

## ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues

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### 1. ABSTRACT

Sialyl-Tn is a simple mucin-type carbohydrate antigen aberrantly expressed in gastrointestinal adenocarcinomas and in the precursor lesion intestinal metaplasia. Sialyl-Tn tumour expression is an independent indicator of poor prognosis. We have previously shown *in vitro* that ST6GalNAc-I and ST6GalNAc-II sialyltransferases can synthesize sialyl-Tn. The aim of the present study was to establish whether ST6GalNAc-I is the major enzyme responsible for the expression of sialyl-Tn. We used a model of CHO-lDL cells producing only MUC1-Tn glycoform and showed that ST6GalNAc-I is the key-enzyme leading to sialyl-Tn biosynthesis. We developed novel monoclonal antibodies specific for ST6GalNAc-I and evaluated its expression in gastrointestinal tissues. ST6GalNAc-I was detected in normal colon mucosa co-localized with *O*-acetylated sialyl-Tn. Expression was largely unaltered in colorectal adenocarcinomas. In contrast, we found that ST6GalNAc-I is weakly expressed in normal gastric mucosa, but over-expressed in intestinal metaplasia, co-localized with sialyl-Tn. In gastric carcinomas ST6GalNAc-I was also associated with sialyl-Tn, but with heterogeneous staining and partial co-localization. Our results showed ST6GalNAc-I as the major enzyme controlling the expression of cancer-associated sialyl-Tn antigen in gastrointestinal tissues.

### 2. INTRODUCTION

Altered glycosylation is a common feature of cancer cells. The sialyl-Tn (STn) antigen (Neu5Ac $\alpha$ 2-6GalNAc $\alpha$ -O-Ser/Thr), also known as CD175s, is a simple mucin-type carbohydrate antigen which has attracted much attention because it is highly expressed in most gastric (1-3), colorectal (4), ovarian (5), breast (6, 7) and pancreatic (8) carcinomas, but has limited expression in normal tissues. The expression of sialyl-Tn is an established indicator of poor prognosis in patients with gastric (9-11), colorectal (12, 13), and ovarian cancer (14) and contributes to the aggressive phenotype of carcinoma cells by altering their behaviour (15-18). Sialyl-Tn expression is also aberrantly expressed in precursor and early lesions of carcinomas of the gastrointestinal tract, such as intestinal metaplasia (IM) of the stomach (2), adenomatous polyps (12), chronic ulcerative colitis (13) and pancreatic intraepithelial neoplasias (8).

The synthesis of the mucin-type carbohydrate antigens depends upon expression of the enzymes that initiate *O*-glycosylation: UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) (19). GalNAc-transferases control the first step in mucin glycosylation and controls the level of complexity of mucin glycans, i.e., the sites and density of *O*-glycan occupancy

of the mucin tandem repeats (20, 21). Processing of GalNAc-*O*-Ser/Thr, also known as the Tn antigen, by different glycosyltransferases is dependent on the cell type and tissue of origin. Monosaccharides are added sequentially to the GalNAc in the Golgi apparatus, forming a variety of *O*-glycan structures that include the Core1 (Gal $\beta$ 1-3GalNAc-*O*-Thr/Ser), also known as T antigen, and the Core2 (GlcNAc $\beta$ 1-6 (Gal $\beta$ 1-3)GalNAc-*O*-Thr/Ser) glycans. These glycoforms may be glycosylated further forming larger and more complex structures. A common feature in carcinoma cells is the activation of an alternative glycosylation pathway that caps the GalNAc-*O*-Thr/Ser structures with  $\alpha$ 2,6-sialic acid. Once sialylated, the resulting sialyl-Tn glycoform, Neu5Ac $\alpha$ 2-6GalNAc-*O*-Ser/Thr, cannot be glycosylated further thereby preventing normal glycan elongation (22-24). The underlying mechanisms responsible for the induction of the sialyl-Tn glycoform in cancer cells are not fully understood. Two  $\alpha$ 2,6-sialyltransferases have been shown to be capable to sialylate the GalNAc residue O-linked on proteins: ST6GalNAc-I, and ST6GalNAc-II (25-27). However, over-expression of the ST6GalNAc-I glycosyltransferase in cells can override the normal *O*-glycosylation pathways leading to the formation of sialyl-Tn *O*-glycans as the dominant mucin phenotype (28-30). We have previously shown that both ST6GalNAc-I and ST6GalNAc-II can synthesize sialyl-Tn, *in vitro*; however, only ST6GalNAc-I could create large amounts of sialyl-Tn when over-expressed in a cancer cell line (28). Previous studies have also correlated the amount of ST6GalNAc-I mRNA with the amount of sialyl-Tn expression in breast cancer (30) and several cell lines (31), while the level of ST6GalNAc-II mRNA did not correlate with the expression of sialyl-Tn in gastric (unpublished results) and breast cancer cell lines (26) or in breast tumours (30). However, the role of each sialyltransferase, ST6GalNAc-I and II, in the synthesis of the cancer-associated sialyl-Tn structure in cells and pathological tissues remains unclear.

A novel monoclonal antibody (MAb) directed to ST6GalNAc-I was used to evaluate its expression in normal gastric and colonic mucosa, in intestinal metaplasia of the stomach, and in gastric and colorectal adenocarcinomas. The results show that over-expression of ST6GalNAc-I in intestinal metaplasia of the stomach co-localizes with the expression of sialyl-Tn. ST6GalNAc-I was not over-expressed in colon adenocarcinomas when compared to normal mucosa, suggesting that the acetylation status of sialyl-Tn may mask this antigen in normal colonic mucosa and exposed in adenocarcinomas. In contrast, in gastric carcinomas expression of sialyl-Tn was partially, but not exclusively, associated with ST6GalNAc-I. Our results demonstrate that ST6GalNAc-I is the major regulator of expression of cancer-associated sialyl-Tn *O*-glycosylation.

### 3. MATERIAL AND METHODS

#### 3.1. Recombinant expression of soluble ST6GalNAc-I

An expression construct of the human ST6GalNAc-I were designed to encode an enzyme lacking the cytoplasmic and the transmembrane region. This soluble construct of ST6GalNAc-I encoding amino acid

residues 43–601 was prepared as described previously (28). The soluble construct of ST6GalNAc-I was expressed in Sf9 cells using the Baculo-virus expression system, and purified to near homogeneity (28).

#### 3.2. Generation of monoclonal antibody

The recombinant ST6GalNAc-I enzyme was used as immunogen. Balb/c mice were immunized with one subcutaneous injection of 10  $\mu$ g native protein in Freund's complete adjuvant, followed by two injections with Freund's incomplete adjuvant, and finally an intravenous boost without adjuvant. Eye bleeds were taken 7 days after the third immunization, and the titre and specificity of antibodies were evaluated by immunocytology with baculo-virus infected Sf9 cells expressing recombinant human ST6GalNAc-I or irrelevant enzymes. Spleen cells from one immunized mouse were fused to NS-1 myeloma cells to produce hybridomas and the cloning procedure was performed as described previously (32, 33). Hybridomas were selected by initial screening on baculo-virus infected Sf9 cells expressing either one of the following human sialyltransferases: ST6GalNAc-I, ST6GalNAc-II or ST3Gal-I. Further characterization of the hybridoma clones was done on human cell lines expressing full-length coding ST6GalNAc-I or ST6GalNAc-II enzymes as previously described (28). Antibodies were also tested by SDS-PAGE Western blot analysis.

#### 3.3. CHO IdID-MUC1F cells stably transfected with ST6GalNAc-I or ST6GalNAc-II

CHO IdID cells were kindly provided by M. Krieger (MIT, USA) (34). Stable CHO IdID cell line expressing full length FLAG-epitope tagged MUC1 (MUC1F) has been previously established (CHO IdID-MUC1F) (35). Based on existing cDNA constructs (28, 30), full length human ST6GalNAc-I and ST6GalNAc-II-Myc constructs were produced by PCR, using PfuUltra™ polymerase (Stratagene). Regions encoding the membrane anchoring (ST6GalNAc-I; aa 1-293 and ST6GalNAc-II; aa 1-80) and catalytic domains (ST6GalNAc-I; aa 294-601 and ST6GalNAc-II; aa 81-374) were generated using two sets of primers. For ST6GalNAc-I membrane anchor ST6I-1 (5'-GCG GAT CCA CCA TGA GGT CCT GCC TGT GGA GAT GCA GGC-3')/ST6I-3 (5'-GAG CGC TCT AGA CCA CAG CGA CTT GGA GGC TTT GAT CTT C-3') and catalytic domain ST6I-2 (5'-GAG CGC TCT AGA AAA CTC TTT CTG CCC AAC CTC ACT CTC-3')/ST6I-4 (5'-GCG GCG GCC GCT CAG TTC TTG GCT TTG GCA GTT CCG GG-3') were used. For ST6GalNAc-II membrane anchor ST6II-1 (5'-GCG GGA TCC ACC ATG GGG CTC CCG CGC GGG TCG TTC TTC-3')/ST6II-3 (5'-GAG CGC TCT AGA GTG GGG GTG CCG CTG AAT GGC CAG-3') and catalytic domain ST6II-2 (5'-GAG CGC TCT AGA GGC CTG TTC AAT CTC TCC ATT CCA GTG-3')/ST6II-4 (5'-GGC GGC CGC GCG CTG GTA CAG CTG AAG GAT GCC GGC C-3'). Single XbaI sites were introduced in the membrane anchoring domain preceding the catalytic domain leading to L293S and Q294R mutations in ST6GalNAc-I and F80S mutation in ST6GalNAc-II-Myc, sites underlined in primers shown above. Dual insert (membrane anchor and catalytic domain) were cloned

**Table 1.** Monoclonal antibodies, their specificity and references

MAb	Specificity	Dilution	Reference
2C3	ST6GalNAc-I	1:2	This paper
1C9	ST6GalNAc-I	1:2	This paper
HB-STn	sialyl-Tn	1:15	Dako
B72.3	sialyl-Tn	1:50	(38)
TKH2	sialyl-Tn	1:10	(39)
PMH1	MUC 2	1:10	(40)
CLH2	MUC 5AC	1:10	(41)
5E5	MUC1-Tn/STn	1:10	(42, 43)

directionally into the BamHI and NotI sites of the pcDNA3-zeo (Invitrogen™). For ST6GalNAc-II the C-terminal Myc-tag construct was introduced by inserting a double stranded Myc-oligo into the Not-I site of ST6GalNAc-II-pcDNA3-zeo. All constructs were sequence verified using an ABI Avant sequenator. CHO ldlD-MUC1F cells were transfected with full length human ST6GalNAc-I or ST6GalNAc-II-Myc and grown in the presence of GalNAc.

### 3.4. Patients and tissues

Gastric carcinomas and gastric mucosa adjacent to carcinomas were obtained from individuals undergoing surgery at Hospital S. João, Medical Faculty (Porto, Portugal). Study was performed with the approval of the local HSJ ethical committee (from 05-09-2007). Specimens were frozen at -80°C. Serial sections were cut and used for immunofluorescence. We evaluated 22 gastric mucosas with normal morphology, 24 IM lesions adjacent to the carcinoma cases that were classified as complete IM (n=14) and incomplete IM (n=10) according to the pattern of mucin expression (described below) and 31 cases of gastric carcinoma. Gastric carcinomas were classified according to Laurén's classification (36). Colorectal adenocarcinoma cases were obtained from the KAM cohort based on the screening group of the Norwegian Colorectal Cancer Prevention study in the county of Telemark, Norway (37) and a series of colorectal cancer cases recruited to the KAM cohort from routine clinical work at Telemark Hospital, Skien and Ulleval University Hospital, Oslo. The KAM study is approved by the Regional Ethical Committee and the Norwegian Data Inspectorate. We evaluated 15 colorectal adenocarcinomas. A sample of control tissue was taken from the surgically removed tissue close to the adenocarcinoma or as far away from the tumor as possible. The histology of adjacent normal tissue was examined independently by two specialist histopathologists, and found to be normal. The histology of the adenocarcinomas was also examined independently by two specialist histopathologists in order to determine the tumor stage. All colon cancer cases were histomorphologically classified as moderately differentiated.

### 3.5. Immunofluorescence

Details of MAbs used are shown in Table 1. Cells were fixed in ice-cold acetone for 5 min and kept at -20°C before staining. Tissue slides were preserved at -80°C and fixed in 4% paraformaldehyde for 15 min at RT, prior to procedure.

Double staining was used for co-localization of

ST6GalNAc-I (MAb 2C3) and sialyl-Tn (MAb TKH2 and HB-STn) and ST6GalNAc-I and MUC2 (MAb PMH1). MUC5AC and MUC2 staining was performed for the characterization of IM types (44). Deacetylation (saponification) of selected samples was accomplished by treating slides immediately after fixation with 0.1N NaOH for 20 min at RT prior to performing the immunofluorescence (45). Samples designed for MUC2 detection with PMH1 were pre-treated with neuraminidase as previously described (40).

Samples were washed twice in PBS and incubated for 20 min with rabbit non-immune serum (DAKO) diluted 1:5 in PBS/10%BSA. Samples were incubated overnight at 4°C with the monoclonal antibodies 2C3 or CLH2 (Table 1) diluted in PBS/5%BSA. Sections were washed in PBS and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:70 in PBS/5%BSA, for 45 min. Then the samples were washed in PBS and blocked with non-immune goat serum (DAKO) diluted 1:5 in PBS/10%BSA for 20 min. Sections were incubated with the monoclonal antibody PMH1 or TKH2 (Table 1) diluted in PBS/5%BSA overnight at 4°C. Sections were washed with PBS and incubated for 30 min with Texas-Red-conjugated goat anti-mouse IgM (in the case of PMH1) or Texas-Red-conjugated goat anti-mouse IgG1-specific (in the case of TKH2) (Jackson Immunoresearch) diluted 1:70 in PBS/5%BSA and DAPI diluted 1:100, for 30 min in the dark. Samples were washed in PBS and mounted in Vectashield (Vector Laboratories, Inc).

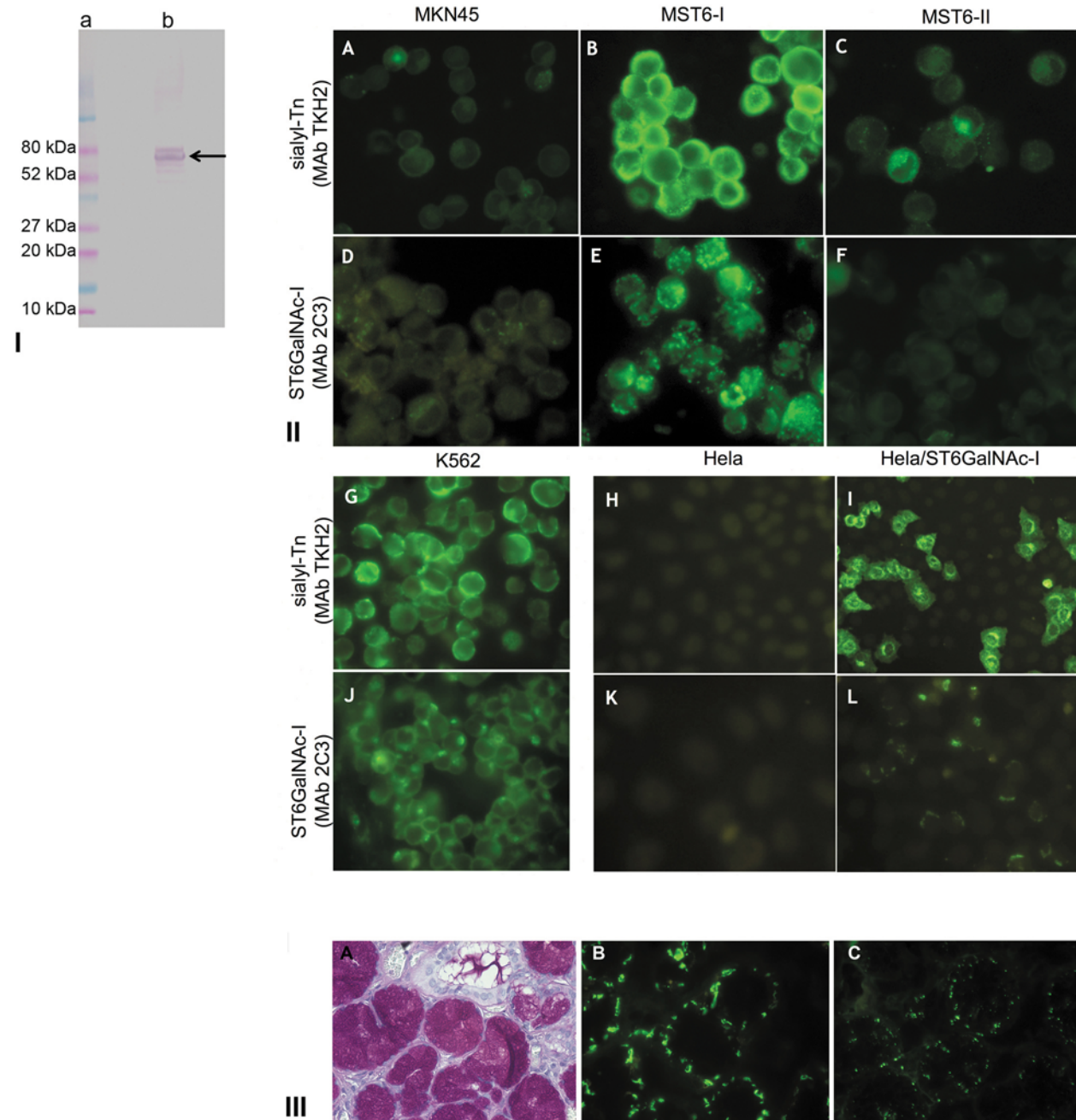
Results are based on assays analyzed by two independent observers. Statistical analysis was performed using the chi-square test with Yates correction using Statview 4.01 software.

## 4. RESULTS

### 4.1 Monoclonal antibodies to ST6GalNAc-I

Two monoclonal antibodies 2C3 (IgG2a) and 1C9 (IgG1) were selected for their specific reactivity with ST6GalNAc-I. MAb 2C3 reacted with Sf9 cells expressing ST6GalNAc-I but no reactivity was observed with Sf9 cells expressing ST6GalNAc-II. MAb 2C3 was also found to react with the human cell line K562, which expresses high levels of ST6GalNAc-I transcripts and sialyl-Tn antigen (31) (Figure 1, Panel II G, J). MAb 2C3 did not show reactivity with the denatured form of ST6GalNAc-I when analyzed by SDS-PAGE Western blot (not shown). On the other hand MAb 1C9 was selected for their specific of the reactivity with ST6GalNAc-I in reduced SDS-PAGE Western blot analysis (Figure 1, Panel I). These results are in agreement with our previous findings that MAbs to glycosyltransferases tend to react either with the native conformation of the protein or with the denatured protein (32, 46). We have generally been unable to produce MAbs that react with both the native protein in unfixed cells or tissues and by SDS-PAGE Western blot analysis.

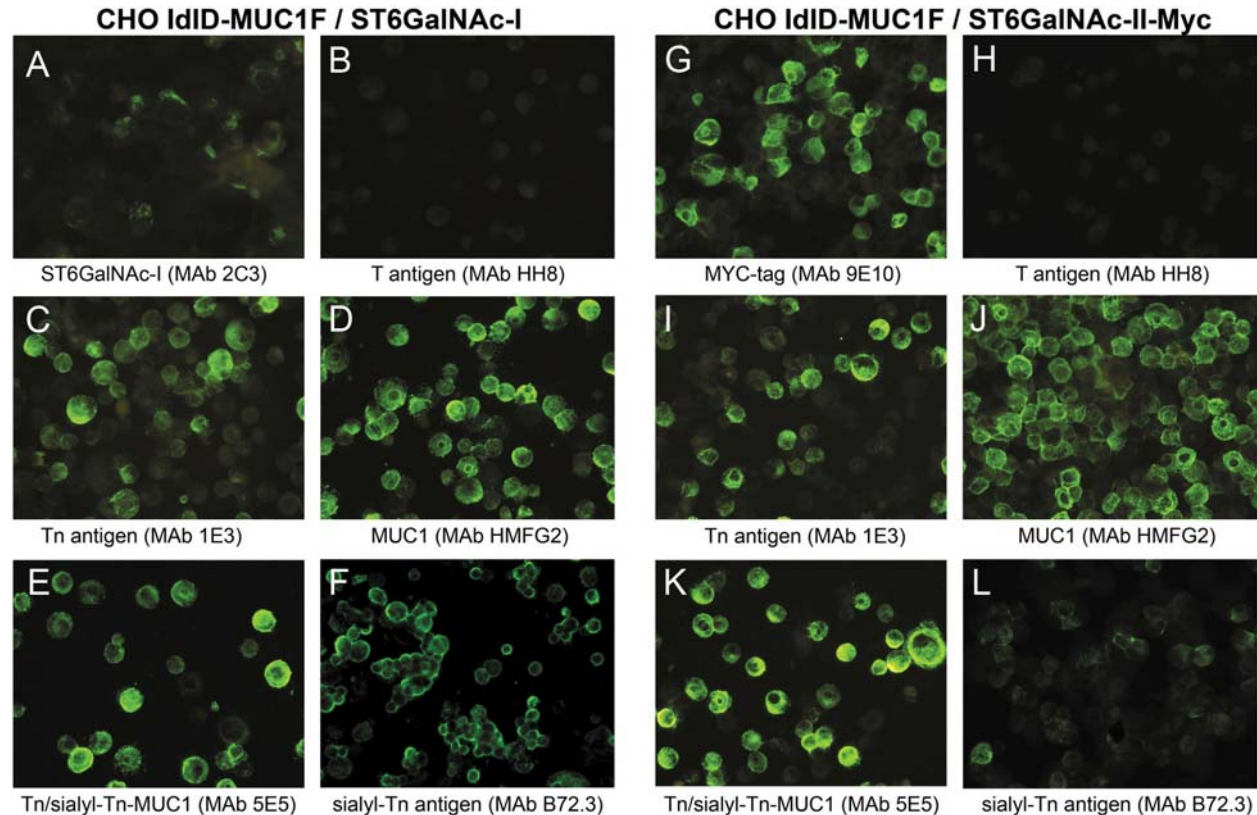
Further analysis of MAb 2C3 showed some immunoreactivity in immunocytochemistry with the human



**Figure 1.** Characterization of monoclonal antibodies to ST6GalNAc-I. Panel-I: SDS-PAGE and Western blot analysis of the immunoreactivity of monoclonal antibody 1C9 with total cell lysate of transfected Sf9 cells expressing a secreted construct of ST6GalNAc-I (b). Molecular weight markers are indicated as (a) in the figure. Arrow indicates the detection ST6GalNAc-I. Additional band corresponds to N-glycosylated form of the protein. Panel-II: Expression of ST6GalNAc-I and sialyl-Tn in cell lines. MAB 2C3 reacts exclusively with MKN45 cells transfected with ST6GalNAc-I (MST6-I) (E) and shows no reactivity with *wt* MKN45 (D) nor with MKN45 cells transfected with ST6GalNAc-II (MST6-II) (F). The same pattern is observed for HeLa cells (K, L). K562 hematopoietic cell line naturally expresses ST6GalNAc-I (J). Sialyl-Tn staining with MAB TKH2 correlates with ST6GalNAc-I (A-C, G-I). Panel-III: Expression of ST6GalNAc-I and sialyl-Tn in minor salivary glands from oral cavity. PAS&E staining (A). MAB 2C3 to ST6GalNAc-I stains salivary glands (B) and co-localizes in the same regions as sialyl-Tn, MAB HB-STn (C). Magnification 200x.

tumour cell lines MKN45 (31) and HeLa, which express low levels of endogenous ST6GalNAc-I. However, we observed strong immunoreactivity with these cells when

transfected with full coding human ST6GalNAc-I (Figure 1, Panel II). The staining pattern observed in these cells was a distinct perinuclear punctuate staining typical of



**Figure 2.** Stably transfected CHO IdID-MUC1F cells with full coding human ST6GalNAc-I and ST6GalNAc-II-MYC grown in the presence of GalNAc. Cell staining images from CHO IdID-MUC1F +ST6GalNAc-I (A-F) or +ST6GalNAc-II (G-L) are shown in left and right panels, respectively. Primary antibodies used are shown in the figure.

Golgi vesicles. Mab 2C3 furthermore produced the same staining pattern in glandular epithelial cells of salivary glands (Figure 1, Panel III), which are known to express ST6GalNAc-I mRNA.

#### 4.2 Glycophenotype of CHO IdID-MUC1F cells stably transfected with ST6GalNAc-I and ST6GalNAc-II

Glycosylation-defective CHO IdID cells lack the epimerase that transforms UDP-Glc and -GlcNAc to UDP-Gal and -GalNAc, respectively (34). This defect blocks *O*-linked glycosylation of proteins. However, the addition of exogenous Gal or/and GalNAc to the media of IdID cells overcame this defect and corrected the abnormal glycosylation phenotype (34). Addition of only GalNAc to the cells produced a truncated mucin limited to Tn. No sialyl-Tn was observed. This indicated that CHO IdID cells have no competing glycosylation pathway after the Tn glycoform was produced. Interestingly, both the parental cell line CHO K1 and the CHO IdID cell line grown in the presence of Gal and GalNAc are known to produce mono- and to a lesser extent di-sialylated T-antigen glycoforms (47), and indicates that an ST6GalNAc-transferase activity must be present. Stably transfected cells (CHO IdID-MUC1F) of a full coding MUC1 have been produced and shown to express MUC1 on the cell membrane (35, 42).

CHO IdID-MUC1F cells cultured in the presence of GalNAc expressed the Tn antigen (GalNAc-*O*-Ser/Thr)

(Figure 2). There was no sialyl-Tn found indicating that the endogenous sialyltransferases were not capable of producing sialyl-Tn. A lack of the presence of the sialyl-T antigen was confirmed by treating the cells with neuraminidase and then staining the cells using controlled conditions with an anti-T antibody. Stable transfection of ST6GalNAc-I into these cells resulted in production of the sialyl-Tn glycoform as detected by B72.3 (Figure 2) and other sialyl-Tn Mabs (not shown). In contrast, stable transfection with ST6GalNAc-II did not result in substantial production of sialyl-Tn, although a few faint cells (<25%) could be visualized (Figure 2). Interestingly, while the cytolocalization of ST6GalNAc-I detected by Mab 2C3 was clearly supranuclear and Golgi-like, the staining of myc-tagged ST6GalNAc-II was localized throughout the cell with no apparent Golgi localization. While tagged constructs may mislocalize recombinant proteins, previously we have shown co-localisation of native and tagged constructs of several other glycosyltransferases. A 6X His tagged ST6GalNAc-II construct has also been expressed as a secreted functional enzyme in insect cells (unpublished). We are currently trying to develop Mabs to ST6GalNAc-II and characterize the cytolocalization in more detail.

#### 4.3 Expression of ST6GalNAc-I and sialyl-Tn in normal gastric mucosa

Gastric mucosa with normal morphology showed



**Table 2.** Immunoreactivity of Mab 2C3 in normal gastric mucosa, intestinal metaplasia, gastric carcinoma, and colon carcinoma

Gastric mucosa and Intestinal metaplasia	ST6GalNAc-I (Mab 2C3 immunoreactivity)			P value
	Negative	Weakly positive	Strongly positive	
Normal gastric mucosa (n=22)	0	21	1	
IM complete <sup>a</sup> (n=14)	0	0	14	
IM incomplete <sup>a</sup> (n=10)	0	0	10	
Gastric Carcinomas (n=31)	ST6GalNAc-I (Mab 2C3 immunoreactivity)			P value
	Negative	< 50% of pos. cells	> 50% of pos. cells	
Histologic type <sup>b</sup>				
Intestinal (n=18)	4	10	4	0.600
Diffuse (n=9)	2	6	1	
Atypical (n=4)	1	1	2	
Sialyl-Tn expression				
Negative (n=4)	3	1	0	0.009
< 50% of positive cells (n=9)	1	8	0	
> 50% of positive cells (n=18)	3	8	7	
Colon Carcinomas (n=15)	ST6GalNAc-I (Mab 2C3 immunoreactivity)			P value
	Negative	< 50% of pos. cells	> 50% of pos. cells	
Clinical stage				
A (n=4)	0	2	2	0.897
B (n=4)	0	3	1	
C (n=7)	0	3	4	
Sialyl-Tn expression				
Negative (n=1)	0	1	0	0.005
< 50% of positive cells (n=7)	0	7	0	
> 50% of positive cells (n=7)	0	0	7	

(a) Classification based on MUC2 and MUC5AC mucin expression, (b) Classification according to Laurén (34)

a complete absence of expression of sialyl-Tn antigen, as expected. Immunodetection of ST6GalNAc-I was observed in all normal gastric mucosa (Table 2) and was characterised by a weak immunofluorescence signal limited to the perinuclear area of cells from the foveolar epithelium, corresponding to a Golgi-like staining (Figure 3A-G). The process of deacetylation did not alter the expression of sialyl-Tn in normal gastric mucosa, which remained negative after treatment (data not shown).

#### 4.4 Expression of ST6GalNAc-I and sialyl-Tn in intestinal metaplasia

The 24 foci of IM, 14 of complete IM and 10 of incomplete IM, were classified according to the pattern of expression of mucins (44). Cases with metaplastic areas which co-expressed gastric mucin MUC5AC together with intestinal mucin MUC2 were classified as incomplete IM, whereas cases with MUC2 but lacking MUC5AC were classified as complete IM.

Expression of sialyl-Tn antigen was observed in the mucin vacuoles of goblet cells of all cases of complete and incomplete IM (Figure 3C and 3D, respectively). All IM cases showed a strong expression of ST6GalNAc-I (Table 2, Figure 3A-D), clearly contrasting with the faint staining of normal mucosa areas. The staining was also restricted to goblet cells and the pattern was perinuclear (Golgi-like). Therefore, both in complete and incomplete IM, ST6GalNAc-I showed increased staining and co-localized with MUC2 (Figure 3A and 3B) and sialyl-Tn (Figure 3C and 3D).

#### 4.5 Expression of ST6GalNAc-I and sialyl-Tn in gastric carcinoma

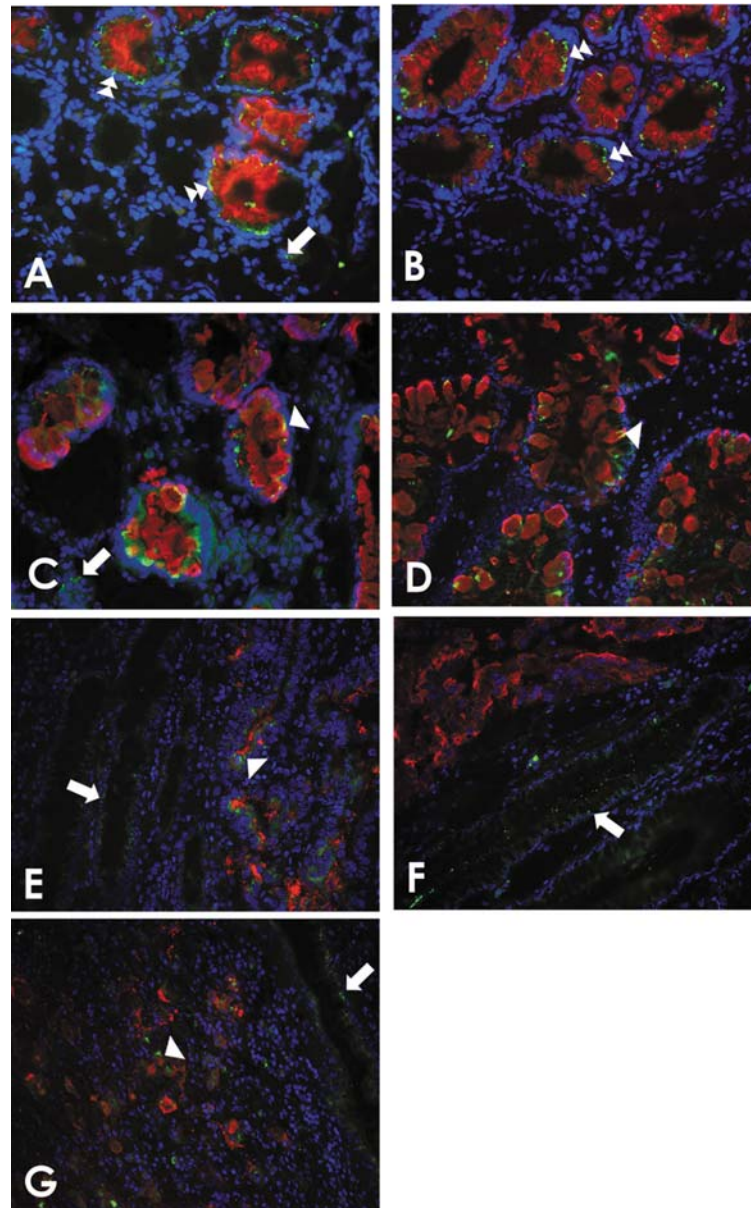
Expression of ST6GalNAc-I was observed in 24/31 (77%) of cases of gastric carcinoma (Table 2; Figure 3 E-G). The expression levels of enzyme (intensity) could

not be assessed due to the heterogenous intensity observed within each gastric carcinoma case. However, differences were observed between the pattern of expression in gastric carcinoma and normal mucosa. A punctuated Golgi-like staining restricted to foveolar epithelium was seen in normal mucosa, opposite to the widespread perinuclear and diffuse cytoplasmic staining observed in gastric carcinoma. Due to this fact and to the focal and patchy nature of sialyl-Tn and ST6GalNAc-I expression in carcinomas, a semi-quantitative scale of percentage of positive cells was used to achieve a more informative classification.

We observed a significant association between ST6GalNAc-I expression and sialyl-Tn expression, but not with the histopathologic type of gastric carcinoma (Table 2). In the majority of carcinoma cases where co-expression of sialyl-Tn and ST6GalNAc-I was observed, both sialyl-Tn and ST6GalNAc-I were largely expressed in the same cells. However, in 8 cases the score for sialyl-Tn expression exceeded the score observed for ST6GalNAc-I. In addition, there were 4 cases with expression of sialyl-Tn where we did not observe ST6GalNAc-I (Table 2).

#### 4.6 Expression of ST6GalNAc-I and sialyl-Tn in normal colorectal mucosa

In all 15 cases, normal appearing colorectal mucosa showed a strong perinuclear, Golgi-like, staining pattern of ST6GalNAc-I throughout all compartments of the colonic crypts (Figure 4B). Before deacetylation with sodium hydroxide, sialyl-Tn was only expressed sporadically in lower compartments of the crypts in about half of the cases (Figure 4C). After deacetylation, all cases expressed sialyl-Tn intracellularly in lower compartments; however, in some cases the expression of sialyl-Tn was also observed in goblet cells throughout the crypts (Figure 4D).



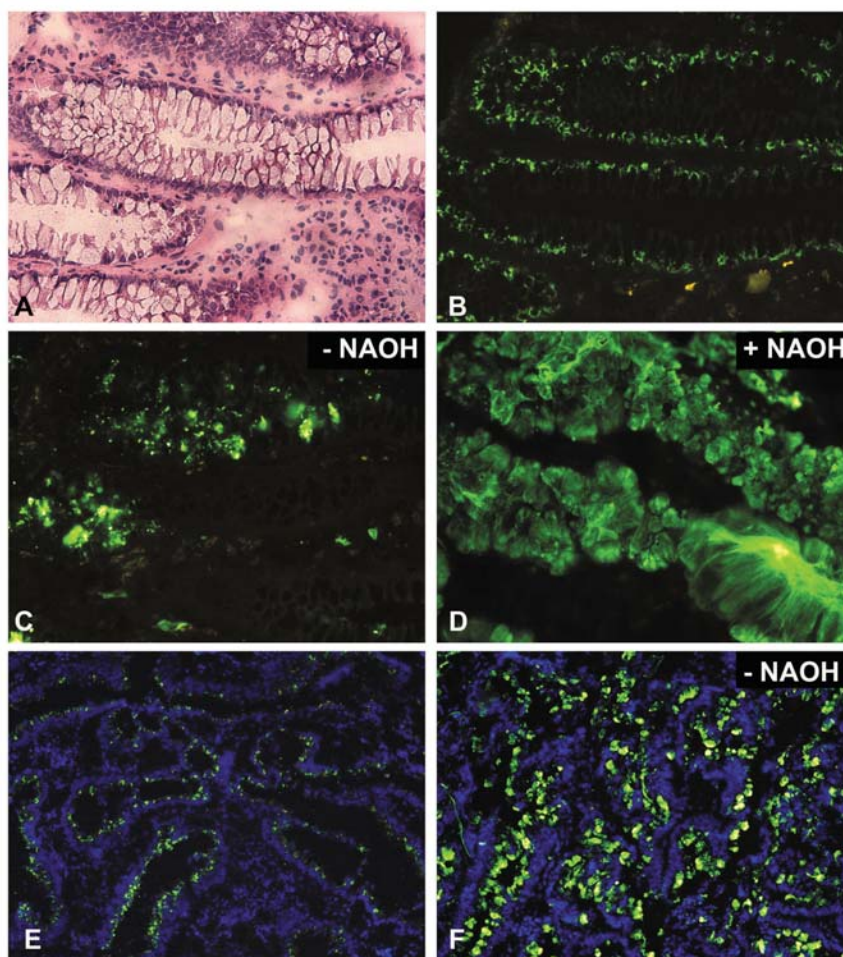
**Figure 3.** Double labelling of ST6GalNAc-I (Mab 2C3) and sialyl-Tn (Mab TKH2) or MUC2 (Mab PMH1) in gastric tissues. Normal gastric mucosa adjacent to intestinal metaplasia and carcinomas shows weak expression of ST6GalNAc-I in the foveolar epithelium (A, C, E, F, G, arrows). MUC2 (red) and ST6GalNAc-I (green) are overexpressed in the goblet cells in complete intestinal metaplasia (A, double arrowheads) and incomplete intestinal metaplasia (B, double arrowheads). Sialyl-Tn (red) and ST6GalNAc-I (green) are overexpressed in the goblet cells in complete intestinal metaplasia (C, arrowhead) and incomplete intestinal metaplasia (D, arrowhead). Gastric carcinoma of the intestinal type showing co-localization of sialyl-Tn and ST6GalNAc-I (E, arrowhead). Gastric carcinoma of the intestinal type showing expression of sialyl-Tn and absence of expression of ST6GalNAc-I (F). Gastric carcinoma of the diffuse type showing co-expression of sialyl-Tn and ST6GalNAc-I (G, arrowhead). Magnification 200x.

#### 4.7. Expression of ST6GalNAc-I and sialyl-Tn in colorectal adenocarcinoma

ST6GalNAc-I was observed in 15 cases of colorectal adenocarcinoma (Table 2, Figure 4E and 4F). Expression of ST6GalNAc-I was observed in 50% of cases and in >50% of the tumour cells. Generally the staining intensity was weaker in tumour cells than in the normal

cells. Exceptionally strong staining of both ST6GalNAc-I and sialyl-Tn was observed in transitional tissue, a tissue which is immediately adjacent to tumour edge but with no histological features of malignancy. Transitional tissue was observed in 4 of the colon biopsies (not shown).

Sialyl-Tn was expressed in the same cases as



**Figure 4.** Normal colon stained with H&E (A), with MAb 2C3 (ST6GalNAc-I) (B) and MAb TKH2 (Sialyl-Tn) before deacetylation (C) and after deacetylation by treatment with NAOH (D) as described in Mat. & Methods. Colon carcinoma stained with MAb 2C3 (ST6GalNAc-I) (E) and MAb TKH2 (Sialyl-Tn) (F). ST6GalNAc-I and sialyl-Tn co-localize in colon carcinomas. In normal colon this co-localization is observed only when the tissue sample is submitted to deacetylation, exposing the Sialyl-Tn antigen. Magnification 200x.

ST6GalNAc-I and in the same areas. Deacetylation of the tissue enhanced the staining of sialyl-Tn in most of the cases, but this effect was far less pronounced than in normal colon samples. Sialyl-Tn was observed in the apical cell membranes, cytoplasm, and luminal contents of the colorectal adenocarcinomas. There was no correlation of Duke's clinical staging with the expression of ST6GalNAc-I; however, a strong association with sialyl-Tn expression was observed (Table 2). We could not detect tumour cells that expressed sialyl-Tn and does not express ST6GalNAc-I. On the other hand we could occasionally detect few tumour cells that expressed ST6GalNAc-I and lacking expression of sialyl-Tn.

## 5. DISCUSSION

Sialyl-Tn antigen is a simple mucin-type carbohydrate antigen whose aberrant expression is common in several human carcinomas and precursor lesions of

cancer (1-8, 12, 13). Sialyl-Tn antigen is the product of an abnormal glycosylation pathway, corresponding to the early  $\alpha$ 2,6-sialylation of GalNAc-*O*-Ser/Thr. The molecular mechanism leading to the activation of this pathway in carcinomas remains to be clarified.

We have previously shown *in vitro* that ST6GalNAc-I and ST6GalNAc-II can synthesize sialyl-Tn, but only ST6GalNAc-I can induce high amounts of sialyl-Tn when over-expressed in cancer cell lines (28). In most cells multiple glycosylation pathways compete for the same substrate and hence concordance between *in vitro* activity and *in vivo* function may not be evident. We used the CHO 1d1D cell system to determine if the function of ST6GalNAc-II was inhibited by other glycosyltransferases competing for the same substrate. Indeed, ST6GalNAc-II did not produce substantial amounts of sialyl-Tn in a cell capable of only producing the enzymes substrate, the Tn glycoform as part of the abundant MUC1 mucin. In



contrast, ST6GalNAc-I produced sialyl-Tn under the same conditions. Therefore, it may be concluded that the low activity of ST6GalNAc-II at producing sialyl-Tn in cells was not due to competing glycosylation. Different molecular mechanisms leading to sialyl-Tn biosynthesis have been previously described. In the LS174T colon carcinoma cell line model, Brockhausen and co-workers demonstrated that the expression of sialyl-Tn in a subset of cells is associated with a lack of core1  $\beta$ 3Gal-transferase activity, and not with  $\alpha$ 2,6Sialyl-transferase activity (22). Whereas in the LMCR colon carcinoma cell line, they showed that the reverse was true, i.e., sialyl-Tn expressing cells had increased  $\alpha$ 2,6Sialyl-transferase activity (23). Therefore, using two cell lines from colonic origin, it was shown two different mechanisms leading to sialyl-Tn synthesis: one due to ST6GalNAc-transferase activity overexpression, and another due to the inability of synthesizing the core1 glycoform (T-antigen), as a result of inactivation of the molecular chaperone, *Cosmc* (48). The *Cosmc* chaperone is required for expression of an active  $\beta$ 3Gal-transferase and, subsequently, core1 synthesis and proper *O*-glycosylation (49). *Cosmc* mutations have been found in cancer cell lines and primary cervical cancers where its inactivation has been associated with sialyl-Tn expression (48, 49). *Cosmc* mutations in gastric carcinomas remain unidentified. In fact, the action of competing glycosyltransferases, namely core 1  $\beta$ 3Gal-transferase that uses the same substrate as ST6GalNAc-I (GalNAc-*O*-Ser/Thr), and core 3 ( $\beta$ 3GnT6), may be a mechanism interfering with sialyl-Tn biosynthesis. Another factor may be the sub cellular localization and Golgi compartment localization of these glycosyltransferases. Previous studies have shown that sialyl-Tn synthesis in colorectal adenocarcinoma was localized throughout the Golgi apparatus, including the early compartments (50). This result was further supported by another study showing that ST6GalNAc-I was found in all Golgi sections in carcinoma cells (30). In fact, the biosynthetic pathway leading to the production of disialyl-T structures (27-29) involves the assembly of the monosialyl-T glycoform (NeuAc $\alpha$ 2-3Gal $\beta$ 1-3GalNAc-*O*-Thr/Ser) prior to the action of ST6GalNAc-transferase. This sequence of glycan addition to the T-antigen is supported by the topological location of ST6GalNAc-I in the Golgi compartment; whereas, core 1  $\beta$ 3Gal-transferase and  $\alpha$ 2,6sialyltransferases, are located in earlier cellular compartments. Studies showing that transfection of cells with ST6GalNAc-I can result in overriding of the natural *O*-glycosylation pathway with resulting truncated sialyl-Tn *O*-glycans therefore suggest that it is the overexpression and likely the resulting altered topology of the enzyme that drives the premature  $\alpha$ 2,6sialylation of Tn before core 1 extension (30). This interpretation would be in agreement with the recent finding that ST6GalNAc-II, while capable *in vitro* of using Tn substrates, is incapable of inducing substantial amounts of sialyl-Tn synthesis in cells with abundant Tn substrates and without competitive alternative glycosylation pathways (28). Presumably this is due to an entirely different subcellular topology (Figure 2).

In the present study, we have produced and characterized two novel MABs that specifically react with

either the native or the denatured ST6GalNAc-I enzyme protein and have utility in immunohistology applications and SDS-PAGE Western blot. Although the homologous enzymes ST6GalNAc-I and ST6GalNAc-II share a degree of sequence similarity we did not identify clones with cross-reactivity between the enzymes. This result is similar to our past experience with the immunogenicity of homologous glycosyltransferases (32). In agreement with previous studies we have observed the absence of sialyl-Tn expression in normal gastric mucosa. Nevertheless, weak immunoreactivity for the ST6GalNAc-I enzyme was observed in the foveolar epithelium of gastric mucosa. The ST6GalNAc-I staining was restricted to the perinuclear region suggesting Golgi localization (Table 2; Figure 3). A recent study has shown that ST6GalNAc-I mRNA is weakly expressed in various normal tissues despite that these tissues generally do not express the sialyl-Tn antigen (30). In addition, we have previously shown that gastric cell lines devoid of sialyl-Tn, still express basal levels of ST6GalNAc-I transcripts (31). It is therefore clear that expression of low levels of ST6GalNAc-I mRNA as well as basal enzyme levels, as shown here, may not be sufficient *per se* to induce sialyl-Tn expression and further supports the importance of the levels of expression of the ST6GalNAc-I enzyme as well as the Golgi compartment localization.

The expression of sialyl-Tn in colonic mucosa appears to represent a different scenario. ST6GalNAc-I is expressed in normal colon both at the mRNA and protein levels. Nevertheless, sialyl-Tn is not normally detectable at the surface colon cells, being restricted to perinuclear staining at the base of the crypt in few cases (Figure 4). In normal colonic epithelial cells, sialic acid residues are often modified by *O*-acetyl groups, thereby precluding antibody recognition of the sialyl-Tn antigen. Deacetylation leads to sialyl-Tn exposure and consequent detection, and MAb staining largely increases after this procedure, as can be seen in Figure 4. Yet, this mechanism is not true for normal gastric mucosa. We observed no sialyl-Tn expression after deacetylation of normal gastric mucosa (data not shown). *O*-acetylation in the human stomach is very rare and limited to pathological conditions (51) so the absence of sialyl-Tn detection in normal gastric mucosa cannot be explained by such mechanism.

In the human stomach, Intestinal Metaplasia is a pre-malignant lesion characterized by a global trans-differentiation of gastric epithelium into intestinal epithelium and is associated with an increased risk for gastric carcinoma development. Intestinal metaplasia expresses several intestinal markers that are foreign to normal gastric epithelial cells, such as MUC2 and sialyl-Tn, which co-localize at the mucinous vacuoles of goblet cells (44, 52). We observed that ST6GalNAc-I was expressed in all intestinal metaplasia cases (24/24), and this expression co-localizes both with MUC2 and sialyl-Tn (Figure 3). The ST6GalNAc-I staining was in the perinuclear area of goblet cells observed in all metaplastic glands, regardless of the histological sub-type, and was noticeably more intense than in normal gastric mucosa (Figure 3). It is therefore clear that an overexpression of

ST6GalNAc-I exists in intestinal metaplasia when compared to normal gastric mucosa, and supports the hypothesis that overexpression of ST6GalNAc-I is a mechanism leading to sialyl-Tn antigen expression in intestinal metaplasia.

In the present study, the abundant sialyl-Tn expression observed in intestinal metaplasia without deacetylation indicated that *O*-acetylation did not interfere with sialyl-Tn detection in intestinal metaplasia as it does on normal colon (51). Nevertheless, our results showed that the intestinal metaplasia model recapitulates normal colon as ST6GalNAc-I and sialyl-Tn are co-expressed in the same cells.

In gastric carcinoma, sialyl-Tn expression was detected in 87% of the cases similarly to previously published series (2), and ST6GalNAc-I expression was found on 77% of gastric carcinoma cases. ST6GalNAc-I showed a heterogeneous staining, either clustered in focal areas or widely dispersed, and showed variable intensity of staining within each case, thus justifying for a different classification criteria than in intestinal metaplasia cases, based on the percentage of positive cells in the tumour. Using this classification, ST6GalNAc-I expression was significantly associated with sialyl-Tn expression, independent of the histologic type of the tumour. Expression of ST6GalNAc-I in >50% of tumour cells was observed in 7/31 cases, all of which also expressed sialyl-Tn in >50% of cells. This equals saying that all gastric carcinoma cases that were high enzyme expressers were also so for sialyl-Tn. However, the inverse was not true, i.e. there were several cases that expressed sialyl-Tn in >50% of their tumour cells but have <50% cells with ST6GalNAc-I (8/18) or are even negative (3/18). This means that, although the majority of gastric carcinoma cases expressed sialyl-Tn and ST6GalNAc-I in the same cells, there were areas/cases where sialyl-Tn was expressed without ST6GalNAc-I detection. Therefore, although there was an association between sialyl-Tn and ST6GalNAc-I at the case level, the expression of both did not always co-localize at the cellular level. This apparent discrepancy at the cellular level may be explained by different hypothesis. One possibility is that ST6GalNAc-I may be present but technically undetectable due to low levels of expression. Supporting this hypothesis, Sewell and co-workers have found that, in breast carcinomas, ST6GalNAc-I expression correlated at the mRNA level with sialyl-Tn expression. Nevertheless, cases that were weak to moderate positives for sialyl-Tn (50% of cases) did not express any detectable ST6GalNAc-I RNA by Northern Blot analysis, but were positive by RT-PCR (30). These results suggest that enzyme detection at the protein level may only occur in cases with abundant sialyl-Tn expression. Another possibility is that ST6GalNAc-I may not be constitutively expressed in the tumour cells, resulting in a temporary presence of the enzyme and longer expression of sialyl-Tn bearing glycoprotein. Finally, we could not exclude the possibility that ST6GalNAc-II, which was demonstrated to be enzymatically capable of synthesizing sialyl-Tn in this study and in a previous one (28), may co-adjuvate ST6GalNAc-I in tumour cells.

A clear scenario was observed in colon adenocarcinomas where a major overlap of enzyme and glycan staining was observed. Colon adenocarcinomas showed a significant association between ST6GalNAc-I expression and sialyl-Tn expression that is independent of the clinical stage (Table 2). The presence of both enzyme and product was similar to normal colon mucosa with the difference that for a complete overlap, deacetylation procedures must be applied. In line with this, a previous study has shown that normal and cancerous colon tissues have similar ST6GalNAc enzymatic activity (53). Interestingly enough, transitional tissues have been shown to display increased enzymatic activity when compared to normal mucosa and cancer (54). We found identical results reflected by the exceptionally strong staining of ST6GalNAc-I in our transitional colon tissues.

In conclusion our results demonstrate that the novel monoclonal antibody 2C3 is highly specific for the ST6GalNAc-I enzyme and detects this protein in cells and tissues expressing it. We also demonstrate that ST6GalNAc-I expression is associated with sialyl-Tn expression in gastrointestinal tissues supporting the ST6GalNAc-I as the major regulator of expression of cancer-associated sialyl-Tn antigen on *O*-linked carbohydrate chains.

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**Key Words:** ST6GalNAc-I, Sialyl-Tn, Glycosylation, Gastric Carcinoma, Intestinal Metaplasia

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