Characterization of Sialidases Neu1, Neu2, and Neu4 in a Canine Model of Breast Cancer

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ABSTRACT: Sialidases are enzymes that catalyze the removal of sialic acids from glycoproteins and glycolipids. Previously, we have studied the effect of sialidase inhibition as a modulator of sialylation-related mechanisms of invasion and found that it induces aggressiveness in canine mammary tumors (CMTs). In this study, we aimed to assess the expression of glycoprotein-acting sialidases, Neu1, Neu2, and Neu4, in the complex multistage process of cancer metastasis. Thus, we examined their expression in a series of spontaneous malignant CMTs, CMT cell lines, and nude mice xenografts. All malignant CMT lesions expressed mammalian sialidases, although overall decreased when compared to normal adjacent mammary tissues. This difference was statistically significant regarding Neu4. In accordance, CMA07 adenoma cell line expressed higher levels of sialidase protein expression when compared with the CMT-U27 carcinoma cell line. Finally, with few tumor subpopulation exceptions, Neu1 and Neu4 expression was also overall low in primary and metastatic CMT xenografts. Thus, overall loss of sialidases seems to be an important feature for CMT progression and invasion.

KEYWORDS: mammalian sialidases, mammary tumors, metastasis

CITATION: de Oliveira et al. Characterization of Sialidases Neu1, Neu2, and Neu4 in a Canine Model of Breast Cancer. *Glycobiology Insights* 2016:6 11–19 doi:10.4137/GBI.S30034.

TYPE: Original Research

RECEIVED: October 12, 2015. **RESUBMITTED:** April 3, 2016. **ACCEPTED FOR PUBLICATION:** April 5, 2016.

ACADEMIC EDITOR: Hafiz Ahmed, Editor in Chief

PEER REVIEW: Two peer reviewers contributed to the peer review report. Reviewers' reports totaled 933 words, excluding any confidential comments to the academic editor.

FUNDING: This work was funded by FEDER funds through the Operational Programme for Competitiveness Factors-COMPETE and National Funds through the FCT. This paper was published under the framework of the European Social Fund, Human Resources Development Operational Programme 2007e2013 (POSDRU/159/1.5/S/136893). This work was supported by FCT under the Programa Operacional Ciência e Inovação 2010 (POCI 2010) do Quadro Comunitário de Apoio III and Project grant no. PTDC/CVT/117610/2010. RB (SFRH/BPD/68276/2010) and CG (SFRH/BPD/96510/2013) acknowledge the Portuguese Foundation for Science and Technology. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Introduction

Sialidases, or neuraminidases, are glycohydrolytic enzymes that remove sialic acid residues from the terminal end of glycoproteins, oligosaccharides, and glycolipids.[1–3] There are four known mammalian sialidases and they vary in their subcellular patterns of expression, enzymatic properties, and chromosomal localization of the encoding genes. Neu1, Neu2, Neu3, and Neu4 classical subcellular localizations are lysosomes, cytosol, plasma membranes, and the lysosomal or mitochondria lumina and intracellular membranes, respectively. Regardless, it was shown that the subcellular localization of sialidases may change upon stimuli from the microenvironment.[4] Sialidases play key roles in various physiological functions, such as cell differentiation, cell growth, membrane function, and antigen masking. Their involvement in such phenomena could be either direct or secondary to desialylation of many different substrates.[1] In cancer, sialidases are reported to be present in several different types of malignancies, although not well characterized.[5] Sialidase activity, for instance, was found to be highly elevated in the sera of breast cancer patients.[6] In an apparent contradiction, most tumor-associated antigens (TAAs) are sialylated, and increased sialylation is a well-known feature of transformed cells.[7,8] In breast cancer, expression of TAA such as Sialyl-Lewis(x) (SLe(x)) or sialyl-Tn is strongly associated with poor prognosis and decreased overall patient survival.[7] Using exogenously added sialidases, our group has shown overall sialylation masking of galectin-3 ligands in primary canine mammary tumor (CMT) and an apparently microenvironment-dependent uncloaking of such ligands in invasive tumor cell subpopulations.[9] In view of this, in a cancer patient, endogenous sialidases present in the serum, at the surface of immune cells or even at that of tumor cells themselves, might thus also, upon microenvironment stimuli, be able to cleave the α-bound sialic acid from galectin-3-ligands. In a subsequent study, we have shown that inhibiting mammalian sialidases with the anti-influenza sialidase inhibitor, oseltamivir phosphate, led to increased mammary tumor aggressiveness both in vitro and in vivo.[10] It was

thus deemed of utmost importance to identify the sialidases that are putatively responsible for modulating CMT biological behavior, in line with the crucial role of sialylated glycoproteins, such as mucins and the lectins, which recognize them during mammary tumorigenesis and progression.[11,12] Therefore, the present study aimed to evaluate the expression of sialidases, which could alter the function of glycoproteins, Neu1, Neu2, and Neu4 (Neu3 is active only on glycolipids). To do so, in the complex multistage process of cancer metastasis, we examined the expression of enzymes in a series of CMTs, CMT cell lines, and nude mice xenografts.

Material and Methods

Animal tissues and dogs. A series of 40 spontaneous malignant CMTs were obtained from female dogs, bearing only one CMT lesion, submitted to surgery at the Small Animal Clinic of ICBAS-UP (UPVET) with curative intents. The specimens were fixed in 10% neutral buffered formalin. After dehydration and paraffin wax embedment, serial sections of 3 μm were cut. One section was stained with hematoxylin and eosin (H&E), and the other sections were used for immunohistochemical studies. Animals were evaluated for disease recurrence every three months (through physical examination, three-view thoracic radiographies, and abdominal ultrasound) during a follow-up postoperative period of two years. All dogs were followed up until death or until the end of the preestablished observation period. Animals that died were submitted to complete necropsy and histological confirmation of local recurrences or metastatic lesions.

Cell lines and culture conditions. In this study, the following two different cell lines were used: (1) a highly metastatic CMT cell line, CMT-U27, kindly provided by Professor Eva Héllmen, from Sweden[13] and (2) a benign cell line, CMA07 (benign CMA07 cell line, established at our laboratory from a primary complex adenoma excised from a six-year-old female dog, which has undergone more than 50 passages and does not form tumors upon subcutaneous injection into nude mice).[10] The two cell lines were cultured at 37°C in a humidified 5% CO_2 incubator and maintained in RPMI 1640 medium (with glutamax and 25 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid [HEPES]), supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (10.000 units/mL penicillin and 10.000 μg/mL streptomycin; Gibco Life Technologies).

Experimental nude mice. A group of CMT-U27 tumors experimentally induced in four nude mice were retrieved from the archives of the Veterinary Pathology Department of ICBAS-UP. Tumor inoculations had been performed for an earlier published research purpose. All mice had been subjected to tumor inoculation, growth, and euthanasia according to the established Humane Endpoints and necropsied. The ethical committee of the University of Porto[10] approved all studies with animals. Specimens had been fixed in 10% neutral buffered formalin. Three micrometer sections were cut and stained with H&E for histopathological and immunohistochemical analysis.

Immunohistochemistry. Expression of canine sialidases Neu1, Neu2, and Neu4 was evaluated in the formalin-fixed, paraffin-embedded sections of both spontaneously occurring CMT and malignant CMT-U27 xenografts, using standard immunohistochemistry protocols. Briefly, 3 μm thick paraffin sections were deparaffinized, then hydrated, and endogenous peroxidase was blocked with H_2O_2. Sections were then blocked with the appropriate normal sera nonimmune swine (for anti-Neu1) and rabbit serum (for anti-Neu2 and Neu4) and diluted in 10% bovine serum albumin (BSA) at 1:5 for 20 minutes. Normal serum was replaced with the primary antibodies: rabbit anti-Neu1 polyclonal antibody (J-23; Santa Cruz Biotechnology, diluted at 1:50), mouse anti-Neu2 polyclonal antibody (37Y; Santa Cruz Biotechnology, diluted at 1:300), and goat anti-Neu4 polyclonal antibody (L-15; Santa Cruz Biotechnology, diluted at 1:300), and slides were incubated overnight at 4°C. After two washes with 1× phosphate-buffered saline (PBS), sections were incubated with the appropriate biotinylated secondary antibodies swine antirabbit, rabbit antimouse, and rabbit antigoat (Dako, diluted at 1:200), respectively, for 30 minutes at room temperature. After two more washes with 1× PBS, sections were incubated with avidin–biotin complex system (Vector, diluted at 1:100), a peroxidase-based detection system, for 30 minutes at room temperature. Peroxidase activity was examined using 3,3′-diaminobenzidine tetrahydroxychloride (Dako) as the substrate. Sections were counterstained with hematoxylin and then were mounted in scientific mounting medium (National Diagnostics). Negative controls in which the primary antibody was replaced by BSA 5% in PBS were performed in all series, and sections of human carcinomas were used as positive controls.

Scoring of immunostaining and statistical analysis. Sialidase expression in CMT was accessed in a semiquantitative way. Tumors were grouped into four classes: less than 25% of immunoreactive cells (low), 25–50% of immunoreactive cells (medium low), 50–75% of immunoreactive cells (medium high), or more than 75% of immunoreactive cells (high). Initially, a descriptive study of the data was realized. Then association hypotheses were tested, using the Student's *t*-test for continuous variables and Fisher's exact test or chi-square test for discrete variables. SPSS software (version 22.0) was used for statistical analysis.

RNA extraction and quantitative real-time polymerase chain reaction. To perform a quantitative real-time polymerase chain reaction, total RNA was isolated from the CMT cell lines using TRI Reagent (Sigma) according to manufacturer's instructions. Briefly, 1 μg of RNA were primed with random hexamers and reverse transcribed using Superscript II (Invitrogen) in a final volume of 20 μL. Two microliters of a 1:10 dilution of this mixture were amplified with SYBR Green (Applied Biosystems) and primers at a final concentration of 300 nM each, in a fluorescence reader (ABI Prism 7000), and duplicates were run for each RT product. The level of 18S and GAPDH RNA in each sample was measured and

used for normalization of target genes abundance (the level of two housekeeping genes was used to improve accuracy and avoid potential differences due to the metabolic state of cells). The primer sequences are listed subsequently.

Neu1: 5′GGGGATAAAGAACGACTTCAG3′/
5′GTGTGCACATTACCGATCTGC3′
Neu2: 5′GAACGAGCAAGAAGGATGAGC3′/
5′CTGTCTTCTCATCGTACAGG3′
Neu4: 5′CACGCCTTCACCTTCTATAGG3′/
5′GTTGCAGTAGAGGATGCTGC3′.

Protein extraction and Western blot. Cells were washed twice in PBS, centrifuged at 350 *g*, resuspended in lysis buffer (12 mM Na_2HPO_4, 8 mM NaH_2PO_4, 0.2% sodium dodecyl sulfate, 4 mM EDTA, 0.3 M NaCl, 2% NP-40, 0.1 M NaF, and 50 mM sodium deoxicolate) with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and complete protease inhibitor cocktail (Roche), and incubated on ice for 15 minutes. The lysates were then centrifuged, the supernatants collected, and protein content was measured using the bicinchoninic acid method (Pierce), according to the manufacturer's instructions. Total extracts were boiled for five minutes at 95°C in Laemmli sampling buffer. Next, samples were run in standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (pre-cast 4–20% tris-glycine gel; Invitrogen), transferred to a nitro-cellulose membrane (Amersham Biosciences), and blotted with the primary antibodies 1:200, rabbit anti-Neu1 polyclonal antibody (J-23; Santa Cruz Biotechnology); 1:200, mouse anti-Neu2 polyclonal antibody (37Y; Santa Cruz Biotechnology); and 1:200, goat anti-Neu4 polyclonal antibody (L-15; Santa Cruz Biotechnology), and α-actin (Santa Cruz Biotechnology, 1:8000) in 5% BSA, in 5% nonfat milk, and in 1× Tris-buffered saline with 0.02% Tween-20 (Sigma). The primary antibodies were revealed using the appropriate peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:2000 in 5% BSA) and the enhanced chemiluminescence detection kit (BioRad).

Immunocytochemistry by fluorescence. Cells were grown for 24 hours on cover glasses precoated for 3 hours with RPMI 1640 medium supplemented with 10% FBS. Cells were then fixed for 10 minutes in ice-cold methanol and blocked with nonimmune donkey and rabbit serum diluted in 10% BSA at 1:10 for 20 minutes. Normal serum was replaced with the abovementioned primary antibodies diluted in rabbit anti-Neu1 polyclonal antibody at 1:50, mouse anti-Neu2 polyclonal antibody at 1:200, and goat anti-Neu4 polyclonal antibody at 1:100, in 5% BSA, and slides were incubated overnight at 4°C. After two washes with 1× PBS, slides were incubated with the appropriate Alexa Fluor 594-conjugated secondary antibodies (Thermo Fisher Scientific) diluted in 5% BSA at 1:200 for 30 minutes in the dark at room temperature. Incubation with 1:100 DAPI in PBS was followed, and then sections were mounted in Vectashield (Vector). Slides were analyzed with a Leica DMIRE2 fluorescence microscope.

Results

Malignant CMTs were classified according to histological types and grades (Table 1). A total of 10 cases presented distant metastases. Metastases were found in the lungs, liver, spleen, and bone. In 32 cases, regional lymph nodes were available for assessment. Ten of these presented tumor invasion (Table 1). In this series, Neu1, Neu2, or Neu4 expression was not found to be statistically different, concerning the tumors' histological type and grade, mode of growth, or presence of distant and lymph node metastases.

Neu1 expression in normal adjacent mammary tissue, primary tumors, and distant metastases. In 30 (75%) of 40 malignant CMT cases, normal adjacent mammary tissue was available for comparative study. Seventy-three percent of normal adjacent mammary tissues presented less than 25% Neu1 immunoreactive cells. In normal adjacent mammary tissues, cytoplasmic Neu1 staining pattern with apical accentuation was mainly observed (Fig. 1A). The great majority of primary malignant CMTs, 33 cases (84.6%), also presented less than 25% Neu1 immunoreactive tumor cells. Five (12.8%)

Table 1. Clinicopathological features of the malignant canine mammary tumors.

CLINICOPATHOLOGICAL FEATURES	NUMBER OF CASES (%)
Histological type (n = 40)	
In situ carcinoma	1 (2.5%)
Complex carcinoma	5 (12.5%)
Tubulopapillary carcinoma	10 (25%)
Solid carcinoma	9 (22.5%)
Carcinosarcoma	4 (10%)
Mucinous carcinoma	2 (5%)
Carcinoma in benign tumor	3 (7.5%)
Micropapillary carcinoma	2 (5%)
Anaplasic carcinoma	1 (2.5%)
Papillary carcinoma	2 (5%)
Tubular carcinoma	1 (2.5%)
Histological grade (n = 40)	
I	10 (25%)
II	20 (50%)
III	10 (25%)
Lymph node metastases (n = 32)	
No	22 (68.8%)
Yes	10 (31.2%)
Distant metastases (n = 38)	
No	28 (73.7%)
Yes	10 (26.3%)

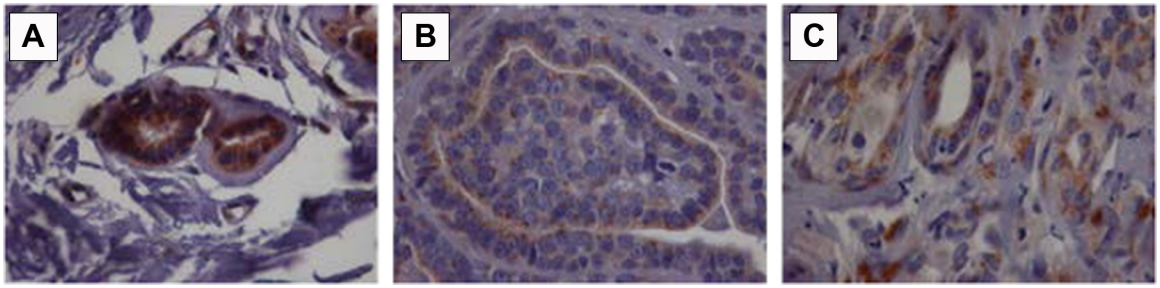

Figure 1. Neu1 pattern of expression in primary CMT specimens. Photomicrographs depict Neu1 (brown color) expressed in (**A**) normal adjacent mammary tissue showing a vesicular pattern with apical accentuation; (**B**) in a tubulopapillary carcinoma showing predominantly an apical vesicular pattern; and (**C**) in a carcinossarcoma a perinuclear vesicular pattern.

malignant CMTs presented Neu1 in between 25 and 50% of immunoreactive tumor cells, while only one (2.6%) malignant CMT presented 50–75% immunoreactive tumor cells. No malignant CMT cases presented more than 75% Neu1 immunoreactive tumor cells. In malignant CMTs, a vesicular cytoplasmic Neu1 staining pattern was mainly observed (Fig. 1B). Often intense perinuclear unilateral accentuation was found (Fig. 1C). No significant differences were observed when comparing Neu1 expression in primary CMTs and normal adjacent mammary tissue ($P = 0.18$; Fig. 2A). All distant metastatic lesions analyzed (four cases) presented less than 25% of Neu1-stained tumor cells; two of them were lung metastases (Fig. 2B).

Neu2 expression in normal adjacent mammary tissue, primary tumors, and distant metastases. In 48.4% CMT cases, normal adjacent mammary tissues presented more than 75% Neu2 immunoreactive cells. In normal adjacent tissue, both vesicular and diffuse cytoplasmic Neu2 staining patterns were observed (Fig. 3A). Thirteen primary CMT cases (36.1%) presented Neu2 staining in between 50 and 75% of the cells. Eleven cases (30.6%) presented Neu2 expression in more than 75% of tumor cells, while seven (19.4%) presented staining in between 25 and 50% of tumor cells. Only five (13.9%) cases presented less than 25% Neu2-stained cells. In malignant CMTs, diffuse cytoplasmic Neu2 was mainly found (Fig. 3B and C). In complex areas, loss of Neu2 staining in myoepithelial cells was observed (Fig. 3D). Primary malignant CMTs and normal adjacent mammary tissue presented no significant differences in Neu2 expression ($P = 0.08$; Fig. 2A). Most distant metastases, three lesions (75.0%) presented Neu2 in 25–50% of tumor cells (Fig. 2B).

Neu4 expression in normal adjacent mammary tissue, primary tumors, and distant metastases. Over 40% (42.9%) of normal adjacent mammary tissues that were evaluated

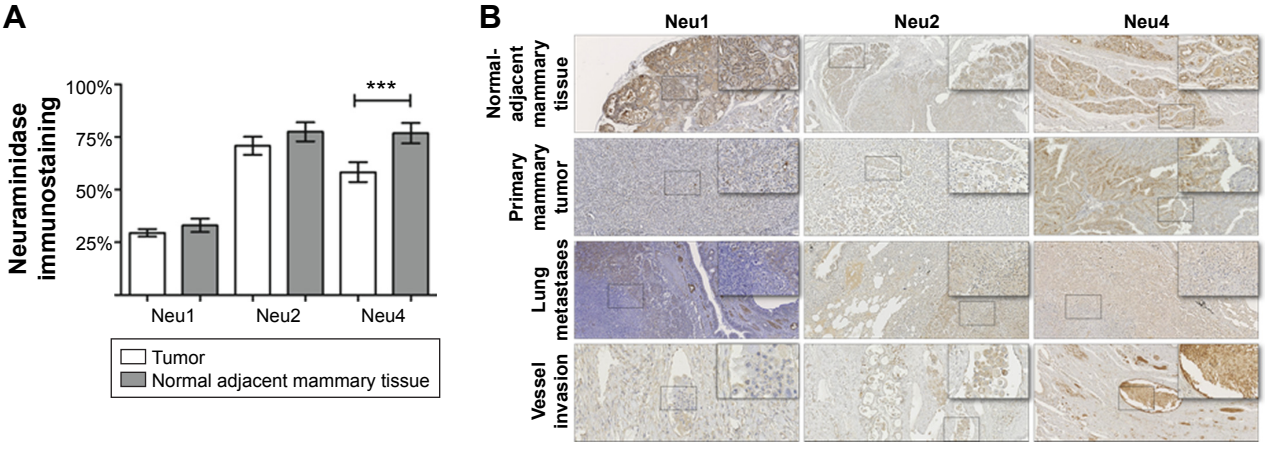

Figure 2. Expression of sialidases in primary and metastatic CMT lesions. (**A**) Neu1, Neu2, and Neu4 immunohistochemical staining was analyzed in a series of 40 CMTs. Primary tumors and, respectively, normal adjacent tissues were compared according to the percentage of neuraminidases-expressing tumor cells. No differences between primary tumors and normal adjacent tissues were observed for Neu1 and Neu2. However, the majority of these tumors presented low levels of Neu4 expression when compared with normal adjacent tissues. ***There was a significant difference ($P < 0.05$) by Pearson's chi-square test. (**B**) Photomicrographs depict Neu1, Neu2, and Neu4 immunostaining (brown color) with hematoxylin counterstain in malignant CMTs and lung metastasis. Low expression of Neu1 was observed both in primary malignant CMTs and their metastases. No expression of Neu1 was observed in vessel-invading cells. Primary tumors and lung metastasis expressed in similar levels of Neu2 when compared with normal adjacent tissues. Vessel-invading cells also expressed Neu2. Neu4 was expressed in all lesions in a heterogeneous subpopulation-related manner. Vessel-invading CMT cells expressed Neu4.

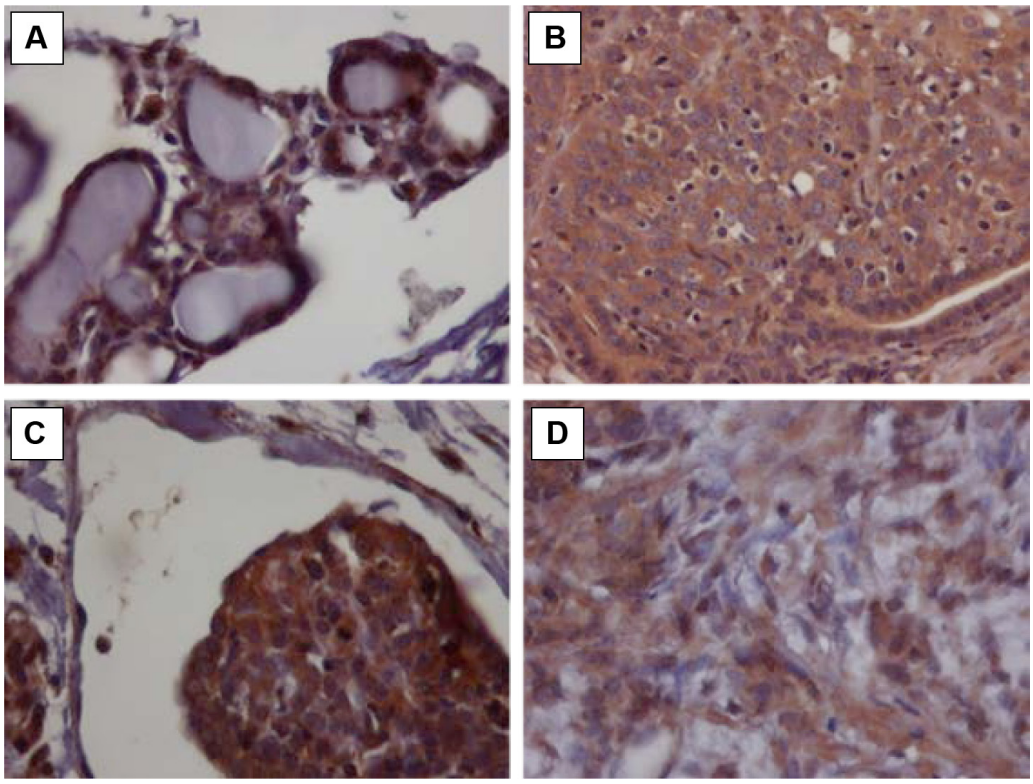

Figure 3. Neu2 pattern of expression in primary CMT specimens. Photomicrographs depict Neu1 (brown color) expressed (**A**) in normal adjacent mammary tissue showing an intense cytoplasmic pattern; (**B**) in a tubulopapillary carcinoma; (**C**) in intravascular cells showing a predominantly diffuse cytoplasmic pattern; and (**D**) in a complex carcinoma, although decreased in myoepithelial tumor cells.

presented more than 75% of Neu4 immunoreactive cells. In those tissues, Neu4 was expressed diffusely in the cytoplasm (Fig. 4A). Most of the primary malignant CMTs, 19 cases (57.6%), presented Neu4 staining in less than 50% of tumor cells. Nine (27.3%) primary malignant CMT cases presented Neu4 expression in less than 25% of the cells, while 10 (30.3%) presented staining in 25–50% of the neoplastic cells, 8 (24.2%) in between 50 and 75% of the cells, and only 6 cases (18.2%) expressed Neu4 in more than 75% of the tumor cells. In malignant CMTs, cytoplasmic Neu4 expression was observed. Basal staining pattern (Fig. 4B) and vesicular staining (Fig. 4C) were also found in different tumor subpopulations. Significant differences ($P < 0.0001$) were observed when comparing Neu4 expression in normal adjacent mammary tissue to that of primary malignant CMTs (Fig. 2A). Concerning the subpopulation pattern of expression, Neu4 was consistently found in intravascular CMT cells (Fig. 2B). Finally, Neu4 was expressed in more than 50% cells of metastatic CMT lesions in two (50%) of distant metastases cases. However, both Neu4-positive and -negative metastatic lesions were observed in the same case (Fig. 2B).

Metastatic CMT-U27 shows decreased levels of Neu1 when compared with benign CMA07 cell line. In order to further assess the expression of sialidases in CMT, we used two

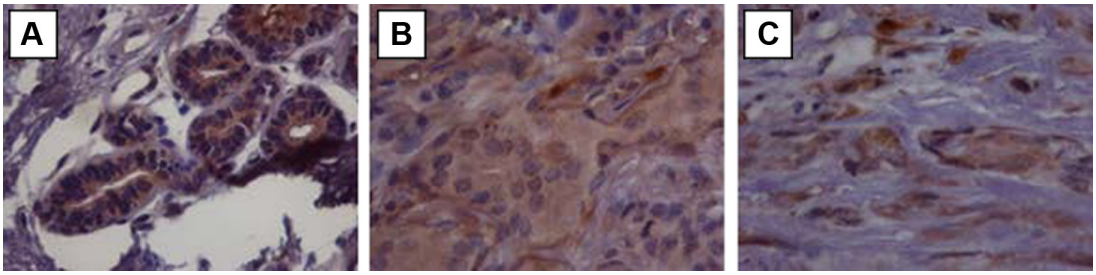

Figure 4. Neu4 pattern of expression in primary CMT specimens. Photomicrographs depict Neu4 (brown color) expressed in (**A**) normal adjacent mammary tissue showing a cytoplasmic pattern; (**B**) in a tubulopapillary carcinoma showing a pattern of markedly basal accentuation; and (**C**) in a carcinossarcoma showing a perinuclear slightly vesicular pattern.

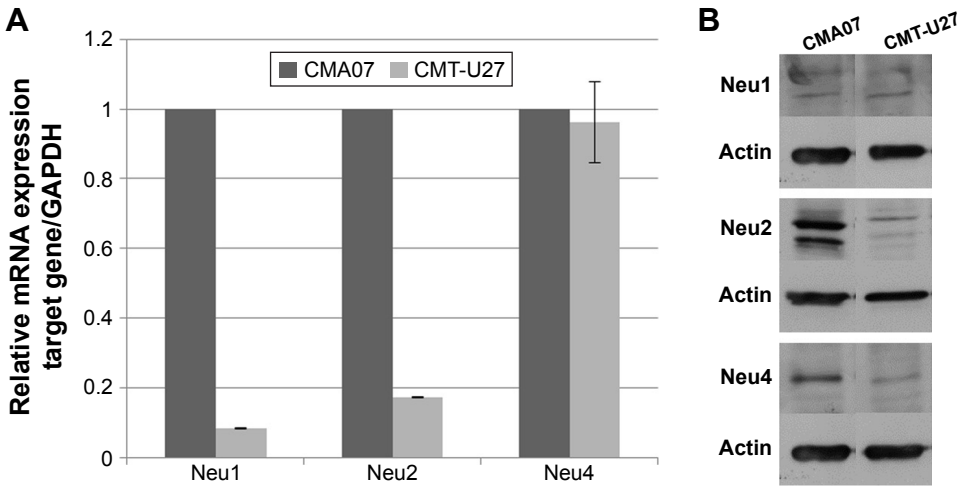

Figure 5. mRNA and protein expression of sialidases in CMA07 and CMT-U27 cell lines. (**A**) mRNA extracted from benign CMA07 and malignant CMT-U27 cell lines was converted to cDNA and analyzed by real-time polymerase chain reaction (RT-PCR) to observe the expression of Neu1, Neu2, and Neu4. Neu1 and Neu2 mRNA expression were considerably decreased in CMT-U27 cell line when compared to CMA 07 benign cell line. Malignant CMT-U27 and benign CMA07 cell lines expressed Neu4 mRNA in very similar levels. cDNA contents were normalized on the basis of predetermined levels of GAPDH. (**B**) Western Blot analyses show low levels of all sialidases in the CMT-U27 cell line when compared with CMA07. Relative intensity of the indicated protein level bands was normalized to actin and was measured.

CMT cell lines as models: the CMA07 (nontumorigenic) and CMT-U27 (highly metastatic) cell lines. First, the levels of sialidases were compared between the two cell lines. CMT-U27 showed decreased expression of both Neu1 and Neu2 when compared to CMA07 at the mRNA level (Fig. 5A). On the other hand, the levels of Neu4 mRNA expression were not decreased in the metastatic CMT-U27, being in fact similar in the two cell lines (Fig. 5A). At the protein level, Neu2 and Neu4 presented decreased expression in the malignant CMT-U27 when compared to benign CMA07 cell line (Fig. 5B). On the other hand, Neu1 level of protein expression was found to be similar in the malignant CMT-U27 and the benign CMA07 cell lines (Fig. 5B).

CMT-U27 xenografts display a pattern of sialidases expression similar to that found in both primary and metastatic lesions of CMT. In order to investigate the importance of sialidases in mammary tumor progression and a potential microenvironment role in their expression, further in vitro and in vivo expression studies were performed. CMT-U27 immunostaining was observed by immunofluorescence and immunohistochemistry, upon orthotopic inoculation into the mammary fat pad of nude mice. In vitro, Neu1, Neu2, and Neu4 presented similar vesicular and diffuse cytoplasmic patterns of expression (Fig. 6). Due to technical concerns regarding specificity issues of using mouse anti-Neu2 in nude mice studies, levels and staining patterns of expression of only Neu1 and Neu4 were assessed in four primary tumor xenografts and their spontaneous metastases. Neu1 expression was found to be low in primary tumors xenografts and in their metastases (Fig. 7). Neu4 was expressed in primary tumor xenografts; however, it was decreased in their corresponding distant metastases (Fig. 7).

Discussion

Sialidases have been implicated in cancer progression. However, most studies performed to date have been mostly done using cell lines.[4] Hence, a study of sialidase expression in the metastatic process, namely in that naturally occurring in mammary tumors, was thought to be of the utmost importance. In this study, we assessed the expression of sialidases acting on glycoproteins, namely Neu1, Neu2, and Neu4 using the model of spontaneously occurring CMT, CMT cell lines, and CMT nude mice xenografts. In the present study, we have focused on the putative role of sialidases during mammary tumor progression and have demonstrated that distinct sialidases seem to be differentially involved in CMT progression.

Most normal mammary tissues adjacent to malignant CMT presented Neu1 expression, often in apical secretory vesicles. In our CMT series, overall Neu1 expression was found to be low. This is in accordance with the literature since decreased expression of Neu1 has been associated with a malignant phenotype in both murine cancer models and human cancer studies.[4] Moreover, Neu1 has been found to be overexpressed by colonic cells experimentally forced to differentiate.[14] More importantly, decreased Neu1 expression was also observed in human colon carcinoma when compared to the normal colonic mucosa.[5] In fact, in human colon adenocarcinoma HT-29 cells, Neu1 overexpression suppressed cell migration and invasion, whereas its knockdown resulted in the opposite effects.[15] In addition, induced overexpression of Neu1 suppresses cell migration and invasion in murine melanoma cells lines.[16] In accordance to the literature, which suggests Neu1 to be associated with decreased tumor aggressiveness, despite generally downregulated in our malignant CMT series, well-differentiated tumor areas presented Neu1

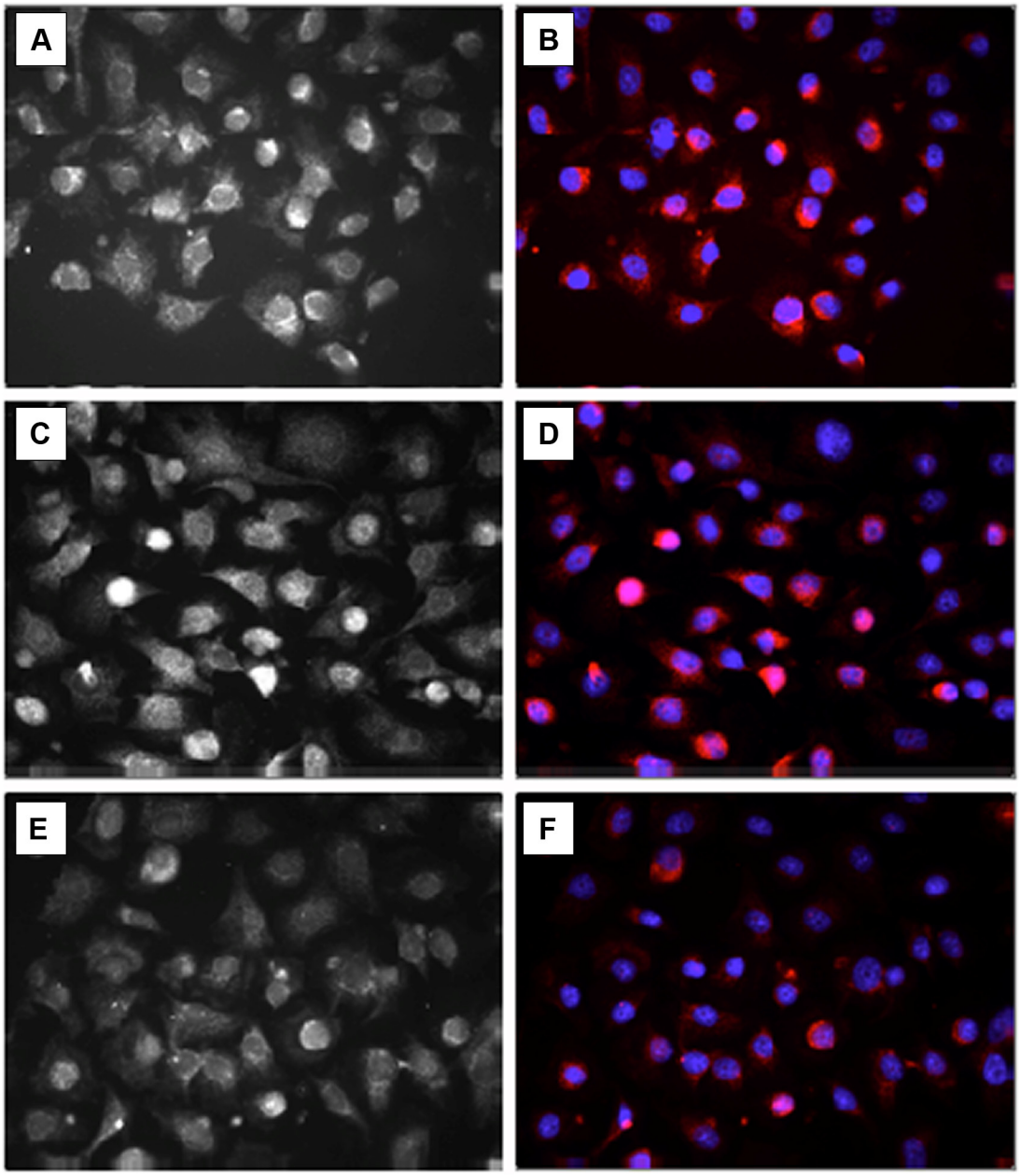

Figure 6. Subcellular patterns of sialidase expression in the CMT-U27 cell line. Photomicrographs depict Neu1, Neu2, and Neu4 immunostaining assessed by immunofluorescence (red color) without and with DAPI (blue color) nuclear staining, respectively. (**A** and **B**) Neu1 cytoplasmic vesicular staining pattern with perinuclear accentuation was observed in CMT-U27. (**C** and **D**) Neu2 showed a diffuse cytoplasmic staining pattern. (**E** and **F**) Neu4 revealed a diffuse and only slightly vesicular cytoplasmic staining pattern.

upregulation further supporting a role for this enzyme in normal mammary differentiation. The fact that Neu1 expression is detrimental to tumor progression is even more strongly hinted by our findings that lung metastases seems to present a lower level of Neu1 expression when compared to their corresponding primary tumors.

In accordance, Neu1 expression was found to be low in primary tumor xenografts and almost absent in their metastases. In other experimental models, transformation of mouse fibroblasts leads to decreased expression of Neu1 and murine melanoma cell lines with increased metastatic potential that reveals less Neu1 activity.[17] Therefore, our present study together with the literature suggests that loss of Neu1 expression confers survival advantage and invasive capacity to mammary tumor cells.

In the present work, Neu2 was strongly expressed in malignant CMTs. Neu2 has been suggested to participate in regulatory desialylation because of its ability to act on native glycoproteins and gangliosides at neutral pH.[18] Neu2 was expressed throughout all of the primary malignant lesions and in metastatic lesions. In line with this, recently, Neu2 expression was found to be elevated in prostate cancer cells.[19]

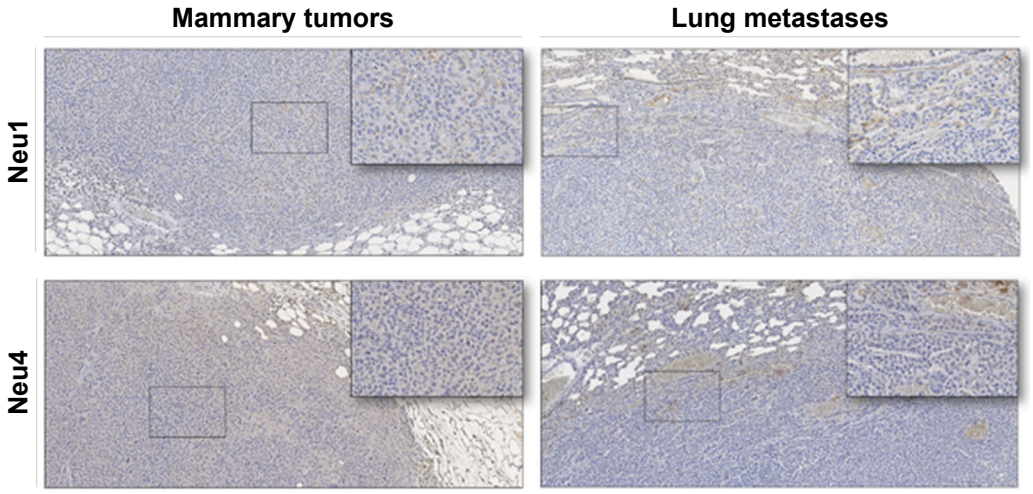

Figure 7. Expression of sialidases in primary tumors and lung metastases of CMT-U27 xenografts. Immunohistochemistry was performed to study the expression (brown color) of sialidases (Neu1, Neu2, and Neu4) in primary tumors and lung metastases of female. N: NIHY(s)II-nu/nu mice inoculated subcutaneously in the mammary fat pad with a suspension of 10^6 cells of the malignant CMT-U27 cell line. CMT-U27 xenografts displayed similar patterns of Neu1 and Neu4 expression.

Neu2 forced overexpression in vitro in mouse adenocarcinoma cells that led to cell invasion suppression due to decreased cell motility.[20] Overexpressed Neu2 also led to increased invasion in a murine melanoma cell line but not to altered ECM adhesion or cellular proliferation rate. Interestingly, Neu2 overexpression did not alter cell surface carbohydrates or intracellular glycoproteins.[21] Given that we have consistently found Neu2 expression in malignant and also normal mammary tissues, its contribution to tumor progression and whether or not it would be glycosylation related warrant further studies.

In all studied cases, normal mammary tissues adjacent to CMT presented Neu4 expression. On the other hand, malignant CMTs presented a statistically significant decrease in the expression of Neu4 when compared with the normal adjacent tissue. This is in accordance to the literature, Neu4 is decreased in colon cancer when compared with normal colon mucosa.[22]

CMT-U27 and CMA07 presented similar mRNA levels in vitro but Neu4 protein levels were lower in the malignant cell line. This suggests that Neu4 undergoes a posttranscriptional type of regulation. In line with this hypothesis, recent experiments have suggested an important role for the microenvironment in the regulation of Neu4 expression.[23] Also pointing to a microenvironment role in Neu4 regulation, despite its low levels of protein expression in vitro, Neu4 was, although tenuously, expressed in primary tumor xenografts but not in all metastatic lesions. Experimental findings in the literature regarding the role of Neu4 in malignancy are somewhat contradictory. On the one hand, Neu4 siRNA inhibition was reported to be involved in promotion of cell migration and invasion while its overexpression led to a decrease in both.[22] On the other hand, apoptosis induced by either the death ligand TRAIL or serum deprivation led to Neu4 upregulation in the early stage of apoptosis in cultured human colon cancer cells. However, Neu4 siRNA inhibition led to decreased apoptosis and caspase-3 activity and its overexpression by transfection led to apoptosis.[22] In the present study, intravascular malignant CMT cells expressed Neu4. Thus, our CMT data suggest that certain tumor cell subpopulations such as circulating tumor cells may express it. This might be involved in the cells' ability to homotypically aggregate and/or survive in the blood stream. SLe(x) and SLe(a) were recently shown to be substrates for Neu4.[23] Thus, Neu4 might downregulate the expression of SLe(x) observed in primary CMTs,[24] important for tumor cell detachment. Neu4 has important substrate specificity since it is the only sialidase that acts on mucins with high efficiency.[5] Mucins are expressed in CMTs.[12,25] In particular, MUC1 is associated with distant metastases in these tumors as shown by our group.[12] This mucin contributes to cell–cell deadhesion in primary tumors, among other reasons, due to the negative charges of its carried glycans such as SLe(x).[26] However, once inside vessels, cells need to be able to adhere to one another in order to avoid anoikis.[27] MUC1 clustering has been involved in such increased adherence in circulating tumor cells.[28] We have also been able to show that MUC1 is expressed in circulating CMT cells.[29] Interestingly, in intravascular CMT cells, the presence of an unsialylated form of the MUC1-carried T-antigen was observed and confirmed to be physically interacting with antianoikis galectin-3 and also found to be upregulated in this specific tumor cell subpopulation by others[9,11] and by us.

Although endogenous mammalian sialidases have differently been implicated in the progression of various types of cancers, mechanisms by which and how they influence metastasis remain unclear. As other pet models of human cancer, the incidence of CMT is three times higher than that of human breast cancer, and the time course of the disease is

much shorter, often less than two years. This allowed us to collect and analyze primary and metastatic tumors from the same dogs, which would be far too complicated to carry out in humans.[30] The multiple features of Neu1, Neu2, and Neu4 found in the present work suggest that each type of sialidase may play a unique role in mammary cancer according to its properties and available substrates. It is noteworthy that previous data from our group show that indeed the CMA07 cell line appears to be less sialylated than the CMT-U27 cell line,[10] which is in accordance to the pattern of overall increased sialidase contents found in the benign when compared to the malignant cell line. However, these results must be interpreted with caution given that only two CMT cell lines were analyzed in the present work. In this study, we have hinted important opposing roles for mammalian sialidases differentially influencing mammary tumor invasion and metastasis. Further studies are necessary in order to fully understand their role in malignant CMTs.

Acknowledgments

The authors would like to thank Nuno Mendes and Conceição Magalhães for all their help in experimental animal studies, Fátima Martins and Alexandra Rema for very important technical support and Prof. E. Helmén for supplying the CMT-U27 cell line. IPATIMUP integrates the i3S Research Unit, which is partially supported by the Portuguese Foundation for Science and Technology (FCT).

Author Contributions

Conceived and designed the experiments: JTO, ALS, RB, and CG. Analyzed the data: JTO, ALS, CR, and FG. Wrote the first draft of the manuscript: JTO and ALS. Contributed to the writing of the manuscript: JTO, ALS, RB, CR, CG, and CAR. Agree with manuscript results and conclusions: JTO, ALS, RB, CR, CG, AJM, CAR, and FG. Jointly developed the structure and arguments for the paper: JTO, RB, AJM, CAR, and FG. Made critical revisions and approved final version: JTO and RB. All authors reviewed and approved of the final manuscript.

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