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An α2,3 sialyltransferase (ST3Gal I) is elevated in primary breast carcinomas

Joy Burchell[1], Richard Poulsom[4], Andrew Hanby[3], Caroline Whitehouse[2], Lucienne Cooper, Henrik Clausen[5], David Miles and Joyce Taylor-Papadimitriou

Imperial Cancer Research Fund Breast Cancer Biology Group and [3]Hedley Atkins/ICRF Breast Pathology, Guy's Hospital, London SE1 9RT, UK, [4]*In situ* Hybridization Service and Histopathology Unit, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK, [5]Department of Oral Diagnostics, School of Dentistry, University of Copenhagen, DK2200 Copenhagen, Denmark

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The MUC1 mucin is expressed on the luminal surface of most simple epithelial cells but in carcinomas, especially those of the breast and ovary, MUC1 is upregulated and aberrantly glycosylated. MUC1 contains a large amount of O-linked glycans which, in the mucin expressed by normal mammary epithelial cells, consist mainly of core 2 based structures carrying polylactosamine chains. However, the mucin expressed by breast carcinomas has shorter side-chains, often consisting of sialylated core 1 (Galβ1–3GalNAc). *in situ* hybridization of primary breast tissue showed that a sialyltransferase (ST3Gal I), responsible for adding sialic acid to core 1 thereby terminating chain extension, is elevated in primary breast carcinomas when compared to normal or benign tissue. Furthermore, the level of mRNA expression encoding ST3Gal I is correlated to the intensity of staining seen with the antibody SM3, which specifically recognises underglycosylated, tumour associated MUC1. Thus, the aberrant glycosylation of MUC1 seen in breast carcinomas appears to be due, at least in part, to the elevation of ST3Gal I.

Key words: MUC1/sialyltransferase/core 2 β1,6N-acetylglucosaminyltransferase/O-linked glycosylation/breast cancer

Introduction

Changes in the structure of glycan chains attached to glycolipids and glycoproteins are a common feature of the progression to malignancy (Hakomori, 1989). This has a profound effect on the structure of mucin glycoproteins as they carry multiple O-linked glycans. The MUC1 mucin is an integral membrane protein with a large extracellular domain made up of tandemly repeated amino acids (Gendler *et al.*, 1988), the number of which varies with the individual. Each repeat contains five potential sites for O-linked glycosylation all of which may be utilised (Muller *et al.*, 1997). MUC1 is aberrantly glycosylated in 95% of breast carcinomas, the side-chains being shorter with a relatively higher sialic acid content than those found on the mucin expressed by normal mammary epithelial cells (Lloyd *et al.*, 1996). This change in O-glycan structure results in the exposure of peptide epitopes, such as that recognised by the monoclonal antibody SM3, which are masked in MUC1 expressed by normal mammary epithelial cells (Burchell *et al.*, 1987), and in the appearance of novel carbohydrate epitopes. This makes the MUC1 expressed by breast carcinomas antigenically distinct from that expressed by normal mammary cells. Studies with breast cancer cell lines have indicated that changes in O-glycan structure correlate with changes in the profile of expression of specific glycosyltransferases (Brockhausen *et al.*, 1995).

Mucin-type O-linked glycosylation is initiated in the Golgi apparatus (Rottger *et al.*, 1998) by the addition of N-acetylgalactosamine to the hydroxyl group of serines and threonines. The oligosaccharide side chains are then built up by the sequential addition of individual sugars, via various core structures, each reaction being catalyzed by specific glycosyltransferases (Brockhausen, 1996). Thus the final structure of the O-glycans is determined by the activity of individual glycosyltransferases and by their position relative to each other in the Golgi pathway.

In the MUC1 mucin, galactose is added to the initial GalNAc to form the core 1 structure, which in normal breast epithelial cells is then converted to core 2 by the addition of N-acetylglucosamine; the reaction being catalysed by the enzyme core 2 β1,6 N-acetylglucosaminyltransferase (C2GnT). Core 2 is then extended by the addition of polylactosamine units (Hanisch *et al.*, 1989; Lloyd *et al.*, 1996). However, in breast carcinomas the core 1 to core 2 conversion is reduced, resulting in the O-glycans on the tumour associated MUC1 being shorter and less complex (Hull *et al.*, 1989; Lloyd *et al.*, 1996). ST3Gal I, which catalyzes the addition of sialic acid in an α2,3 linkage to Gal β1–3 GalNAc, terminating chain extension, uses the same substrate (core 1) as C2GnT. Thus, changes in the expression and activity of these enzymes could lead to changes in the structure and length of the O-glycans attached to MUC1 and to the exposure of peptide epitopes such as that recognised by SM3. We have previously shown that, when compared to SM3 negative normal mammary epithelial cell lines, SM3 positive breast cancer cell lines have an 8–10 fold elevation in the enzymic activity responsible for transferring sialic acid in α2,3 linkage to the core 1 substrate (Brockhausen *et al.*, 1995). In contrast, C2GnT activity is absent or decreased in the tumor cell lines.

To determine whether similar changes in the expression of glycosyltransferases are also seen in primary breast cancers we

[1]Correspondence to: Dr Joy Burchell, ICRF Breast Cancer Biology Group, 3rd Floor, Thomas Guy House, Guy's Hospital, London SE1 9RT, UK
[2]Present address: Cancer Genetics Laboratory, 8th Floor, Guy's Tower, Guy's Hospital, London SE1 9RT, UK

Table I. *In situ* hydridization results

Tumor type	C2GnT				ST3Gal I			
	Level of expression				Level of expression			
	0	+	+++	++++	0	+	++	+++
Benign	55%	33%	6%	6%	56%	38%	6%	0%
Ductal Ca	33.3%	33.3%	0%	33.3%	0%	66%	17%	17%
Grade I								
Ductal Ca	0%	60%	40%	0%	0%	0%	83.5%	16.5%
Grade II								
Ductal Ca	17%	33%	17%	33%	0%	0%	67%	33%
Grade III								
Mucoid	64%	36%	0%	0%	0%	36%	36%	28%

Sections of formalin fixed and paraffin-embedded tissue were analyzed by *in situ* hybridization using antisense probes for ST3Gal I or C2GnT. Ductal carcinoma n = 18, grade I n = 5, grade II n = 6, grade III n = 7; lobular carcinoma n = 2; mucinous carcinoma n = 10; fibroadenoma n = 2; lactating adenoma n = 2. Note the increased expression of ST3Gal I with increased grade of ductal carcinomas.

have used *in situ* hybridization to detect levels of mRNA encoding a specific sialyltransferase, ST3Gal I. Our results show that ST3Gal I mRNA is indeed more abundant in carcinoma than benign breast epithelium and the level correlates with the intensity of staining with the monoclonal antibody SM3.

Results

Expression of ST3Gal I and C2GnT transferases by breast tissues

Expression of mRNA encoding for ST3Gal I or C2GnT was analyzed in 34 breast tissue sections (see Table I). In 22 of the breast carcinoma sections, normal or benign epithelial tissue was observed, and these areas were evaluated separately. ST3Gal I expression was detected exclusively in epithelial cells and was stronger in the carcinomas compared to normal or benign breast tissue (Figure 1). When a scoring system was used to analyze the intensity of the staining (see *Materials and methods*), it became clear that the expression of ST3Gal I was elevated in breast carcinomas when compared to normal and benign lesions. Furthermore in ductal carcinomas, the level of expression appeared to be related to tumor grade (Table I).

The same breast tissue samples were analyzed by *in situ* hybridization for C2GnT mRNA expression (see Figure 1). Again C2GnT RNA was mostly observed in the epithelial tissue and, as to be expected from the presence of extended core 2 based structure identified on MUC1 isolated from human milk (Hanisch *et al.*, 1989), was quite strongly expressed by lactating breast (Figure 1F). However, as can be seen from Table I, there appears to be no obvious correlation in the expression of this glycosyltransferase with the type of breast tissue.

Elevated level of ST3Gal I is correlated with the staining intensity of SM3

The monoclonal antibody SM3 was raised to MUC1 largely stripped of its carbohydrate by exposure to hydrogen fluoride (Burchell *et al.*, 1987). This antibody reacts with a peptide epitope (Burchell *et al.*, 1989) which is selectively exposed in carcinomas (Girling *et al.*, 1989). Sections parallel to those used for *in situ* hybridization were stained with SM3 by indirect immunoperoxidase (Figure 1C,G) and the staining intensity scored 0 to +++ without knowledge of the ST3Gal I results. Table II shows the staining intensity observed with SM3 in comparison to the level of expression of ST3Gal I. To analyze if the apparent correlation was indeed statistically significant, a Spearman's Correlation test was performed. This gave a positive correlation with a p value of 0.0053, indicating that there is a statistically highly significant correlation between ST3Gal I mRNA expression and the intensity of staining of the monoclonal antibody SM3.

Table II. Staining intensity of SM3 compared to expression levels of ST3Gal I mRNA as determined by *in situ* hybridization (P = 0.0053)

SM3 intensity	ST3Gal I mRNA expression			
	0	+	++	+++
0	1	2	0	0
+	0	7	4	0
++	0	1	7	1
+++	0	3	6	2

Increased ST3Gal I mRNA expression is correlated to increased α2,3 sialic acid structures

In an attempt to correlate increased ST3GalI mRNA expression with increased α2,3 oligosaccharides parallel sections of nineteen of the tumors were stained with *Maakia amurensis* lectin (Table IIIA) which recognizes α2,3 linked sialic acid (Konami *et al.*, 1994). When these were sections were scored for staining intensity and compared to ST3Gal I expression a significant positive correlation (p = 0.0015) was found as defined by

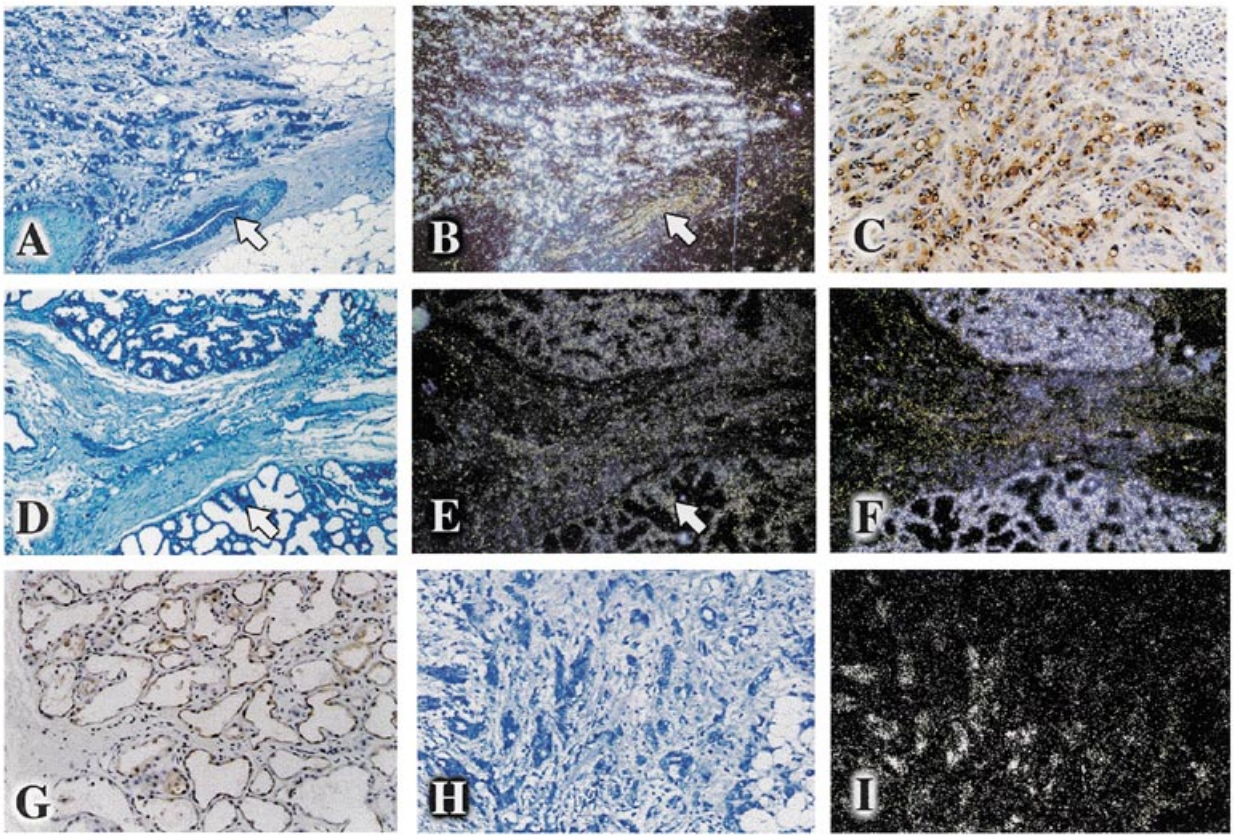

Fig. 1. Expression ST3Gal I and C2GnT mRNA and the SM3 epitope on MUC1 by breast tissue. Sections of formalin fixed and paraffin-embedded tissue, (**A**–**C**, **H**, **I**) ductal carcinoma; (**D**–**G**) lactating adenoma, were analyzed by *in situ* hybridization using antisense probes for ST3Gal I (**A**, **B**, **D**, **E**); (**A**, **D**) light field; (**B**, **E**) dark field; or for C2GnT (**F**, **I**). The same tissues were analyzed for the expression of the SM3 epitope by immunohistochemistry (**C**, **G**). The arrow points to a blood vessel which shows no expression of ST3Gal I. Note the relatively low expression of ST3Gal I by lactating breast (**E**) relative to ductal carcinoma (**B**).

Spearman's correlation test. Furthermore, Maackia amurensis staining was also strongly correlated with SM3 epitope expression ($p = 0.0001$) demonstrating that the staining of this lectin was an indication of the presence of α2,3 sialic acid on MUC1 (Table III B).

Discussion

Although it is well documented that changes in the composition of O-glycans occur in malignancy (Hakomori, 1989), it has rarely been possible to relate these changes with changes in the expression and/or activity of relevant glycosyltransferases (Yang *et al.*, 1994). We have previously shown (Brockhausen *et al.*, 1995) that, in breast cancer cell lines the truncated O-glycans carried by the cancer-associated MUC1 mucin correlates with an increase in sialyltransferase activity responsible for the addition of sialic acid in an α2,3 linkage to core 1 (Galβ1–3GalNAc). There are three sialyltransferases, ST3Gal I, ST3Gal II and ST3Gal IV that could be responsible for this increase in activity (Chang *et al.*, 1995; Recchi *et al.*, 1998), and recently, a PCR method has been developed that can distinguish between at least two of these enzymes in cell lines (Recchi *et al.*, 1998). However, this methodology cannot establish which cell types within a particular specimen express the mRNAs *in vivo*. We now show by *in situ* hydridization that mRNA encoding a specific sialyltransferase, ST3Gal I, is elevated in primary breast carcinoma cells compared to normal tissue, and that in ductal carcinomas the level of mRNA is related to the grade of the tumour. In addition, ST3Gal I mRNA expression was correlated with Maackia amurensis lectin binding. Although this lectin recognizes α2,3 linked sialic acid on O-linked core 1 glycans (Konami *et al.*, 1994) it is not specific for O-glycans (Wang and Cummings, 1988). However, its positive correlation with the expression of a peptide epitope (recognized by the monoclonal antibody SM3) on the MUC1 mucin, which is masked in MUC1 expressed by normal mammary epithelial cells but exposed when O-glycan chain extension is inhibited (Burchell and Taylor-Papadimitriou, 1993), suggests that it is reflecting an increased in α2,3 sialic acid structure on MUC1. Furthermore, we have previously shown that overexpression of ST3Gal I does indeed result in the increased sialylation of MUC1 (Whitehouse *et al.*, 1997). As ST3Gal I expression is also positively correlated with the expression of the SM3 epitope, the increased expression of ST3Gal I may, at least in part, explain the truncation of O-glycans carried by the carcinoma associated mucin which results in the exposure of the SM3 epitope.

Table III. Staining intensity of *Maackia amurensis* versus A, ST3Gal I expression as determined by *in situ* hybridization and B, SM3 staining intensity

A

		ST3Gal I expression (p = 0.0015)			
		0	+	++	+++
Macckia	0	2	2	1	0
lectin intensity	+	0	7	1	1
	++	0	0	3	1
	+++	0	0	0	1

B

		SM3 intensity (p = 0.0001)			
		0	+	++	+++
Macckia	0	3	2	0	0
lectin intensity	+	0	4	3	2
	++	0	0	2	2
	+++	0	0	0	1

In normal breast epithelium, the core 1 glycan is acted upon by C2GnT which initiates chain extension involving the formation of polylactosamine side-chains (Hanisch *et al.*, 1989; Lloyd *et al.*, 1996). Thus, C2GnT could compete with ST3Gal I for the core 1 substrate. In the cell lines previously examined, the nonmalignant cell line (MTSV1-7) exhibited reasonable levels of the C2GnT, while two of the three breast cancer lines studied appeared to have lost expression of this enzyme at the level of the mRNA. In the third cell line, the message level was higher than in the normal cell line but the activity was lower (Brockhausen *et al.*, 1995), possibly suggesting posttranscriptional control. Thus, levels of mRNA encoding C2GnT may not reflect the activity of the enzyme and indeed, *in situ* hybridization of the core 2 transferase mRNA in primary breast cancers showed there was no consistent pattern of reduction in the level of message expressed. An accurate comparison of glycosyltransferase activity would be extremely difficult to achieve in breast tissue as normal specimens consist mainly of stroma with very little epithelium, in contrast to tumor samples which can contain a very high proportion of epithelial cells. However, by transfecting ST3Gal I into a cell line expressing active C2GnT, we have shown that overexpression of ST3Gal I, even in the presence of active core 2 enzyme, can result in shorter sugar side-chains being found on MUC1 (Whitehouse *et al.*, 1997). Furthermore, by transfection of the core 2 enzyme into the breast cancer cell line T47D, we have shown that the SM3 epitope is masked by the core 2 branch (Whitehouse, 1998). Thus the positive correlation of SM3 staining intensity with ST3Gal I expression levels suggests that in primary breast carcinomas, overexpression of ST3Gal I allows this glycosyltransferase to compete with C2GnT for the core 1 substrate, resulting in sialylation of core 1, inhibiting further chain extension. Monoclonal antibodies to ST3Gal I are now being developed which will permit the analysis of the larger number of specimens required to confirm the correlation with SM3 binding, and to confirm the association of ST3Gal I with increased grade of ductal carcinomas.

Materials and methods

In situ hybridization

Specific localization of the mRNAs for ST3Gal I and human core 2 β1,6 GlcNA was accomplished by *in situ* hybridization using antisense riboprobes synthesized with ^{35}S-UTP (~800 Ci/mmol; Amersham, UK). Tissue blocks were from the archives of the Histopathology Unit and Guy's Hospital.

The template for synthesis of the ST3Gal I riboprobe was *Sac*I linearized pcDNA1 plasmid containing the full coding region of human ST3Gal I (Chang *et al.*, 1995), generously provide by Dr. Joseph Lau. The riboprobe was produced with Sp6 RNA polymerase and contained 942 bases complementary to ST3Gal I mRNA. For human core 2 N-acetylglucosaminyltransferase (C2GnT) (Bierhuizen and Fukuda, 1992), *Hae*II linearized pBluescript plasmid containing a 1 kb HindIII fragment of C2GnT, obtained by PCR from the breast cancer cell line MCF-7, was used with T7 RNA polymerase to generate a riboprobe containing 965 bases complementary C2GnT transferase mRNA. Hybridization was essentially as described by Senior *et al.* (1988), for formalin-fixed paraffin-embedded tissue.

The presence of hybridizable mRNA in all compartments of the tissues studied was established in near serial sections using an antisense β-actin probe generated with SP6 RNA polymerase and *Dra*I linearized phβA-10, prepared by subcloning a ~450 bp region of a human β-actin cDNA into pSP73.

Autoradiography was at 4°C (two exposures per section; 11 d and 14 d for the enzyme mRNA targets and 11d for β-actin mRNA), before developing in Kodak D19 and counterstaining by Giemsa's method. Sections were examined under conventional or reflected light dark-field conditions (Olympus BH2 with epi-illumination) that allowed individual autoradiographic silver grains to be seen as bright objects on a dark background.
Evaluation of RNA expression was carried out by assessing the intensity of the silver grains giving a score of 0 for negative, 1+ for weak, 2++ for moderate, and 3+++ for strong intensity. When a section contained areas of morphologically defined malignant and benign tissue, these areas were scored separately.

Histochemistry

Dewaxed and rehydrated 3 μm sections from the primary tumors of the selected patients were incubated in tissue culture supernatant containing the mouse monoclonal antibody SM3 for 1 h at room temperature and the binding detected by incubation with a peroxidase conjugated rabbit antimouse and a standard streptavidin–biotin complex method (DAKO Denmark). Staining was visualized with diaminobenzidine (Sigma UK) and lightly counterstained with hematoxylin. The primary antibody was omitted and replaced with Tris buffer pH 7.6 on sections used as negative controls.

Evaluation of SM3 immunostaining was carried out by assessing the intensity of apical and cytoplasmic staining, giving a score of 0 for negative, 1+ for weak, 2++ for moderate, and 3+++ for strong staining.

The binding of *Maackia amurensis* was determined by incubating dewaxed and rehydrated sections with biotinylated *Maackia amurensis* (Vector Laboratories, UK), followed by streptavidin–biotin horseradish peroxidase. Staining was visualized with diaminobenzidine (Sigma UK) and the sections lightly counterstained with hematoxylin. The scoring system

was the same as that used for SM3. Due to specimen availability, only 19 sections were analyzed for *Maackia amurensis* staining.

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