microwave in citrate buffer (pH 6) for 10 min, after blocking the endogenous peroxidase. After washing the slides in PBS, sections were incubated with BSA 10% in PBS for 20 min. Sections were then incubated with biotinylated lectins (Table 1), diluted in PBS, for 1 h. Slides were washed with PBS and incubated with the avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min. After washing with PBS, 3,3'-diaminobenzidine was used as the chromogen, followed by hematoxylin counterstaining. Slides were dehydrated and mounted.

Lectins reactivity was evaluated by three independent observers and semi-quantified as follows: –, no staining; ±, few weak labeled positive cells; +, few positive cells; ++, well-defined areas with positive cells; +++, almost every cell stained. The negative control sections were treated with PBS instead of the biotinylated lectin.

Results

Characterization of E-cadherin *N*-glycosylation profile from adenoma and carcinoma cell lines

The expression of E-cadherin evaluated by Western-blot was observed in all cell lines at approximately 120 kDa (Fig. 1A).

Immunoprecipitated E-cadherin from adenoma and carcinoma cell lines was also detected at 120 kDa range (Fig. 1B).

Characterization of E-cadherin glycosylation profile from adenoma and carcinoma cell lines was further evaluated using lectins and mAbs. As shown in Fig. 2A the reactivity profile of E-cadherin from both cell lines revealed marked differences in glycosylation.

The carcinoma cell line showed the presence of sialic acid residues α2,6-linked and α2,3-linked in the oligosaccharides structure of E-cadherin. These sialylated structures were not observed in E-cadherin from the adenoma cell line (Fig. 2A).

In addition, we observed that E-cadherin from carcinoma cell line display a GlcNAc β1,6- branched tri- and/or tetra-antennary complex type glycans as detected by L-PHA lectin. No evidence for the presence of GlcNAc β1,6- branched was observed in E-cadherin from adenoma cell line (Fig. 2A).

Furthermore, E-cadherin from carcinoma cell line also showed the presence of high mannose/hybrid type oligosaccharides as indicated by the positive reaction with GNA lectin. E-cadherin from adenoma cell line did not show this high mannose structure (Fig. 2A).

Finally, terminal α1,2 fucosylated residues on E-cadherin were not observed in both cell lines (negative reaction with UEA-I), Fig. 2A.

Table 1
Carbohydrate binding specificities of lectins.

Lectin*	Lectin-specific glycan structure	Dilution
<i>Sambucus nigra</i> agglutinin (SNA)	NeuAcα2,6Gal- or NeuAcα2,6GalNAc-	1:250
<i>Maackia amurensis</i> lectin (MAL-I)	NeuAcα2,3Gal-	1:250
<i>Phaseolus vulgaris</i> agglutinin (PHA-L)	Galβ1,4GlcNAcβ1,6 (Galβ1,4GlcNAcβ1,2)Manα-	1:150
<i>Galanthus nivalis</i> agglutinin (GNA)	Manα1,2Man- Manα1,6Man- Manα1,3Man-	1:150
<i>Ulex europeas</i> agglutinin (UEA-I)	Fucα1,2Galβ1-(3)4GlcNAc-	1:50

* Lectins from vector laboratories.

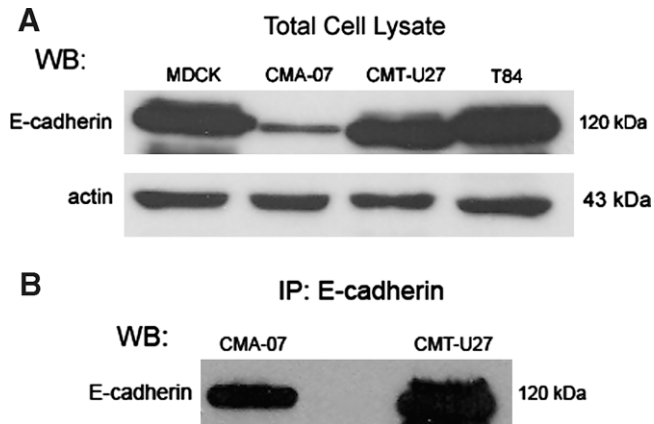


Fig. 1. Expression of E-cadherin molecule in adenoma and carcinoma cell lines. (A) The four cell lines revealed in the 120 kDa range one main band that corresponded to E-cadherin molecule. Actin was used as loading control. (B–E) Cadherin from carcinoma and adenoma cell lines was immunoprecipitated and Western-blot analysis showed the identification of the protein at 120 kDa.

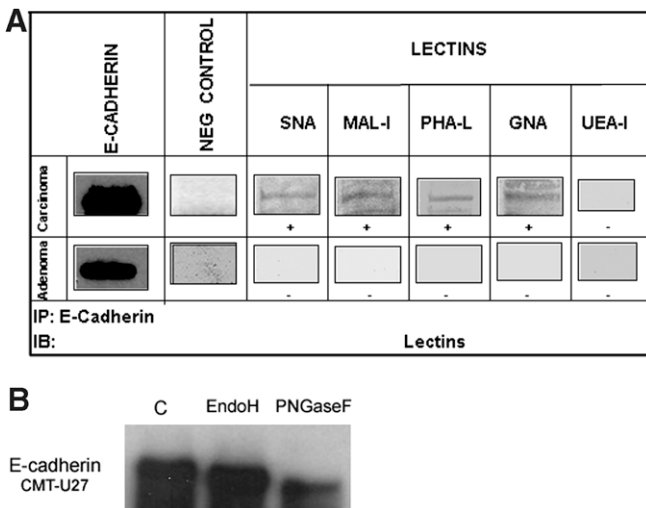


Fig. 2. (A) N-glycan chain profile of canine E-cadherin from carcinoma and adenoma cell lines. The positive reaction of canine E-cadherin from carcinoma cell line with SNA, MAL-I, PHA-L and GNA indicated the presence of α 2,6; α 2,3 linked sialic acid; β 1,6 GlcNAc branched and the presence of high mannose/hybrid type oligosaccharides, respectively. (B) N-glycosylation of E-cadherin in carcinoma cell line. Total cell lysate from carcinoma cell line was treated with either EndoH or PNGaseF. E-cadherin from carcinoma cell line is modified primarily with complex N-glycans and some high mannose/hybrid structures, comparing with the control (C) without PNGaseF or EndoH digestion).

Structural characterization of E-cadherin N-glycans from carcinoma cells

Evaluation of E-cadherin N-glycosylation from CMT-U27 cell line was carried out detecting PNGaseF and EndoH sensitivity. Based on the mobility shifts before and after EndoH and PNGaseF treatments, E-cadherin from carcinoma cell line displayed a clear PNGaseF sensitivity with little EndoH sensitivity, indicating that E-cadherin was modified with a major complex N-glycans and some high mannose/hybrid structures (Fig. 2B). The finding that E-cadherin from carcinoma cell line was more extensively modified with complex N-glycans and some high mannose/hybrid structures confirm the results obtained with lectin-blot using L-PHA and GNA lectins (Fig. 2A).

Further analysis of immunoprecipitated E-cadherin from carcinoma cell line was subjected to SDS-PAGE separation and silver stained. Two major bands were observed at approximately 120 kDa (band A) and 92 kDa (band B), Fig. 3A. Both bands (A and B) were excised from the gel, and their tryptic digests were analyzed by MALDI-TOF mass spectrometry in order to confirm the identity by peptide mass fingerprinting (Fig. 3). Band A was identified as E-cadherin (gi|73957458) with a confidence interval (CI) of 99.4%, Mascot score of 74. Thirty-two peptides (Fig. 3C) were associated with this identification, which correspond to a protein sequence coverage of 21%. This result was supported by MS/MS analysis of the peak 16 and 17 (Fig. 3B and C) with a best ion score of 99.9%.

Detailed analysis of mass spectrum using the Glycomod software [16,17], a tool that can predict possible N-glycosylation of proteins from their experimentally determined masses, indicated that the Asn-775 is N-glycosylated (Fig. 3). Peaks 30–32, as well as their glycosylated forms (a–c), correspond to the same amino-acid sequence. Peaks (b) and (c) correspond to N-glycan variants of peak number 31, and peak (a) correspond to a N-glycan of peak number 32.

Carbohydrate structures of the major peaks were deduced from the m/z values of protonated ions. The presence of $[(\text{Hex})_1(\text{HexNAc})_1+(\text{Man})_3(\text{GlcNAc})_2+\text{H}]^+$ (m/z 1257), peak (c), suggests that this N-glycan is a hybrid/complex type.

Taking into consideration the L-PHA lectin-blot, the enzymatic digestion pattern, and the MALDI-TOF MS analysis of E-cadherin from carcinoma cell line, we have evidences that the N-glycan in Asn 775 is a β 1,6 branched GlcNAc structure.

Band B (Fig. 3A) was identified as β -catenin (gi|47523792) with a CI of 100%, Mascot score of 128 and a sequence coverage of 32%, which corresponded to 21 associated peptide peaks (data not shown).

Characterization of glycosylation profiles in canine mammary adenoma and carcinoma tissues

Glycosylation profiles in canine mammary adenoma and carcinoma are shown on Table 2 and illustrated in Fig. 4.

Canine mammary carcinomas displayed positive SNA staining in most cells. The staining was predominantly apical/membranar. Adenoma cases also displayed a positive staining though in a lower number of cells.

MAL-I also stained all canine mammary carcinoma cases, with a membranar pattern of expression.

Mammary gland adenoma and carcinoma tissue samples were stained with L-PHA. All tumors exhibited a positive staining but the pattern of expression was clearly different between adenomas and carcinomas. In adenomas, the staining was predominantly observed in the basal cells of glandular structures that correspond to the myoepithelial layer (Fig. 4H). On the contrary, carcinomas showed a staining in epithelial cells at the luminal surface. In some regions of the carcinomas (Fig. 4C) and adjacent proliferative lesions, like hyperplasias (Fig. 4H, insert), some glandular structures also exhibited a myoepithelial pattern of expression. Interestingly, we observed increased expression of β 1,6-branched structure in a lung metastasis of a positive primary tumor (Fig. 4K and L).

The presence of high mannose/hybrid N-glycans was observed in canine mammary carcinomas and adenomas which showed a positive staining mainly in the luminal surface of the cell membrane (Fig. 4D and I; Table 2).

The presence of α 1,2 fucosylated residues was not observed either in carcinomas or in adenomas (negative reactivity with UEA-I lectin).

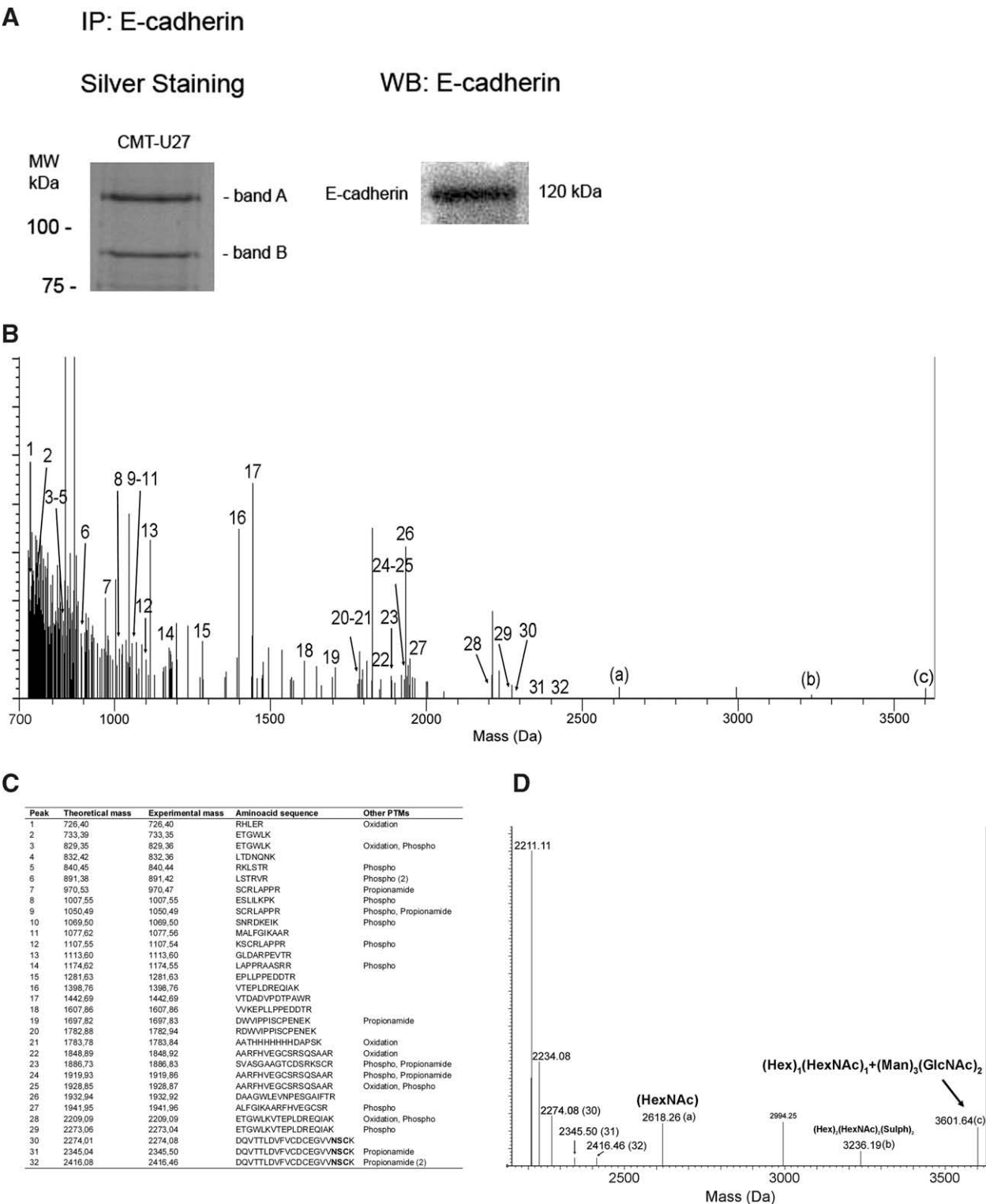


Fig. 3. MALDI-TOF analysis of immunoprecipitated E-cadherin from carcinoma cell line. (A) Immunoprecipitated E-cadherin revealed band A at 120 kDa and band B at 92 kDa. Western-blot control was performed with E-cadherin antibody. Identity of band A and B was confirmed by mass spectrometry as being E-cadherin and β -catenin, respectively. (B) shows the mass spectra obtained from the tryptic digest of band A from 700 to 3625 Da with the indication of E-cadherin peaks (1–32). (C) shows the list of E-cadherin peaks, their respective theoretical mass, experimental mass, aminoacid sequence and the detection of different post-translational-modifications. (D) shows an amplified mass spectrum region containing peaks (a–c) corresponding to E-cadherin N-glycosylated peptides. Peaks 30–32, as well as their glycosylated forms (a–c), correspond to the same aminoacid sequence. Peaks (b) and (c) correspond to N-glycan variants of peak number 31 and peak (a) correspond to a N-glycan of peak number 32.

Table 2
Glycosylation profile in canine mammary tumors using lectin histochemistry.

Lectins	Degree of reactivity	
	Carcinoma tissue samples(n = 6)	Adenoma tissue samples(n = 6)
SNA	+++	++
MAL-I	++	+/-
L-PHA	++	+
GNA	++	++
UEA-I	—	—

Discussion

Aberrant glycosylation expressed in glycoproteins in tumor cells has been implicated as an essential mechanism in defining stage, direction and fate of tumor progression [6].

Our study shows that E-cadherin displays a modified *N*-glycosylation pattern in cancer cells. In agreement with our study, Yoshimura [11] have demonstrated that in murine melanoma B16-hm cells overexpressing GnT-III, the altered glycosylation of E-cadherin was accompanied by a reduced protein turnover rate, increased expression of E-cadherin at the intercellular junctions and a reduced metastatic capacity of the cells [11,19]. On the contrary, the aberrant *N*-linked $\beta(1,6)$ branching (catalyzed by GnT-V) that occurs in cancer cells reduces homotypic cell–cell adhesion, contributing to increased cellular motility and invasiveness [12,20–21].

The present study, revealed that $\beta(1,6)$ -GlcNAc branched complex type glycan was present in E-cadherin from carcinoma cell line, while absent in E-cadherin from non-malignant adenoma cell line. Indeed, the high PNGaseF sensitivity of E-cadherin from carcinoma cell line further support the presence of complex *N*-glycans on E-cadherin. Our observation is in agreement with the hypothesis that an increased in $\beta(1,6)$ branching of *N*-glycans catalyzed by GnT-V regulates cadherin-associated homotypic cell–cell adhesion and is associated with the acquisition of the malignant phenotype [4,12].

In the present study, we observed a slight increase of $\beta(1,6)$ branched structures in carcinoma tissues, compared with either adenomas, benign lesions as hyperplasias and with adjacent normal mammary tissues, which are in agreement with other studies in woman breast cancer [20]. Interestingly, the tissue localization of $\beta(1,6)$ branched structures expression in carcinomas was different from adenomas. These results may suggest that cells in pre-malignant lesions expressing $\beta(1,6)$ branched oligosaccharides are those with the highest malignant potential. Furthermore, carcinoma cells with higher branched structures are the ones with the highest metastatic potential and thus, the main progenitors of metastases [20,21]. Our findings showing the increase expression of $\beta(1,6)$ branched in the metastatic cells of positive primary tumor supports that hypothesis (Fig. 4K and L).

Concerning the expression of high mannose/hybrid type *N*-glycans, only E-cadherin from carcinoma cell line exhibited this structure, though the lower EndoH sensitivity when comparing with PNGaseF indicates low amounts of these high mannose/hybrid structures.

N-glycans can be terminally sialylated and have also been associated with cancer cell behavior. In our study, we observed that E-cadherin from carcinoma cell line has both types of linked sialic acids ($\alpha(2,6)$ and $\alpha(2,3)$), while E-cadherin from adenoma cell line did not contain these sialylated structures. We also showed a general increase in overall sialylation in carcinoma tissues when compared with adenomas. Our results are in agreement with previous studies showing that increase expression of $\alpha(2,6)$ linked sialic acid in a bone colonizing variant of a human breast cancer cell line has been demonstrated to increase the migration properties of the cells [8].

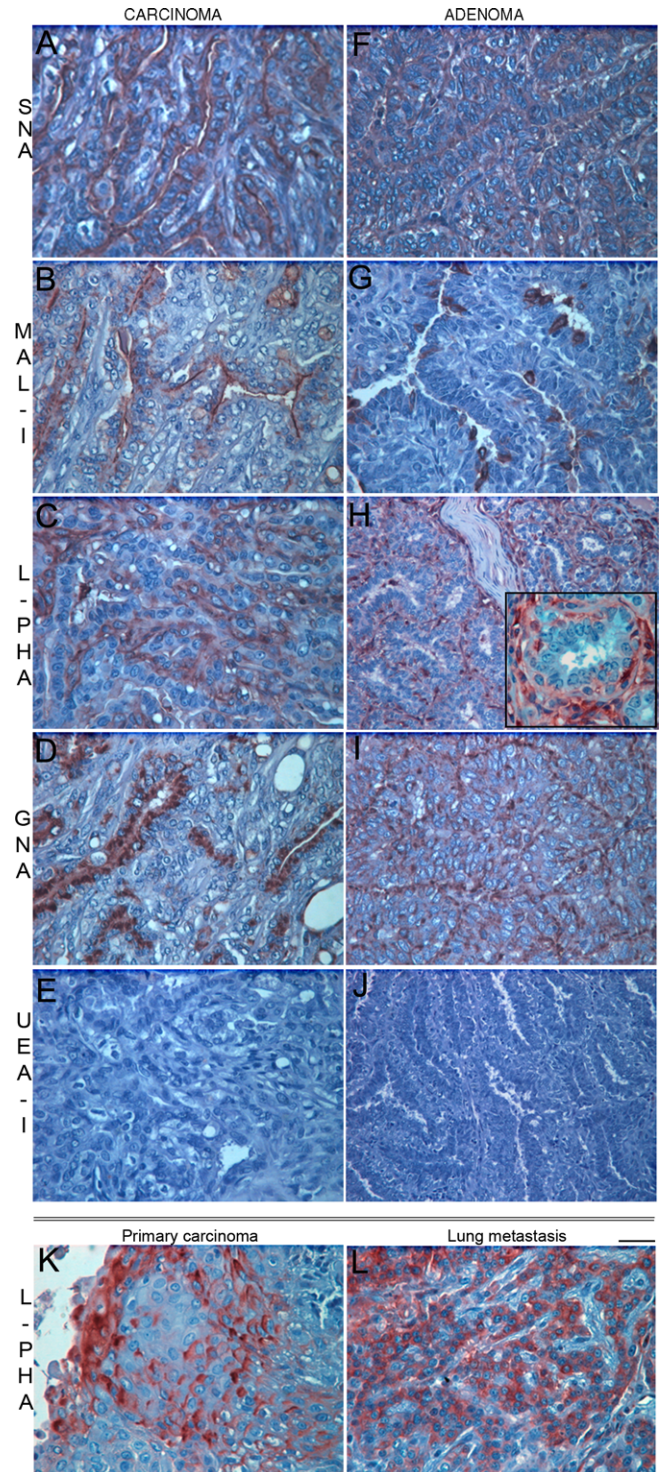


Fig. 4. Tissue glycosylation profiles of mammary gland carcinoma and adenoma tissues using lectin histochemistry. The figure illustrates different lectins labeling results of one carcinoma case (A–E) and one adenoma case (F–J). K and L shows a case of primary carcinoma and its lung metastasis, respectively, labeled with L-PHA. The figure H intends to illustrate at the left side of the conjunctive tissue band, the adenoma and at the right side with a higher magnification insert, an adjacent proliferative lesion—hyperplasia. Bar $\approx$ 25 μ m.

Our results corroborate the evidences regarding the importance of sialylation of terminal oligosaccharide structures not only in the carcinogenic process but also in tumor progression by interfering in cell adhesion machinery mediated by E-cadherin molecule.

Further mass spectrometry characterization of E-cadherin *N*-glycosylation from malignant cell line was able to confirm the identity of E-cadherin with a high confidence interval and include 32 peptides (Fig. 3B and C). Detailed analysis of mass spectrum using the Glycomod software [16,17] detected three peaks potentially corresponding to *N*-glycans in Asn-775 in the sequence Asn-Ser-Cys [5]. From the glycan composition predicted from peak (c) [(Hex)₁(HexNAc)₁(Man)₃(GlcNAc)₂+H]⁺ we deduced that this *N*-glycan is a hybrid/complex type. Furthermore, the L-PHA lectin-blot reactivity and the PNGase F sensitivity of E-cadherin led us to conclude that Asn-775 in sequence Asn-Ser-Cys [5] is *N*-glycosylated with a β 1,6 branched GlcNAc *N*-glycan form.

Three putative sites of *N*-glycosylation, shared by human, mouse and canine have been described in the extracellular domain of E-cadherin [2,3]. Our results demonstrate, for the first time, that canine E-cadherin can have a fourth site (Asn-775) that is *N*-glycosylated most likely with a complex type *N*-glycan, located on the fifth extracellular domain of E-cadherin. This site is also present in mouse but not in human E-cadherin. In conclusion, canine E-cadherin has four *N*-glycosylation sites in the extracellular domain, one in EC4 and 3 in EC5. The demonstration that E-cadherin is *N*-glycosylated by mass spectrometry analysis corroborates the pattern of glycan profile on E-Cadherin using specific lectins.

In summary, our results showed for the first time the characterization of *N*-glycans of E-cadherin molecule and demonstrated modifications in the glycosylation profile of E-cadherin from malignant and non-malignant canine mammary gland tumor cell lines. The cancer related changes in the pattern of E-cadherin *N*-glycosylation included the synthesis of highly branched *N*-glycans, an increase in sialylation and an expression of some high mannose/hybrid structures. These studies support that changes in glycosylation play crucial roles in the process of malignancy of mammary gland and in regulation and modification of E-cadherin-associated cell–cell adhesion which could lead to the malignant behavior of tumor cells. We also demonstrated a new site that is *N*-glycosylated in the EC5 domain of canine E-cadherin, containing a complex type *N*-glycan, possibly a β 1,6 branched GlcNAc form. These results in a model of canine mammary gland tumor open new perspectives in the identification of targets of therapeutic applications.

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Chapter 5

Role of N-acetylglucosaminyltransferase III and V on E-cadherin Post-Translational Modifications

Paper IV

The Role of N-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin

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The role of N-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin

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It has long been recognized that E-cadherin dysfunction is a major cause of epithelial cell invasion. However, very little is known about the post-transcriptional modifications of E-cadherin and its role in E-cadherin mediated tumor progression. N-acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting GlcNAc structure in N-glycans, and has been pointed as a metastasis suppressor. N-acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of β 1,6 GlcNAc branching of N-glycans, and has been associated to increase metastasis. The regulatory mechanism between E-cadherin expression and the remodeling of its oligosaccharides structures by GnT-III and GnT-V were explored in this study. We have demonstrated that wild-type E-cadherin regulates *MGAT3* gene transcription resulting in increased GnT-III expression. We also showed that GnT-III and GnT-V competitively modified E-cadherin N-glycans. The GnT-III knockdown cells revealed a membrane de-localization of E-cadherin leading to its cytoplasmic accumulation. Further, the GnT-III knockdown cells also caused modifications of E-cadherin N-glycans catalyzed by GnT-III and GnT-V. Altogether our results have clarified the existence of a bidirectional crosstalk between E-cadherin and GnT-III/GnT-V that was, for the first time, reproduced in an *in vivo* model. This study opens new insights into the post-transcriptional modifications of E-cadherin in its biological function, in a tumor context.

INTRODUCTION

The remodeling of cell surface glycoproteins oligosaccharides by glycosyltransferases has been revealed to be crucial for several functional aspects of glycoproteins. These oligosaccharides play important roles in cell physiology, such as differentiation, adhesion, cell proliferation and in pathological conditions such as cancer where it has been shown to contribute to cell invasion and metastases (1). The oligosaccharides of glycoproteins are produced via catalysis by several glycosyltransferases and most of the cancer-associated changes of sugar chains of glycoproteins are due to the changes of glycosyltransferases. An increasing body of evidence suggests that phenotypic changes observed in cancer cells and tissues seem to be due

to a direct effect of glycosyltransferases genes or an indirect one (1,2). Functional glycomics, which uses sugar remodeling by glycosyltransferases, is a promising tool for the understanding and characterization of glycan roles (3).

N-glycosylation represents an important protein post-translational modification, where N-glycans are attached to asparagine residues within the consensus sequence NX(S/T), where X can be any amino acid other than proline (4). N-glycans can be divided in three major groups: High Mannose-type; Complex-type and Hybrid-type (5). Two of the major glycosyltransferases involved in N-glycan biosynthesis are N-acetylglucosaminyltransferase III (GnT-III) and N-acetylglucosaminyltransferase V (GnT-V).

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GnT-III is a glycosyltransferase, product of the human *MGAT3* gene, which transfers N-acetylglucosamine (GlcNAc) in β 4-linkage to the core mannose of complex or hybrid N-glycans forming a bisecting GlcNAc linkage. The addition of this bisecting GlcNAc residue alters not only the composition, but also the conformation of the N-glycan (6). GnT-III is considered a key glycosyltransferase in N-glycan biosynthetic pathway since the introduction of the bisecting GlcNAc residue results in the suppression of further processing and elongation of N-glycans, precluding the formation of β 1,6 GlcNAc branching, catalyzed by GnT-V since it is unable to use the bisected oligosaccharide as a substrate (7,8). GnT-III has been shown to be a metastases suppressor. When GnT-III gene was transfected into melanoma B16 cells with high metastatic potential, the sugar chains on the cell surface were remodeled and the metastatic potential impaired. Additionally, it was shown that lung colonization after mice intravenous administration of the GnT-III transfectants was significantly decreased (9).

Contrary to the function of GnT-III, GnT-V activity and its product, the β 1,6 branched N-glycans, were significantly increased in highly metastatic tumors (10). Consistently, in GnT-V knockout mice cancer metastasis is greatly suppressed (11). Thus, the enzymatic activity of GnT-III has been proposed to have an antagonistic effect to the one of GnT-V (7).

The enzymatic competition between GnT-III and GnT-V, where GnT-III has been shown to exhibit a priority activity, contributes to the suppression of cancer metastasis. In fact, overexpression of GnT-III in highly metastatic melanoma cells reduced β 1,6 GlcNAc branching in cell surface N-glycans, which has been shown to be accompanied with an increase of bisecting GlcNAc N-glycans (9,12). Along with this enzymatic competition between GnT-III and GnT-V, cells expressing GnT-III showed an enhanced cell-cell adhesion through the prolonged turnover of E-cadherin on the cell surface (13). Conversely, GnT-V overexpression, accompanied with an increase of β 1,6 branched structures, resulted in a decrease in the rates of N-cadherin mediated cell-cell adhesion (14). Overall, these results suggest the existence of a putative co-modulation of distinct cadherins controlled by glycosyltransferases GnT-III and GnT-V.

It is a long-last interest of our group to identify molecular mechanisms underlying E-cadherin dysfunction in cancer development and progression. Most studies mainly focus on the role of epi/genetic alterations in its promoter region and in its coding sequence or in its transcriptional repressors (15–20). However, N-glycans have been shown to be essential for E-cadherin folding and to assure its biological functions (21,22). Since E-cadherin is a glycoprotein that can be post-transcriptionally modified by phosphorylation, O-glycosylation and N-glycosylation, in the present work, we aimed to elucidate the role of GnT-III and GnT-V glycosyltransferases and its products, in E-cadherin expression.

We found that GnT-III knockdown resulted in a remarkable modification of cell morphology accompanied by a membranar de-localization of E-cadherin leading to an increase of aberrant cytoplasmic expression. Further, we verified that E-cadherin regulates the transcription levels of GnT-III which through competition with GnT-V glycosylate E-cadherin molecule. In primary gastric carcinoma, we validate our *in vitro*

model and showed the mutual regulatory mechanism between E-cadherin and GnT-III/GnT-V.

RESULTS

Stable transfection of E-cadherin wild-type in MDA-MB435 cells induced alterations in GnT-III mRNA transcription levels

The stable transfection of a full-length human E-cadherin in MDA-MB435 cells, that endogenously lack E-cadherin, induces an increase in transcription levels of *MGAT3* gene (Fig. 1A). Quantitative real-time PCR analysis further confirmed that GnT-III mRNA transcription levels in MDA-MB435 transfected with E-cadherin wild-type (wt) were $\sim$ 2-fold higher than those in MDA-MB435 mock cells (Fig. 1B). The same was not verified for GnT-V mRNA expression levels since no alterations of this enzyme were verified, upon stable transfection of wt E-cadherin (data not shown).

Increase GnT-III mRNA expression after E-cadherin transfection altered the overall protein glycosylation in general and E-cadherin glycosylation in particular

Due to the significant increase of GnT-III mRNA expression mediated by E-cadherin transfection in MDA-MB435 cells, we evaluated the N-linked glycosylation in the total cell lysates from MDA-MB435 E-cadherin negative mock cells and from MDA-MB435 transfected with wt E-cadherin and on E-cadherin immunoprecipitated from these later MDA-MB435 cells. L-PHA lectin, that specifically recognizes β 1,6 branched structures (product of GnT-V), and E-PHA lectin, which recognizes bisecting GlcNAc structure (product of GnT-III), were used in order to analyze the alterations in the N-glycosylation pattern of glycoproteins and on E-cadherin immunoprecipitated. Figure 2A shows that the introduction of E-cadherin in MDA-MB435 cells resulted in a notable decrease of GnT-V product (β 1,6 branched structures) in the whole glycoprotein lysate, as revealed by decrease L-PHA reactivity in MDA-MB435 wt E-cadherin derived cells. The differences in GnT-III product (E-PHA blot) were not easily detected between mock and transfected cells, however, a slight increase in E-PHA reactivity in MDA-MB435+E-cadWT cells could be observed.

In order to evaluate whether E-cadherin is targeted for modifications by GnT-III and GnT-V, E-cadherin isolated from MDA-MB435 wt E-cadherin transfected cells was immunoprecipitated and probed with L-PHA and E-PHA lectin (Fig. 2B). The Figure 2B shows that E-cadherin was targeted by both GnT-V and GnT-III enzymes, being modified with β 1,6 branched structures (L-PHA positive reactivity) and with bisected GlcNAc structures (E-PHA positive reactivity).

Knockdown of GnT-III by siRNA caused no alteration in expression levels of E-cadherin mRNA and protein, but induces alterations on E-cadherin cellular localization in MCF-7/AZ cells

Assuming that E-cadherin has a role in the regulation of GnT-III expression which in turn glycosylates E-cadherin,

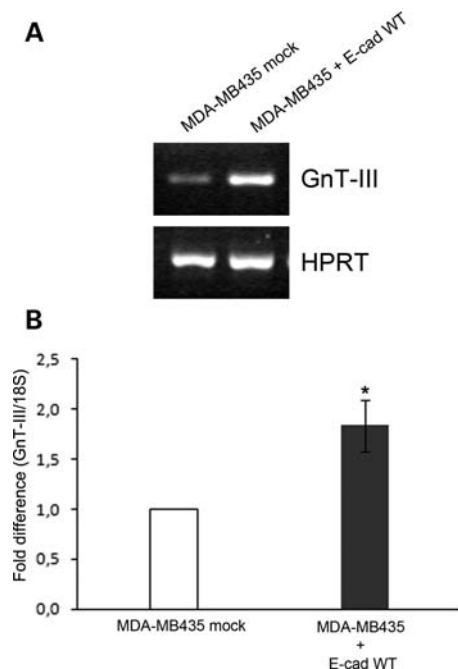


Figure 1. Stable transfection of E-cadherin wild-type in MDA-MB435 cells resulted in a significant increase of GnT-III mRNA transcription levels. (A) PCR for mRNA expression of GnT-III in MDA-MB435 mock cells (left lane) and in MDA-MB435 stably transfected with E-cadherin wild-type (WT) cells (right lane). The figure illustrates a remarkable increase of GnT-III mRNA expression after E-cadherin WT transfection; HPRT was used as the housekeeping gene. (B) Quantitative real-time PCR analysis of GnT-III, normalized to 18S housekeeping gene. The mRNA expression levels of GnT-III in MDA-MB435+E-cadWT are expressed as the fold increase, compared with the expression level of GnT-III in MDA-MB435 mock cells, which was taken as 1 (Student's *t*-test: **P* = 0.005).

we silenced, by siRNA, GnT-III in order to breakdown this loop and in this way evaluate the role of GnT-III on E-cadherin glycoprotein. For that we used MCF-7/AZ cell line which endogenously expresses wild-type E-cadherin. The levels of GnT-III mRNA expression in MCF-7/AZ cells are similar with MDA-MB435+E-cadWT cells (Fig. 3).

As shown in Figure 4A, there was no alteration in E-cadherin mRNA transcription levels after GnT-III knockdown compared with parental cells (MCF-7/AZ) and with controls cells (mock cells and non-silencing siRNA cells). Moreover, the levels of GnT-V mRNA transcription levels were also not affected (data not shown) by GnT-III knockdown. Additionally, no alteration in E-cadherin protein expression was observed after GnT-III knockdown cells compared with parental and controls cells, as illustrated in Figure 4B.

However, the same was not verified concerning E-cadherin localization at cell membrane when cells were treated with siRNA for GnT-III. E-cadherin cellular localization was evaluated by immunofluorescence with an antibody that recognizes the cytoplasmic domain of E-cadherin. In GnT-III knockdown MCF-7/AZ cells, E-cadherin modified its pattern of expression exhibiting an increase of the cytoplasmic staining, compared with non-silencing cells. The membranar expression was limited only to focal points of intercellular contact. Figure 4C illustrates that GnT-III knockdown leads to a de-localization of E-cadherin membranar expression.

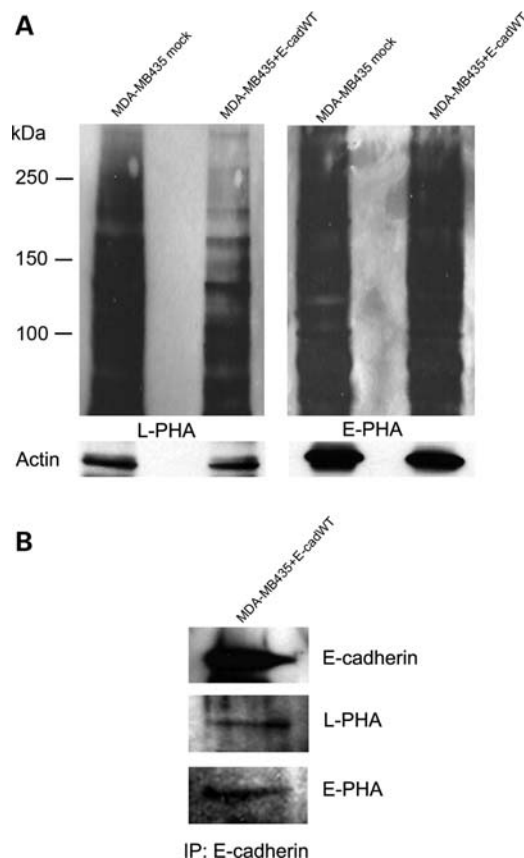


Figure 2. Analysis of GnT-III and GnT-V product on total cell lysates and on E-cadherin immunoprecipitated from MDA-MB435 cells. (A) Lectin blot analysis with L-PHA and E-PHA lectins in total cell lysates from MDA-MB435 mock and MDA-MB435+E-cadWT cells. (A) illustrates a significant decrease of L-PHA reactivity after E-cadherin transfection in whole glycoprotein lysate. (B) E-cadherin immunoprecipitated from MDA-MB435+E-cadWT was subjected to L-PHA and E-PHA lectin blot analysis. The figure shows a positive reactivity of both L-PHA and E-PHA on E-cadherin glycoprotein.

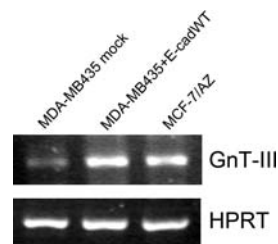


Figure 3. GnT-III mRNA expression of MCF-7/AZ cell line compared with MDA-MB435 mock and with MDA-MB435+E-cadWT.

Moreover, MCF-7/AZ cells treated with siRNA for GnT-III showed notable modification of the cellular phenotype. Figure 4C (bottom) shows a disruption of the cell-cell contact with an increase of lamellipodia and filopodia formations. When MCF-7/AZ cell line which endogenously expresses E-cadherin are treated with siRNA for GnT-III, the cells maintain the contact with neighboring cells only by cytoplasmic extensions. In control cells (non-silencing siRNA cells, subjected to the same experimental conditions

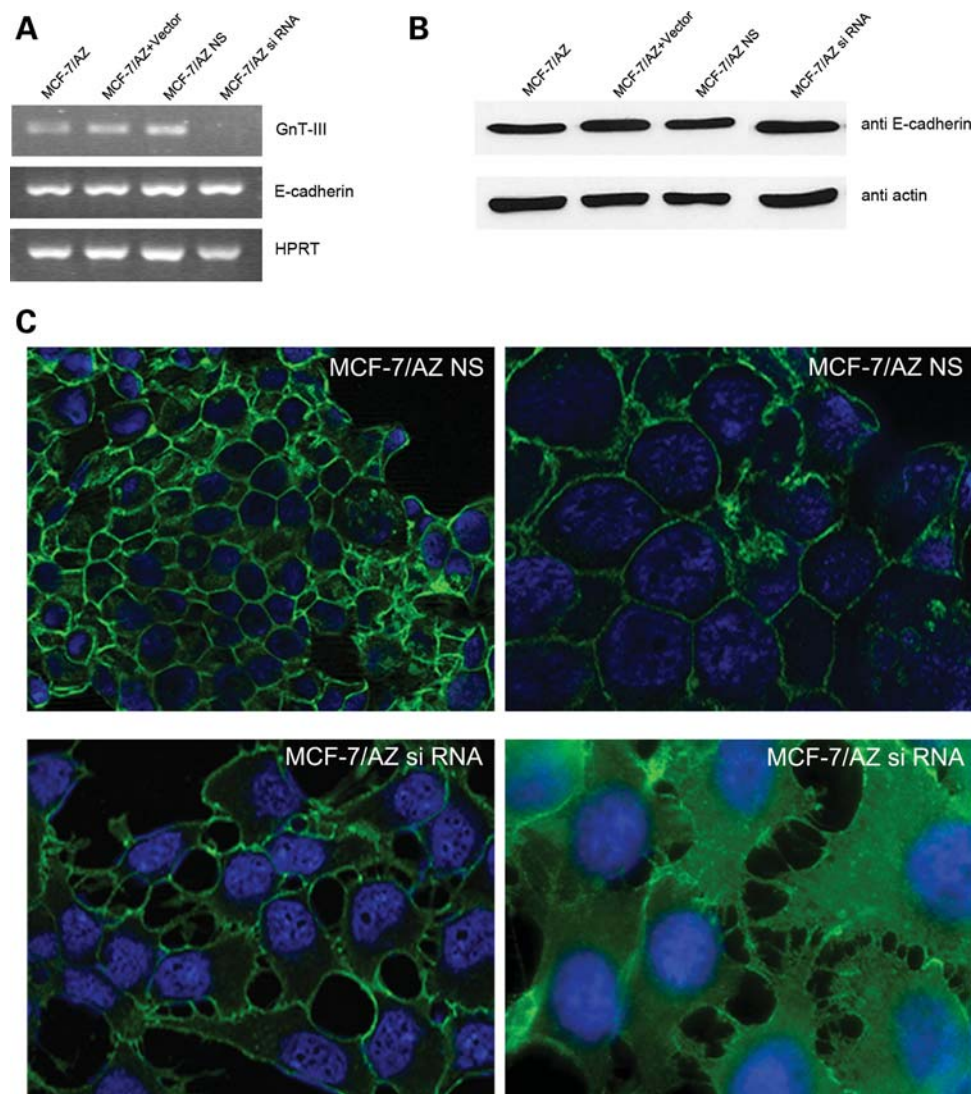


Figure 4. E-cadherin expression (A, RNA; B, protein and C, cellular localization) after GnT-III siRNA transfection at MCF-7/AZ cells. (A) PCR for mRNA expression of E-cadherin in MCF-7/AZ cells after GnT-III siRNA. The figure shows that there is no alteration of E-cadherin mRNA transcription levels after GnT-III silencing. (B) Western blot analysis from parental cells (MCF-7/AZ), controls [vector and non-silencing (NS)] and GnT-III knockdown cells probed with antibody against E-cadherin and actin (loading control). The figure demonstrates that no alterations in E-cadherin protein levels were observed following GnT-III silencing. (C) Immunofluorescence labeling of E-cadherin in non-silencing (NS) cells (top) and GnT-III knockdown cells (bottom). In MCF-7/AZ NS, cells form a compact monolayer with E-cadherin present at the cell–cell contacts, exhibiting a typical membranar pattern of expression. In MCF-7/AZ GnT-III siRNA, there was a disruption of cell–cell contacts, with an increase of cytoplasmic staining of E-cadherin comparing with non-silencing cells. Right lane represents a higher magnification ($63\times$) of left lane ($40\times$).

as GnT-III knockdown cells), E-cadherin was predominantly expressed in the cell membrane and cells form a compact monolayer (Fig. 4C, top).

Knockdown of GnT-III caused changes in N-glycosylation in the whole glycoprotein lysate and specifically in E-cadherin

We evaluated whether GnT-III silencing could modify the N-glycosylation profile in terms of β 1,6 branched structures (GnT-V product) and bisecting GlcNAc structures (GnT-III product) in glycoproteins using total cell lysates and immunoprecipitated E-cadherin. Total cell lysates and immunoprecipitated

E-cadherin, from both control cells and GnT-III siRNA cells, were probed with E-PHA or L-PHA lectin (Fig. 5). Figure 5A (top) shows that the GnT-III knockdown resulted in a decrease of bisecting GlcNAc structure (E-PHA reactivity) compared with the control cells (Fig. 5A, left). Along with this observation, there was an increase of β 1,6 branched structures (L-PHA blot) after GnT-III knockdown, compared with control cells (Fig. 5A, right). Taking into account these observations in whole glycoprotein lysates, we further evaluated specifically the alterations in E-cadherin N-glycosylation after GnT-III silencing. Figure 5B (bottom) shows that GnT-III knockdown resulted in a decrease of bisecting GlcNAc structure (E-PHA reactivity) accompanied with an

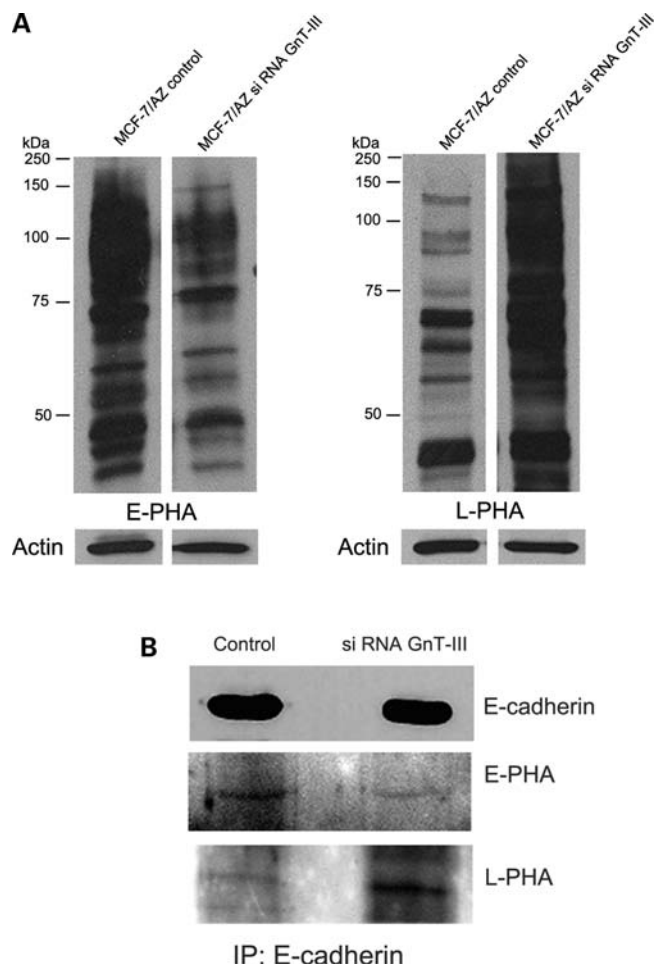


Figure 5. Evaluation of GnT-III and GnT-V products after GnT-III silencing. (A) Total cell lysates from MCF-7/AZ cells and GnT-III Knockdown cells were subjected to blot analysis with E-PHA and L-PHA lectins. As expected there was a decrease product of GnT-III (E-PHA blot) after GnT-III silencing. On the contrary, there was an increase product of GnT-V (L-PHA blot) following GnT-III knockdown. (B) E-cadherin immunoprecipitated from both MCF-7/AZ NS control cells and GnT-III siRNA cells was blotted with E-PHA and L-PHA lectin. There was a significant increase of L-PHA positivity in E-cadherin from GnT-III silencing cells, comparing with NS control cells.

increase of β 1,6 branched structures (L-PHA reactivity) on E-cadherin glycoprotein when compared with control cells.

Relationship between expression of E-cadherin, bisecting GlcNAc and β 1,6 branched N-glycans structures expression in sporadic diffuse gastric carcinomas

In order to examine whether the relationship between E-cadherin, GnT-III and GnT-V observed *in vitro* was also observed *in vivo*, we performed E-cadherin, E-PHA and L-PHA histochemistry in a small series of diffuse gastric carcinoma, that harbor in a large percentage of cases alterations of E-cadherin expression. In all cases studied of diffuse gastric carcinoma, there was a significant loss of expression of E-cadherin which varied between <5% and 5–25% of tumor cell expression (Fig. 6, top). E-PHA reactivity, detecting

bisecting GlcNAc structures, also shows a lower expression in diffuse gastric carcinomas that varied between <5% and 25–50% of expression. The E-PHA reactivity was mainly observed in the cytoplasm of tumor cells (Fig. 6, middle). On the contrary, L-PHA reactivity was consistently higher in all isolated tumor cells from diffuse gastric carcinomas (50–75% to >75% of L-PHA reactivity). Almost all isolated tumor cells labeled with L-PHA lectin, which stained in the cytoplasm and/or in the cell membrane (Fig. 6, bottom). Figure 6 illustrates in the same case of diffuse gastric carcinoma a low expression level of both E-cadherin and GnT-III product (E-PHA reactivity), and a higher expression level of GnT-V product (L-PHA reactivity), validating our *in vitro* model system.

DISCUSSION

E-cadherin is a cell–cell adhesion molecule that plays a pivotal role in epithelial cell invasion and has been considered the major contributor to cancer progression (23). In spite of a wide variety of studies focusing on the epi/genetic regulation on the E-cadherin-switch-off during tumorigenesis, post-translational mechanisms of regulation of this molecule remain largely unknown (15–20,24).

E-cadherin is a glycoprotein and glycan modifications have been shown to play key roles in the regulation of diverse proteins and also in fundamental cellular processes such as cell differentiation, adhesion and proliferation (25). Recently, the importance of E-cadherin N-glycosylation in the folding, stability and in its adhesive functions has been reported (21,22,26). The aim of the present study was to evaluate the relationship between E-cadherin expression, GnT-III and GnT-V glycosyltransferases and its products, namely, bisecting GlcNAc structures and β 1,6 branched structures, respectively. This knowledge will identify new regulatory mechanisms of E-cadherin that are likely to contribute to cancer progression, namely in diffuse gastric carcinoma negative for E-cadherin genomic alterations.

We have demonstrated for the first time that E-cadherin regulates *MGAT3* gene transcription and leads to a significant up-regulation of GnT-III mRNA transcription levels. Our studies are in accordance to the reports of Iijima *et al.* (12) and Akama *et al.* (27) that suggested that E-cadherin–catenin–actin complex plays an important role in the regulation of GnT-III enzymatic activity, since GnT-III activity increases in confluent culture conditions compared with sparse culture conditions.

Changes in glycosyltransferases expression levels have impact in the remodeling of cell surface glycoproteins oligosaccharides, which may consequently affect several cellular mechanisms such as intercellular adhesion (1–3). Thus, it would be expectable that the increase of GnT-III expression observed after E-cadherin transfection would change the N-glycosylation profile of the glycoproteins. Accordingly, we found that the up-regulation of GnT-III resulted in a significant decrease of β 1,6 branched structures, product of GnT-V, on glycoproteins, showing that GnT-III and GnT-V modify the same target glycoprotein in a competitive fashion, as previously shown by Zhao *et al.* (7).

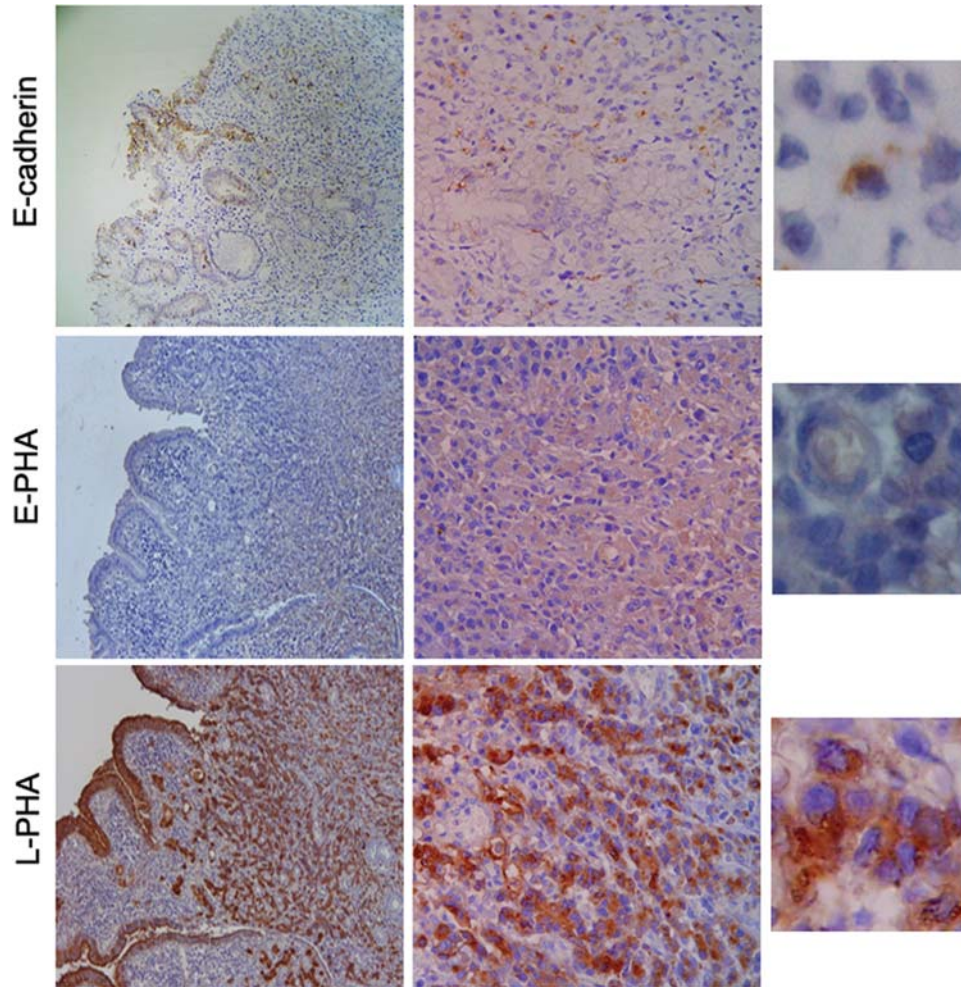


Figure 6. *In vivo* representation of the relationship between expression of E-cadherin, GnT-III product and GnT-V product in a diffuse gastric carcinoma. The figure illustrates the immunoreactivity of E-cadherin, E-PHA and L-PHA in the same tissue sample (diffuse gastric carcinoma). In terms of E-cadherin expression, <5% of tumor cells were positive to E-cadherin. In the same way, <5% of epithelial tumor cells exhibited a positive reactivity for E-PHA lectin. On the contrary, >95% of tumor cells were positive for GnT-V product (L-PHA). Left panel, light microscopy 20 $\times$ magnification; middle panel, 40 $\times$ magnification. The right panel represents magnified images of the middle panel.

In fact, when E-cadherin glycoprotein was stably transfected in MDA-MB435 cells, which are E-cadherin negative cells, was N-glycosylated by GnT-III (as demonstrated by presence of bisecting GlcNAc structures) and by GnT-V (presence of β 1,6 GlcNAc branched structures). This post-translational modification of E-cadherin by GnT-III is functionally relevant since it has been previously suggested that glycosylation of E-cadherin by GnT-III has a role in metastases suppression (13,28).

Taking into account all above-mentioned observations, we can conclude that E-cadherin up-regulates GnT-III expression which in turn modifies E-cadherin N-glycans, adding bisecting GlcNAc structures suggesting the existence of a functional feedback loop between E-cadherin and GnT-III, illustrated in Figure 7A and B.

In order to clarify the effect of this post-transcriptional modification on E-cadherin carried out by GnT-III, we disrupted this functional loop (Fig. 7A and B) by silencing GnT-III expression. To address this question, we used MCF-7/AZ cell line that endogenously expresses E-cadherin and GnT-III, and

verified that GnT-III knockdown did not affect E-cadherin transcriptional or transductional levels. However, when we compared cells silenced for GnT-III with cells treated with a scramble siRNA, there was a remarkable modification of the cellular phenotype. GnT-III knockdown MCF-7/AZ cells extensively lose their intercellular contacts, showing an increase of lamellipodia and filopodia extrusions, and E-cadherin membranar staining was persistent only in the focal points of intercellular contact. Moreover, there was an increase of cytoplasmic staining of E-cadherin comparing with control cells showing that GnT-III knockdown resulted in a membranar de-localization of E-cadherin with repercussions in the cellular phenotype. The cytoplasmic internalization of E-cadherin observed after GnT-III knockdown is in agreement with previous reports that described that the pool of E-cadherin undergoing endocytosis and recycling was markedly increased in cells without stable cell–cell contacts (29).

In response to GnT-III knockdown, we further evaluated the modifications in N-glycosylation profile of glycoproteins in general and on E-cadherin in particular. As expected, we

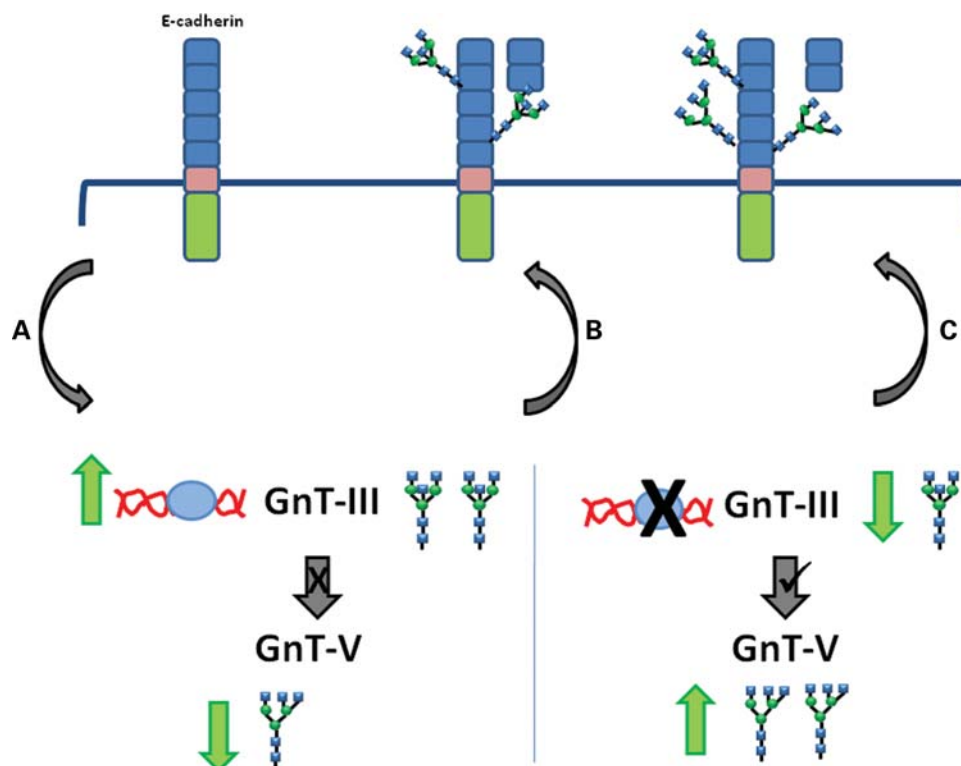


Figure 7. Proposing model of the relationship between E-cadherin glycoprotein, GnT-III and GnT-V glycosyltransferases. E-cadherin glycoprotein regulates *MGAT3* gene transcription levels, increasing GnT-III mRNA expression. This GnT-III up-regulation resulted in an increase of bisecting GlcNAc structures and a decrease of β 1,6 branched structures, through competition with GnT-V glycosyltransferase (A). Concomitantly, E-cadherin is modified with bisecting GlcNAc structures, as a result of GnT-III enzymatic catalysis (B). When this system [(A) and (B)] is perturbed by GnT-III siRNA, there is a decrease of bisecting GlcNAc structures which allows GnT-V activity and thus an increase of β 1,6 branched structures. E-cadherin N-glycans are remodeled by a decrease in bisecting GlcNAc structures and an increase in β 1,6 branched structures (C).

observed that in fact the GnT-III knockdown resulted in a decrease of bisecting GlcNAc structures on glycoproteins. In consequence, GnT-V has gained ability to synthesize β 1,6 GlcNAc branched N-glycans, resulting in an increase of this product on glycoproteins. This remodeling of N-glycans was specifically observed in E-cadherin resulting in an increase of β 1,6 GlcNAc branched structures, product of GnT-V, and a decrease of bisecting GlcNAc structures on E-cadherin molecule as presented in our model (Fig. 7C), leading to alterations of cell morphology and disruption of cell–cell contacts.

In conclusion, our *in vitro* model shows a dynamic regulatory mechanism between E-cadherin and glycosyltransferases, GnT-III and GnT-V supporting the hypothesis that E-cadherin-mediated cell–cell adhesion may occur via remodeling of N-glycans controlled by glycosyltransferases (GnT-III and GnT-V) whose regulation and expression (GnT-III) can also be dependent of E-cadherin expression (see proposed model, Fig. 7).

This crosstalk between E-cadherin and GnT-III/GnT-V reported here *in vitro*, was further confirmed for the first time, in an *in vivo* model. We had chosen a model of Diffuse Gastric Carcinoma, based on the fact that E-cadherin deregulation is a common feature of these tumors (30). Mutations in the E-cadherin gene (*CDH1*) have been found only in Diffuse Gastric Cancer (31). The present study has demonstrated that in all cases of diffuse gastric carcinomas, a decrease or loss of E-cadherin expression was accompanied

with a decrease of bisecting GlcNAc N-glycans, product of GnT-III. Conversely, GnT-V product remarkably increased with almost all isolated carcinoma cells labeled with L-PHA lectin (which looks like a tumor marker). Thus, the down-regulation of E-cadherin expression, observed in those carcinomas, suggest that, therefore, it was unable to regulate the transcription of GnT-III leading to a decrease of bisecting GlcNAc synthesis. Furthermore, the reduced enzymatic competition with GnT-V induced a significant increase of β 1,6 GlcNAc branched synthesis.

In summary, our study provides strong evidences for a regulatory mechanism between E-cadherin, GnT-III and GnT-V (by enzymatic competition) which could underlie a possible novel signaling pathway in carcinogenesis. Moreover, our results open new insights into E-cadherin regulation, emphasizing its post-transcriptional modifications as another possible mechanism that could underlie E-cadherin dysfunction in cancer.

MATERIAL AND METHODS

Cell culture

Human MDA-MB435 cell line, which endogenously lacks E-cadherin expression at both the mRNA and protein level, and MDA-MB435 stably transfected with wild-type E-cadherin (MDA-MB435 + E-cadWT) cells (A.C.F. and J.C. Machado, manuscript in preparation) were maintained at

37°C in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, under a humidified atmosphere containing 5% CO₂. Human MCF-7/AZ breast cancer cell line, which was kindly provided by Prof. Marc Mareel (Laboratory of Experimental Cancerology, Ghent University, Belgium), was maintained under the same culture conditions. MDA-MB435+E-cadWT cells were maintained under blasticidin selection (5 µg/ml; Invitrogen). GnT-III knockdown in MCF-7/AZ cells was generated through siRNA technique.

RNA extraction and analysis of mRNA expression by RT-PCR and real-time PCR

Total RNA from MDA-MB435 mock, MDA-MB435 + E-cadWT and MCF-7/AZ cells was extracted with Tri-Reagent (Sigma) according to the manufacturer's protocol. RNA yield and quality were determined spectrophotometrically, and 5.0 µg of total RNA was reverse transcribed using the Superscript III RNase H Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. Primers used in PCR analysis for GnT-III and hypoxanthine phosphoribosyltransferase (HPRT) mRNA expressions were the following: human GnT-III forward (5'-GCCGCGTCATCAACGCC ATCAA-3') and human GnT-III reverse (5'-CAGGTAGTC GTCGGCATCCA-3'); human HPRT forward (5'-TTCC TCCTCCTGAGCAGTCAG-3') and human HPRT reverse (5'-GCGATGTCAATAGGACTCCAGAT-3'). The product sizes obtained by the PCR were 260 bp for human GnT-III and 756 bp for human HPRT. The PCR conditions used for both gene analyses were 35 cycles of 15 s at 94°C, 30 s at 65°C and 2 min at 70°C. Reaction products obtained were submitted to electrophoresis in 1.6% agarose gels containing ethidium bromide, and bands were quantified using Quantity One software (BioRad). Quantitative real-time PCR analysis of GnT-III mRNA expression was performed using SYBRgreen reagent (Applied Biosystem). Each sample was amplified in triplicate in an ABI Prism 7000 (Applied Biosystem). Expression of 18S was also measured in triplicate for each sample and used for normalization of target gene abundance. Specificity of amplification was confirmed by melt curve analysis. Primers used in the quantitative real-time PCR analysis were as follows: human GnT-III forward (5'-GCGTCATC AACGCCATCAA-3') and human GnT-III reverse (5'-TGG ACTCGCACACCACAAAG-3'); 18S forward (5'-CGCCG CTAGAGGTGAAATTC-3') and 18S reverse (5'-CATTC TTGGCAAATGCTTTCG-3'). The PCR conditions were 45 cycles at 95°C for 5 s and 60°C for 30 s. Results of GnT-III expression levels are presented as the fold increase of MDA-MB435+E-cadWT cells, compared with MDA-MB435 mock cells which was taken as 1. In order to statistically analyze the significance of the GnT-III/18S fold difference between MDA-MB435 mock cells and MDA-MB435 + E-cadWT cells, a Student's *t*-test, with Microsoft Excel, was performed in the triplicates.

Immunoprecipitation, western blot and lectin blot analysis

Cell cultures were washed with phosphate-buffered saline (PBS) and then lysed in cold PBS containing 1% Triton

X-100, 1% NP40, protease inhibitor cocktail (Roche 1 tablet/50 ml buffer) and phosphatase inhibitor cocktail (Sigma, 1:100 dilution). Total protein was quantified using a BCA protein assay kit (Pierce). Equal amounts of total protein (750 µg) from each cell lysate were precleared with 25 µl of protein G-sepharose beads (Sigma) for 1–2 h. After centrifugation, the supernatant was incubated overnight with 5 µg of monoclonal antibody against E-cadherin (BD Biosciences). After that, incubation with protein G-sepharose for 2 h was performed. Next, the beads were washed three times with immunoprecipitation buffer. The immune complexes were released by boiling for 5 min at 95°C in Laemmli sampling buffer and the immunoprecipitates were subjected to 7.5% SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane. The blots were then probed with anti-E-cadherin antibody or biotinylated *Phaseolus vulgaris* erythroagglutinin (E-PHA, which binds to bisecting GlNAc structures) or biotinylated *P. vulgaris* leucoagglutinin (L-PHA, which recognizes β1,6 branched structures) lectins (Vector Laboratories). Immunoreactive bands from lectin blots were then visualized using the Vectorstain ABC kit (Vector Laboratories) and detection was performed by an ECL plus reagent.

Equal amounts of total cell lysates were also subjected to SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated with L-PHA and E-PHA lectin for lectin blot analysis or with anti-Ecadherin antibody for immunoblot analysis. Blots probed with anti-actin antibody (Santa Cruz Biotechnology) were performed for loading control analysis.

siRNA transfection

A set of four different siRNAs targeting GnT-III mRNA were purchased from Dharmacon (IL, USA), and prepared according to manufacturer's instructions. In parallel, non-silencing siRNA duplexes were used as a negative control. Prior to transfection, 60% confluent monolayers of MCF-7/AZ cells plated onto six-well plates were washed with PBS and incubated in serum and antibiotic-free medium. Cells were transiently transfected with 0–200 nM of siRNA, using the Lipofectamine 2000 transfection reagent (Invitrogen). At the end of each transfection, putative cytotoxic effects were evaluated, analyzing cell viability by trypan blue dye exclusion test. Efficiency of GnT-III knockdown was maximum with 150 nM of siRNA at 24 h.

E-cadherin staining

MCF-7/AZ cells were plated on six-well plates with coverslips on the bottom of each well. GnT-III siRNA transfection using the optimized conditions described earlier was performed. After the siRNA experiment, cells were fixed and incubated with anti-E-cadherin monoclonal antibody and with Alexa Fluor® 488 anti-mouse secondary antibody (Invitrogen). The nuclear staining was performed and images were then visualized in a fluorescence microscope (Zeiss).

Histochemistry

Formalin-fixed tissues were used. For E-cadherin staining, heat-induced antigen retrieval was done by microwave in

citrate buffer (pH 6.0) for 10 min, after blocking the endogenous peroxidase. The sections were stained with anti-E-cadherin monoclonal antibody or with biotinylated lectins E-PHA or L-PHA (Vector Laboratories), according to manufacturer's instructions. For E-cadherin staining, slides were incubated for 30 min with a 1:200 dilution of biotin-labeled rabbit anti-mouse secondary antibody (Dako). Incubation with avidin-biotin-peroxidase complex method using a commercial kit (Vectastain ABC Kit, Vector Laboratories) was then performed. 3,3'-diaminobenzidine (DAB) was used as chromogen and hematoxylin was used as a counterstain. Six human gastric adenocarcinoma, classified as diffuse type following the Lauren's classification (32) or as isolated cell type accordingly to Carneiro's classification (33) were analyzed. E-cadherin, bisecting GlcNAc structures and β 1,6 branched structures were labeled using monoclonal antibody against E-cadherin (BD Biosciences), E-PHA and L-PHA lectins, respectively. The degree of E-cadherin and lectins reactivity was evaluated by three independent observers and estimated visually for the entire lesion as follows: <5% of labeled cells; 5–25%; 25–50%; 50–75% and >75% of positive cells. The negative control sections were treated with PBS.

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Conflict of Interest statement. None declared.

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Chapter 6

General Discussion

Summary and Concluding Remarks

GENERAL DISCUSSION

Cancer is one of the leading causes of death worldwide. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007. Cancer can also affect all mammals.

Aberrant glycosylation is a universal feature of cancer cells and certain types of glycan structures are well-known markers for tumour progression. Also, it is now very clear that aberrant glycosylation profoundly affects processes such as apoptosis, angiogenesis, integrin and cadherin function, modulation of invasive phenotype and metastasis, growth factor receptor activation and immune response towards cancer cells. Therefore and given the role of aberrant glycosylation patterns as important markers for certain disease states including cancer, studies in glycoscience are now being revealed very promising. In spite of technically difficult, and simultaneously very challenging, Glycobiology is unequivocally essential for understanding the critical functions of glycans in cancer biology. The present thesis focuses in a specific issue within this large field of research.

It is well known that a large number of proteins undergo post-translational modification, such as glycosylation, with corresponding changes in their structures and functions. In addition, aberrant glycosylation in proteins is known to greatly affect several cellular functions including cell adhesion. Lined up with this, E-cadherin is one of the most important cell-cell adhesion molecule having a central role in cancer. The disruption of E-cadherin-mediated cell adhesion appears to be a crucial event in the transition from noninvasive to invasive carcinomas. Therefore, most studies have been focused on the identification and characterization of the mechanisms of E-cadherin-switch-off during tumourigenesis. Currently, the vast majority of those studies are focusing on the epi/genetic regulation of E-cadherin dysfunction. However, being a glycoprotein, E-cadherin can be N-glycosylated. Therefore and taking into consideration the emerging roles of N-glycans on E-cadherin biology (Liwosz et al. 2006; Zhao et al. 2008a; Zhou et al. 2008), the study of the significance and the function of N-linked glycans on E-cadherin-mediated tumour progression is of major importance, being thereby addressed in the present thesis.

Canine mammary cancer is a very appealing model to study glycosylation alterations and the role of these alterations. It is a spontaneous model of carcinogenesis that gives us a valuable perspective of cancer from the biological point of view. In the present work, the

result of integrating this spontaneous model of canine carcinogenesis in an attempt to uncover the functional roles of glycans in the process of tumour development and progression, has greatly contributed to the increasing knowledge of Glycobiology in cancer science.

Biological role of Sialyl Lewis x antigen in canine mammary carcinoma model and its relationship with E-cadherin adhesion molecule.

Malignant transformation is associated with abnormal glycosylation, resulting in expression of altered carbohydrate determinants (Dube and Bertozzi 2005; Fuster and Esko 2005).

The concept of glycosylation-dependent promotion or inhibition of tumour progression has developed in conjunction with clinicopathological studies. High expression of some glycosyl epitopes promotes invasion and metastasis, leading to shorter survival rates of patients (Hakomori 1996). Along with this, one of the most extensively studied epitopes having prometastatic effects deals with Sialyl Lewis x (sLe^x) antigen (Kannagi et al. 2004; Kannagi 2004).

sLe^x has been identified as a tumour-associated carbohydrate antigen, as already mentioned in general introduction in chapter 1, and we also clearly demonstrated it in chapter 2. The sLe^x expression was consistently demonstrated in all cases of canine mammary carcinoma, being negative in normal mammary gland and in adjacent benign proliferative lesions. Therefore the results presented in chapter 2 support that the *de novo* expression of sLe^x determinant may be considered a marker of the malignant phenotype in canine mammary carcinomas.

Furthermore, we also demonstrated that sLe^x is not only a marker for cancer but also implicated functionally in the malignant behaviour of cancer cells. Being the glycan structure recognized by E-selectin, sLe^x can promote adhesion of tumour cells to endothelial cells, being therefore closely associated with hematogenous metastases (Kannagi 1997). We showed that overexpression of sLe^x was significantly correlated with the presence of lymph node metastases. These observations suggested that sLe^x, apart from having a crucial role on hematogenous metastases, may also play an important role in the process of local lymphatic invasion, metastatic spread and poor prognosis. This important conclusion was further supported in a recent study from Wei et al. where the

authors also found an association between E-selectin and sLe^x expression with lymph node metastases of invasive micropapillary breast carcinoma (Wei et al. 2008).

Altogether, the results reported in chapter 2 demonstrated the importance of sLe^x in malignant phenotype and tumour progression of canine mammary carcinoma. Furthermore the significant relation between sLe^x and lymph node metastases appeal to a practical standpoint because it supports for the first time the use of sLe^x as a prognostic tumour marker in canine malignant mammary gland tumours.

The complexity of the metastatic process has made it difficult to achieve a full understanding of the genesis of this most deadly characteristic of cancer. Several factors take place as important roles, including somatic mutations, epigenetic modulations and interactions with environmental stroma stimuli. However, it is well known that metastasis is a coordinated multistep process encompassing the detachment of cells from a primary tumour to development of a tumorigenic lesion at a distant site (Hart and Saini 1992). A remarkable feature of metastatic cells is the significant flexibility in their adhesive interactions with other cells and/or extracellular matrix.

The role of E-cadherin in invasion and metastasis has become topical in the past few years due to its apparent promise as a prognostic indicator, with loss or reduction of expression correlating with enhanced tumour invasion and metastization of many carcinomas (Birchmeier and Behrens 1994; Mareel et al. 1994; Takeichi 1993; Suriano et al. 2003; Oliveira et al. 2009).

On the other hand, sLe^x is a carbohydrate antigen with also very clear roles in the process of metastization. Extravasation of tumour cells, to form new tumour foci, involves the interaction of E-selectin, cell adhesion molecule expressed at the surface of endothelial cells, with its corresponding carbohydrate ligand, the sLe^x, expressed on the surface of malignant cells (Hakomori 1996; Kannagi 1997).

Therefore, sLe^x and E-cadherin are two adhesion molecules playing crucial roles in the process of tumour progression. Based in this fact, and taking into account the literature that claimed the existence of a cooperative role between E-cadherin and sLe^x in malignant progression (Alpaugh et al. 2002b; Alpaugh et al. 2002a), in chapter 2 we also aimed to explore the relationship between the expression of sLe^x and E-cadherin in a canine mammary carcinoma model. We have found for the first time a statistically significant inverse correlation between E-cadherin and sLe^x expression. Moreover, we further demonstrated this inverse relationship between the two adhesion molecules in the same

tumour cell. When cells expressed sLe^x they do not co-express E-cadherin and vice versa (chapter 2, figure 4).

Together, these results support a coordinated role and a cooperative relationship of expression of both types of adhesion molecules in the process of tumour progression. These hypothesis presented in chapter 2 represented the basis of the research developed in chapter 3.

Molecular plasticity between Sialyl Lewis x and E-cadherin

The major part of cancer morbidity and mortality occurs upon dissemination of carcinoma cells from their original sites of development (Liotta et al. 1991; Woodhouse et al. 1997). Determining the molecular mechanism of invasion/metastasis is of the utmost importance in an attempt to manage and treat cancer.

Tumour cells which show the ability to metastasize should therefore exhibit characteristic features differing not only from normal, but also from the other tumours cells found in primary lesion. Among those features, loss and gain of key adhesion molecules appears to be a significant factor that dictate progression from the invasive to malignant phenotype followed by colonization at a distant site (Couldrey and Green 2000; Laidler and Litynska 1997). Consistently, E-cadherin and sLe^x are two adhesion molecules that may interfere with malignant progression.

The results stated in chapter 2 warranted further investigation addressing the clarification of the inverse relationship between E-cadherin and sLe^x expression in the process of tumour development and progression. For doing so, in chapter 3, we have used two comparative models of mammary carcinogenesis: metastatic canine mammary carcinoma and the highly metastatic inflammatory breast cancer (IBC) model, MARY-X.

MARY-X was established from a patient with IBC and like this type of woman breast cancer, MARY-X grows exclusively within the murine lymphatics and blood vessels, which is the signature phenotype of this highly aggressive malignant breast carcinoma (Alpaugh et al. 1999).

Alpaugh et al. have previously reported that E-cadherin and sLe^x appeared to exhibit a cooperative relationship in the genesis and in the passive dissemination of the lymphovascular embolus of IBC (Alpaugh et al. 2002b; Alpaugh et al. 2002a). Having this in mind and joining the results from chapter 2, we and Alpaugh's group have decided to

further evaluate the inverse correlation between molecules involved in adhesion, E-cadherin and sLe^x, in the process of tumour genesis and dissemination.

In chapter 3 we have confirmed and mutually validated in both models the existence of an inverse relationship of E-cadherin and sLe^x expression in the process of genesis, dissemination and subsequent metastases in both comparative models: MARY-X and canine mammary carcinoma.

The results appeal for the existence of a biologically coordinated mechanism of E-cadherin and sLe^x expression termed molecular plasticity that is essential for tumour establishment and metastatic progression.

In fact, our results suggest a biological regulation of expression of both adhesion molecules in the same tumour cell and this co-expression incompatibility remains to be understood. One hypothesis would be the strong electrostatic repulsions of negative charges known to be present on Sialyl residues which would be likely to disrupt or weaken the E-cadherin mediated cell-cell adhesion (Alpaugh et al. 2002a; Fuster and Esko 2005). However this and other hypothesis need to be tested.

Altogether these results suggest that loss and gain in expression of adhesion molecules is not an aberrant variation but rather an adaptive cellular and molecular mechanism of the cancer cells, which may ultimately allow cancer to escape the natural host defence mechanisms and therefore to achieve successful metastization. Therefore, the results in chapter 3 set the ground for targeting this dynamic mechanism of molecular plasticity between E-cadherin and sLe^x, resulting thus in new therapeutic strategies for metastatic disease.

E-cadherin N-glycosylation profile in the acquisition of the malignant phenotype

Glycosylation is one of the most abundant post-translational modification reactions, and nearly half of all known proteins in eukaryotes are glycosylated. An increasing body of evidences indicates that modifications in cell surface glycosylation affecting cell adhesion are common characteristics of transformed cells (Dwek 1995; Saxon and Bertozzi 2001; Taniguchi et al. 2006; Varki 1993). Glycans participate in major pathophysiological events during tumour progression, such as cell-cell interactions (Dube and Bertozzi 2005; Fuster and Esko 2005).

E-cadherin is a transmembrane glycoprotein, involved in cell-cell adhesion that can be post-translational modified by *N*-glycosylation. In addition, alterations in E-cadherin functions are critical in tumorigenesis, although many of those E-cadherin dysfunctions associated with tumour invasion remain to be explained. Therefore it is imperative to first evaluate the glycosylation modifications undergone by E-cadherin in the transformation to the malignant phenotype.

In the beginning of this work, little was known about the pattern of E-cadherin N-glycans in the malignant and non-malignant phenotype. Thus, the main questions that we have addressed were: 1) Is there any modifications on E-cadherin N-glycosylation status between benign and malignant phenotype?; 2) Which are those N-glycans changes associated with E-cadherin-mediated tumour progression?

In an attempt to answer those questions, in chapter 4, we have characterized the E-cadherin glycosylation profile in two models of canine mammary gland non-malignant (adenoma) and malignant (invasive carcinoma) cell lines, using a panel of lectins (chapter 4, table 1). We further performed a detailed characterization using enzymatic digestion and MALDI-TOF mass spectrometry analysis of E-cadherin *N*-glycans. Finally, lectin histochemistry analysis in canine mammary carcinoma and adenoma tissue samples gave us an *in vivo* perception of the overall glycosylation pattern associated with tumour phenotype (chapter 4, figure 4).

The results presented in chapter 4 clearly show for the first time that E-cadherin displays a modified *N*-glycosylation pattern during the acquisition of the malignant phenotype (answer to question 1 stated above).

In fact, comparing E-cadherin from non-malignant cell line with E-cadherin from malignant and metastatic cell line, in this last aggressive tumour phenotype, E-cadherin undergoes an extensive modification of the *N*-glycans composition. These cancer related changes in the pattern of E-cadherin *N*-glycosylation included the synthesis of highly branched *N*-glycans (β 1,6 GlcNAc branched structures), an increase in sialylation (α 2,3 and α 2,6 linked sialic acids) and an expression of few high mannose *N*-glycan structures (chapter 4, figure 2).

Consistently with these observations, it is very well established that in fact enhanced β 1,6 GlcNAc branching and an increase in sialylation are perhaps the most widely occurring glycosylation changes inducing malignancy (Dennis et al. 1987; Hakomori 1996; Hakomori 2002; Kannagi 1997; Kannagi et al. 2004). Therefore, collectively our results show that *N*-glycans, predominantly highly branched and sialylated structures on E-

cadherin adhesion molecule might play key roles in cell-cell adhesion machinery, affecting thereby cancer development and progression, and in this way we have answered to the above stated question 2.

Having concluded that E-cadherin from the malignant cell line had a modified *N*-glycans profile when compared to the non-malignant cell line, supporting thus a role of these *N*-glycans on E-cadherin-mediated tumour progression, we have performed further structural characterization of E-cadherin *N*-glycans from the carcinoma cell line model.

In fact, the identification of the glycans structures is essential to understand the functions that these glycans play in the biology of the cell (Taniguchi et al. 2001; Zhao et al. 2008c). It is important to mention here that, till the beginning of the present work, very little was known about the structures of E-cadherin *N*-glycans. In spite of being a very attractive area of research that has receiving attention by some recognized groups in the field all over the world and also by our own group, the specific structural characterization of E-cadherin *N*-glycans is far from being an easy task. One of the main critical points that this approach faces is exactly the isolation and purification of E-cadherin molecule. In the cells, E-cadherin is often linked to other different types of molecules forming a complex - the cadherin/catenin complex.

In chapter 4 we were able to identify by mass spectrometry the identity of E-cadherin and β -catenin molecules (chapter 4, figure 3). Furthermore, detailed analysis of mass spectrum of E-cadherin using the Glycomod software allowed us to deduce for the first time a new *N*-glycan structure occupying the Asn-775 of canine E-cadherin. This structure was compatible with a hybrid/complex type *N*-glycan. Additionally and taking altogether the results from L-PHA lectin blot reactivity; the PNGase F sensitivity of E-cadherin (chapter 4, figure 2) and the ones from MALDI-TOF analysis (chapter 4, figure 3), we had strong evidences that this new *N*-glycan structure was a β 1,6 GlcNAc branched *N*-glycan form.

Actually, this β 1,6 branched structure is known to be highly related with malignancy and metastasis (Dennis et al. 1987). Along with this and taking into account the results presented in chapter 4 and those from Guo et al. (Guo et al. 2003), we can now further complete the above sentence suggesting that this role of β 1,6 branched structure in cancer development and metastization is via regulation of E-cadherin mediated cell-cell adhesion mechanisms. This proposed conclusion was further delved and addressed in chapter 5.

In summary, the results presented in chapter 4 supported that changes in glycosylation play crucial roles in the process of malignancy and in regulation and modification of E-cadherin associated cell-cell adhesion which could lead to the malignant behaviour of tumour cells.

E-cadherin Post-Translational Modifications: a potential mechanism in cancer

The biosynthesis of sugar chains in N-glycans is catalyzed by several glycosyltransferases (GT) and most of the cancer-associated changes of sugar chains of glycoproteins are due to the changes of glycosyltransferases (Taniguchi et al. 1998). Functional Glycomics, which uses sugar remodelling by glycosyltransferases, is a promising tool for the characterization of glycans functions (Taniguchi et al. 2001). An increasing bulk of evidence suggests that phenotypic changes observed in cancer cells and tissues seem to be due to a direct or indirect effect of glycosyltransferases genes (Taniguchi et al. 1998; Taniguchi et al. 1999; Taniguchi et al. 1996).

It is well established that glycan modifications are associated with many physiological and pathological events, including cell adhesion, migration, cell growth, cell differentiation and tumour invasion (Dennis et al. 1999; Dwek 1995; Gu and Taniguchi 2008; Ohtsubo and Marth 2006; Saxon and Bertozzi 2001; Taniguchi et al. 2006; Varki 1993).

E-cadherin is a cell-cell adhesion glycoprotein whose post-translational mechanisms of regulation remain largely unknown.

Ever since the classic demonstration that tumour cells adhere to one another less avidly than do non-tumour cells (Brugmans et al. 1978), the question of how adhesive dysfunction contributes to cancer biology has been an active issue in cancer research. In fact, the vast majority of studies have focused on the role of epi/genomic alterations of E-cadherin dysfunction in cancer development and progression. Nevertheless is still unknown in many types of invasive carcinomas which are the mechanisms operating in E-cadherin deregulation.

In chapter 5, we have explored the regulatory mechanism of GnT-III and GnT-V on E-cadherin post-translational modifications, proposing therefore a novel mechanism of E-cadherin dysfunction in cancer which operates at the post-translational level of E-cadherin glycoprotein.

Our interest, in chapter 5, has focused on two of the most important N-acetylglucosaminyltransferases (GnT), GnT-III and GnT-V, both of which play a pivotal role in the branching of Asn-linked N-glycans of glycoproteins. Briefly, and as it was already described in general introduction in chapter 1, GnT-III catalyzes the formation of a bisecting GlcNAc structure (Narasimhan 1982) in N-glycans, and has been pointed as a metastases suppressor whereas GnT-V catalyzes the addition of β 1,6 GlcNAc branching of N-glycans (Brockhausen et al. 1988a; Cummings et al. 1982; Brockhausen et al. 1988b), and has been associated to increase metastasis (reviewed in (Zhao et al. 2008c; Zhao et al. 2008b). Additionally, the enzymatic activity of GnT-III has been shown to have an antagonistic effect to the one of GnT-V (Zhao et al. 2006).

In paper IV (chapter 5), we have clearly demonstrated for the first time that E-cadherin regulates *MGAT3* gene transcription leading to a significant up-regulation of GnT-III mRNA transcription levels (chapter 5, figure 1). In turn, GnT-III post-translationally modified E-cadherin N-glycans adding bisecting GlcNAc structures (chapter 5, figure 2B). These results appeal for the existence of a functional feedback loop between E-cadherin and GnT-III (chapter 5, figure 7A and 7B).

In fact and despite the complexity of factors influencing glycosylation, transcriptional regulation of the glycosyltransferases involved in the synthesis and catabolism of glycans seems to be one of the primary mechanisms to control glycan structures on the cell surface (Abbott et al. 2008). Based on this we can claim that E-cadherin might control its own N-glycans by transcriptional regulating GnT-III expression.

In the light of these results, we have questioned the following: Why E-cadherin being an extremely important tumour suppressor needs GnT-III expression, also described to be a metastases suppressor?; Does it needs bisecting GlcNAc structures to achieve its biological functions?

Therefore and in order to clarify the importance and the role of GnT-III on E-cadherin biology we have disrupted the arm B of the functional loop (chapter 5, figure 7B) by silencing GnT-III expression. Interestingly, GnT-III knockdown did not affect E-cadherin transcription (RNA levels) neither E-cadherin transduction (Protein level). However there was a remarkable modification of the cellular phenotype upon GnT-III silencing. The results presented in chapter 5, figure 4C, showed that GnT-III knockdown resulted in an extensive loss of intercellular contacts with an increase of lamellipodia and filopodia formations. E-cadherin has de-localized from the cell membrane having internalized to the cytoplasm.

These findings suggested that GnT-III expression and the consequent catalysis of bisecting GlcNAc structures seem to be important for E-cadherin expression at the cell border and for the maintenance of a compact cell-cell adhesion monolayer. On another standpoint, and based on the fact that net cadherin internalization is likely to reflect the balance between signals that promote endocytosis and those that inhibit it (Yap et al. 2007), one can speculate that GnT-III and the presence of bisecting GlcNAc structures on E-cadherin *N*-glycans might function as an inhibitor of E-cadherin endocytosis, whereas GnT-V and its product, β 1,6 GlcNAc branched structures, have the opposite role, promoting endocytosis.

In an attempt to explain the changes of the cellular phenotype and the E-cadherin membranar de-localization, another question was raised: What were the modifications on E-cadherin *N*-glycosylation upon GnT-III silencing?

Changes in glycosyltransferases expression levels have impact in the remodeling of cell surface glycoproteins oligosaccharides, which may consequently affect several cellular mechanisms such as intercellular adhesion (Taniguchi et al. 2001). Thus, in chapter 5, we have exactly demonstrated this mechanism. Therefore, we showed that GnT-III knockdown resulted in remodeling of *N*-glycans on glycoproteins in general and specifically on E-cadherin molecule. After GnT-III knockdown, E-cadherin (and also glycoproteins in general) suffered a decrease of bisecting GlcNAc structures with a concomitant increase of β 1,6 GlcNAc branched structures (chapter 5, figure 5). In fact, the silencing of GnT-III enzyme has conferred catalytic advantages to GnT-V, through competition, being therefore able to increase the synthesis of β 1,6 GlcNAc branched *N*-glycan structures on E-cadherin.

Thus, as a consequence of this E-cadherin *N*-glycans remodeling (decrease bisecting GlcNAc structures and increase β 1,6 GlcNAc branched structures), there was a remarkable effect on the E-cadherin cellular localization with repercussions on cellular phenotype, resulting in E-cadherin membranar de-localization and disruption of cell-cell adhesion.

Altogether these results suggested the existence of a dynamic regulatory mechanism that on one hand, E-cadherin mediated cell-cell adhesion is regulated via remodeling of *N*-glycans controlled by glycosyltransferases, GnT-III and GnT-V and, on the other hand, glycosyltransferases such as GnT-III expression is also regulated via an E-cadherin expression dependent manner (proposed model in figure 7, chapter 5).

In summary, modulation of E-cadherin glycosylation significantly alters the biological function of the adhesion molecule.

This bidirectional crosstalk between E-cadherin and GnT-III/GnT-V reported *in vitro* was further confirmed and validated in an *in vivo* model by performing E-cadherin and lectin histochemistry. For doing so, we had chosen a model of sporadic Diffuse Gastric Carcinoma, based on the fact that E-cadherin deregulation is a common feature of these tumours (Becker et al. 1994; Carneiro et al. 2004; Chan 2006; Hamilton and Meltzer 2006; Liu et al. 2006; Machado et al. 2001; Tamura et al. 2000). Furthermore, this type of human sporadic diffuse gastric cancer is also an attractive model since a large number of those cases exhibiting E-cadherin dysfunction are not explained by any of the mechanisms already described to inactivate E-cadherin, including: gene mutations, promoter hypermethylation, presence of transcriptional repressors (Becker et al. 1994; Berx et al. 1998; Graff et al. 1997; Moreno-Bueno et al. 2008; Oliveira et al. 2003; Rosivatz et al. 2002).

The validation of our *in vitro* results on this *in vivo* model opens new insights into E-cadherin regulation, emphasizing its post-translational modification by *N*-glycosylation as another mechanism that could underlie E-cadherin dysfunction in cancer.

In summary, this last paper (chapter 5) demonstrates a potential novel post-translational mechanism of regulation of E-cadherin dysfunction in cancer: the *N*-glycosylation.

Therefore, it is very inspiring and also very challenging to see this paper IV (chapter 5) as an open door that gives us new ways to pursue and so to further understand what we believe to be a new regulatory mechanism of E-cadherin dysfunction in cancer operating at the post-translational level.

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SUMMARY AND CONCLUDING REMARKS

The general aim of the present thesis was to evaluate the role and the mechanism of aberrant glycosylation in cancer, emphasizing the role of the modulation of the *N*-glycans on E-cadherin functionality in a tumour situation. The accomplishment of this goal resulted in the following main conclusions:

1) Aberrant expression of Sialyl Lewis x (sLe^x) carbohydrate antigen is a frequent event in cancer cells. Also, Sialyl Lewis x antigen was found to have a preponderant role in the process of tumour cells metastization. Due to the clear association with cancer cell behaviour, we aimed to study the biological significance of this important cancer related antigen in a model of canine mammary cancer:

We have found that the aberrant expression of sLe^x determinant was considered a marker of the malignant phenotype of canine malignant mammary tumours.

We also demonstrated that sLe^x may also play an important role in the process of local lymphatic invasion and metastization.

Therefore the main conclusion was the potential use of sLe^x determinant as a prognostic tumour marker in canine mammary carcinomas.

1.1) sLe^x and E-cadherin are two adhesion molecules that play crucial roles in the process of tumour development and progression. We aimed to explore the relationship between the expression of sLe^x and E-cadherin in canine mammary carcinoma model:

We have showed that sLe^x and E-cadherin expression were inversely correlated.

2) Taking the close inverse relationship between sLe^x and E-cadherin expression found in the canine mammary carcinoma model, we aimed to further evaluate and clarify this inverse correlation in the process of tumour development and progression using two comparative models of mammary carcinogenesis: metastatic canine mammary carcinoma and the highly metastatic Inflammatory Breast Cancer (IBC) model, MARY-X.

We have found that the inverse relationship of expression of sLe^x and E-cadherin in cancer cells was reproduced and therefore mutually corroborated in both comparative models.

We have concluded about the existence of a biologically coordinated mechanism of E-cadherin and sLe^x expression termed molecular plasticity that is essential for tumour establishment and metastatic progression.

3) E-cadherin functions are critical in tumorigenesis. Nonetheless, the post-translational modifications by *N*-glycosylation of E-cadherin in a tumour milieu remained to be characterized. Based on this, we aimed to characterize the *N*-glycosylation profile of E-cadherin in the process of tumour development and in the transformation to the malignant phenotype.

We have characterized for the first time the pattern of E-cadherin *N*-glycosylation in a benign and in a malignant phenotype from a model of canine mammary tumour. We found that, during the acquisition of the malignant phenotype, E-cadherin underwent an extensive modification of the N-glycans composition, characterized by an increase in β 1,6 GlcNAc branched structures; an increase in sialylation and an expression of few high mannose *N*-glycan structures.

We have identified in canine E-cadherin a new *N*-glycosylation site in Asn-775, containing a potential β 1,6 GlcNAc branched *N*-glycan form.

Our findings support that changes on E-cadherin glycosylation might play a crucial role in the process of tumour malignancy and invasion.

4) A large number of invasive carcinomas display E-cadherin dysfunctions whose mechanism of downregulation remained to be explained. We aimed to understand the regulatory mechanism of GnT-III and GnT-V on E-cadherin post-translational modifications.

We have found that human E-cadherin was capable of regulating *MGAT3* gene transcription. In turn, the up-regulation of GnT-III enzyme resulted in post-translational modification of E-cadherin by adding bisecting GlcNAc structures. Therefore, a functional feedback loop between E-cadherin and GnT-III was proposed.

The GnT-III knockdown resulted in a remarkable modification of cell morphology with loss of intercellular contacts which was accompanied by a membranar de-localization of E-cadherin leading to an increase of aberrant cytoplasmic expression. Concomitantly and upon GnT-III silencing, E-cadherin has suffered a remodelling of *N*-glycans, with an increase of β 1,6 branched structures and a decrease of bisecting GlcNAc structures, controlled by GnT-III and GnT-V glycosyltransferases. The presence of a dynamic

bidirectional crosstalk between E-cadherin-mediated cell-cell adhesion and GnT-III/GnT-V (through enzymatic competition) was suggested.

Also, in human primary gastric carcinoma, we validate our *in vitro* model and showed the mutual regulatory mechanism between E-cadherin and GnT-III/GnT-V.

Our results open new insights into E-cadherin regulation, suggesting its post-translational modifications as another possible mechanism that could underlie E-cadherin dysfunction in cancer. This hypothesis constitutes the background of the future perspectives.

