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Simple mucin-type carbohydrate antigens (T, sialosyl-T, Tn and sialosyl-Tn) in breast carcinogenesis

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Abstract Immunohistochemical analysis of the expression of simple mucin-type carbohydrate antigens (Tn, sialyl-Tn and T) was performed in a series of 43 cases of intraductal hyperplasia without atypia, 9 cases of intraductal hyperplasia with atypia, 54 cases of ductal carcinoma in situ (DCIS) and 26 cases of invasive breast carcinoma. We also studied 36 cases of isolated breast normal epithelium, 20 cases of "normal" breast epithelium adjacent to neoplasms and 14 cases of apocrine metaplasia. All antigens were detected in different frequencies in normal, hyperplastic, metaplastic and neoplastic breast epithelium. Tn and sialyl-Tn are expressed more frequently in malignant than in benign breast epithelium; while Tn expression increases from normal to invasive carcinomas, sialyl-Tn increases until DCIS and drops in invasive carcinomas, suggesting that either there is a failure of a proportion of DCIS to progress to invasive carcinoma or loss of expression of sialyl-Tn when some carcinomas become invasive. The high frequency of Tn and sialyl-Tn expression in breast intraductal proliferations probably reflects incomplete glycosylation in these lesions, which is a well-known tumour-associated phenomenon and supports the assumption that such lesions are putative precursors of breast cancer. T antigen was expressed in all groups studied, but its prevalence differed significantly between normal and neoplastic epithelium. The expression of these antigens in epithelium adjacent to carcinomas is similar to that found in isolated normal breast epithelium, whereas apocrine metaplasia has a pattern of simple mucin-type glycosylation that is specific and distinct from that of the normal breast epithelium, with a high frequency of marked expression of Tn and sialyl-Tn. The similarity of the pattern of expression of simple mucin-type antigens in metaplasia and malignant neoplasia reduces the usefulness of these markers from a diagnostic standpoint.

Key words Breast carcinoma · Hyperplasias · Apocrine metaplasia · Carbohydrate antigens

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Introduction

Epidemiological, histological and experimental studies have suggested that breast carcinoma develops from normal epithelium through several intermediate stages [5, 19]. In a multistep process of carcinogenesis, certain molecular structures become apparent at the same time as some of the normal characteristics are lost. These molecular changes may be regarded as tumour markers that can be used either in diagnosis or to monitor the effects of treatment. In this context, our group has previously demonstrated that breast intraductal proliferations, a putative pre-neoplastic stage of breast carcinoma, express markers, such as carcinoembryonic antigen, which are normally absent in normal epithelium and frequently present in malignant epithelium [18].

In recent years, it has been shown that malignant transformation is frequently associated with abnormal expression of cell surface carbohydrates [1, 2, 4, 7, 8, 10, 13, 17, 20]. This phenomenon of aberrant glycosylation can be detected in most types of human cancers and is also demonstrated in some premalignant lesions [1, 13, 21]. In this vast and complex research field much interest has been devoted to accumulation of simple mucin-type carbohydrates such as Tn, sialyl-Tn and T (Thomsen-Friedenreich) antigens, because these antigens, in general, are widely expressed in carcinomas, but only to a limited degree in normal adult cells [1, 6, 7, 10, 13, 17, 20, 21].

The general biosynthetic pathway of simple mucin-type glycosylation is well established [10, 13, 22]. In summary, Tn antigen is a short carbohydrate structure O-linked to serine and threonine amino acids of the protein backbone (Gal-N-Acα1→O-Ser/Thr). This structure can be extended by a galactose residue forming the T antigen (Galβ1-3Gal-N-Acα1→O-Ser/Thr) or, alternatively, substituted with a sialic acid resulting in the sialyl-Tn antigen. Therefore, Tn, T and sialyl-Tn antigens represent the initial, most immature glycosylation products of serine and threonine of the protein core and are masked in normal tissue due to sialylation and/or chain elongation and branching by the addition of other sugar residues, such as blood group determinants [13, 22].

The demonstration that the accumulation of simple mucin-type carbohydrates in some premalignant lesions of squamous and glandular epithelium [1, 13, 21] is similar to that occurring in the corresponding carcinomas [1, 4, 10, 13, 17, 21] raises the possibility that altered glycosylation may be an early event in the carcinogenesis process.

Recently, Reed et al. [17] showed that simple mucin-type antigens are present in benign and malignant breast lesions and suggested that the expression of T antigen in breast epithelium adjacent to tumours and the loss of sialyl-T in some benign lesions could be a premalignant event. However, these authors did not make a systematic study of the group of proliferative breast lesions that are acknowledged as putative premalignant lesions.

In an attempt to evaluate the distribution of simple mucin-type antigens (Tn, sialyl-Tn and T) at different steps of breast carcinogenesis, we undertook the present immunohistochemical study of normal breast epithelium (adjacent and not adjacent to carcinomas), ductal hyperplasia (with and without atypia), ductal carcinoma in situ and invasive breast carcinomas using specific monoclonal antibodies reacting with Tn, sialyl-Tn and T (before and after treatment with neuraminidase).

Materials and methods

Case selection

Routinely formalin-fixed, paraffin-embedded breast tissue from 106 patients with intraductal epithelial proliferations and 26 with invasive carcinomas were investigated. We also included 36 cases of normal breast tissue obtained from plastic surgery procedures. The 106 cases of intraductal proliferations were subdivided at the histopathological level into ductal hyperplasia without atypia (n=43), atypical ductal hyperplasia (n=9) and ductal carcinoma in situ (DCIS) (n=54). The criteria for classifying a case as typical or atypical hyperplasia were those described by Page and Rogers [15]. The 54 cases of DCIS were classified according to the predominant architectural pattern into: comedo (n=32), cribriform (n=13), micropapillary (n=7) and solid (n=2), following the criteria of Page and Anderson [14]. For the purpose of this study all cases of DCIS were grouped into either "non-comedo" type (n=22) or "comedo" type (n=32), the latter designation being restricted to lesions with centrally necrotic ducts distended by large pleomorphic cells. All invasive carcinomas studied were infiltrating ductal carcinomas classified according to the WHO recommendations [26]. In 20 cases of carcinoma (in situ or invasive), normal breast tissue was present in the sections. These tumour-adjacent regions were examined and grouped separately. Breast epithelium with apocrine metaplasia were examined in 14 cases and also grouped separately.

Tissue from all cases was fixed in buffered 10% formalin, dehydrated and embedded in paraffin; 5-μm sections were cut and stained with haematoxylin and eosin and used to classify all the lesions.

Immunohistochemistry

Antibodies

Mouse monoclonal antibodies with well-defined specificities for simple mucin-type carbohydrate antigens linked through the hydroxyl groups of the amino acids serine and threonine were used. The antibodies, together with their isotype specificities and source, are given in Table 1. All antibodies were used as hybridoma supernatants, in the following dilutions: HB-Tn 1:5; HB-STn 1:8; HB-T 1:10. The detection of T antigen masked by sialic acid was performed with the antibody directed against the T antigen after treatment of the sections with neuraminidase. The finding of masked T antigen in red blood cells was used as an internal positive control for the sections submitted to neuraminidase.

Immunostaining

Paraffin sections from formalin-fixed material were cut, dewaxed and immunostained with the avidin-biotin-peroxidase complex (ABC) method [9]. Sections designed for neuraminidase treatment were preincubated with neuraminidase from *Clostridium perfringens* type VI (Sigma) diluted in a 0.1 M sodium acetate buffer, pH 5.5, to the final concentration of 0.1 U/ml. The incubation, carried out at 37°C for 2 h, was followed by three washings in ice-cold water. All the sections were then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to quench the endogenous peroxidase activity. The sections were incubated for 20 min with normal nonimmune serum to eliminate nonspecific staining. Excess normal serum was removed from the slides. The sections were then incubated with the primary antibodies for 18–22 h at 4°C. This was followed by incubation with a 1:100 dilution of biotin-labelled anti-mouse secondary antibody (Dakopatts, Copenhagen, Denmark) for 30 min and ABC (Dakopatts) for 60 min. Careful rinses were done with PBS between each step of the procedure. The colour was developed with diaminobenzidine and the sections were lightly counterstained with haematoxylin, dehydrated and mounted. All series included positive controls. Negative controls were performed using a mouse monoclonal antibody of irrelevant specificity but of the same subclass and concentration as the respective monoclonal primary antibody.

Evaluation of staining

A case was considered to be positive whenever more than 5% of the cells present in the sections were immunoreactive. Both mem-

Table 1 Structures of carbohydrate epitopes and monoclonal antibodies

Antigen	Structure	Antibody(isotype)[a]
Tn	Gal-N-Acα1→O-Ser/Thr 0	HB-Tn (IgM)
Sialyl-Tn	NeuAcα2→6Gal-N-Acα1→O-Ser/Thr	HB-STn (IgG1)
T	Galβ1-3Gal-N-Acα1→O-Ser/Thr	HB-T (IgM)

[a] All from Dako

branous and cytoplasmic staining were evaluated; membranous staining was defined as distinct linear staining along epithelial cell borders, either luminal or intercellular, whereas cytoplasmic staining was defined as diffuse or granular staining in the cytoplasmic area. Whenever possible, the presence of a supranuclear (Golgi region) pattern of staining was also recorded.

Statistical analysis

Results are expressed as percentages. Statistical analysis was performed using the Chi-square test with Yates' correction. Two values were considered significantly different when p was less than 0.05 and suggestively different when p was less than 0.10.

Results

Normal breast epithelium

The results are summarized in Table 2. Tn antigen was expressed in normal epithelium with a predominantly apical membranous pattern of staining, sometimes together with a granular cytoplasmic staining. Comparison of the expression in normal breast epithelium with that in normal breast epithelium adjacent to carcinomas revealed a higher prevalence of expression in the latter (45.0% vs 25.0%), albeit without statistical significance.

Sialyl-Tn was focally expressed in the cytoplasm of the cells as well as in the luminal apical membrane. No immunoreactivity was detected at the cell membrane. Similar prevalences of expression were observed in the normal epithelium and in the normal epithelium adjacent to carcinomas (22.2% vs 15.0%, respectively).

T antigen was detected in 13.8% of the cases of normal breast epithelium. The immunoreactivity was predominantly observed in the apical membrane and in some cases also in the cytoplasm (Fig. 1). After treatment with neuraminidase, T antigen ("sialyl-T") was detected in every case, indicating that in normal breast epithelium T antigen is masked by sialic acid. Comparison of these results with those observed in normal breast epithelium adjacent to carcinomas revealed a higher prevalence of T expression in the latter (35.0% vs 13.8%), again without statistical significance.

Table 2 Distribution of mucin-type carbohydrate antigens in normal breast epithelium

Antigens	Normal breast epithelium		
	Isolated	Adjacent to carcinomas	p-value
Tn	9/36 (25.0%)	8/20 (45.0%)	0.24
Sialyl-Tn	8/36 (22.2%)	3/20 (15.0%)	0.51
T antigen	5/36 (13.8%)	7/20 (35.0%)	0.07
T antigen after neuraminidase	36/36 (100%)	19/20 (95.0%)	0.17

Apocrine metaplasia

Most cases of apocrine metaplasia exhibited immunoreactivity for all the antigens. Tn and sialyl-Tn staining was intense and predominantly in the apical membrane and supranuclear area ("Golgi pattern"; Fig. 2). Tn was expressed by the apocrine epithelium more often than by the normal breast (64.3% vs 25.0%; P=0.005) and hyperplasias without atypia (64.3% vs 41.7%; P=0.03) and similar to those observed in DCIS and invasive carcinomas. The expression of sialyl-Tn (85.7%) was higher than normal (22.2%), hyperplastic (41.7%) and neoplas-

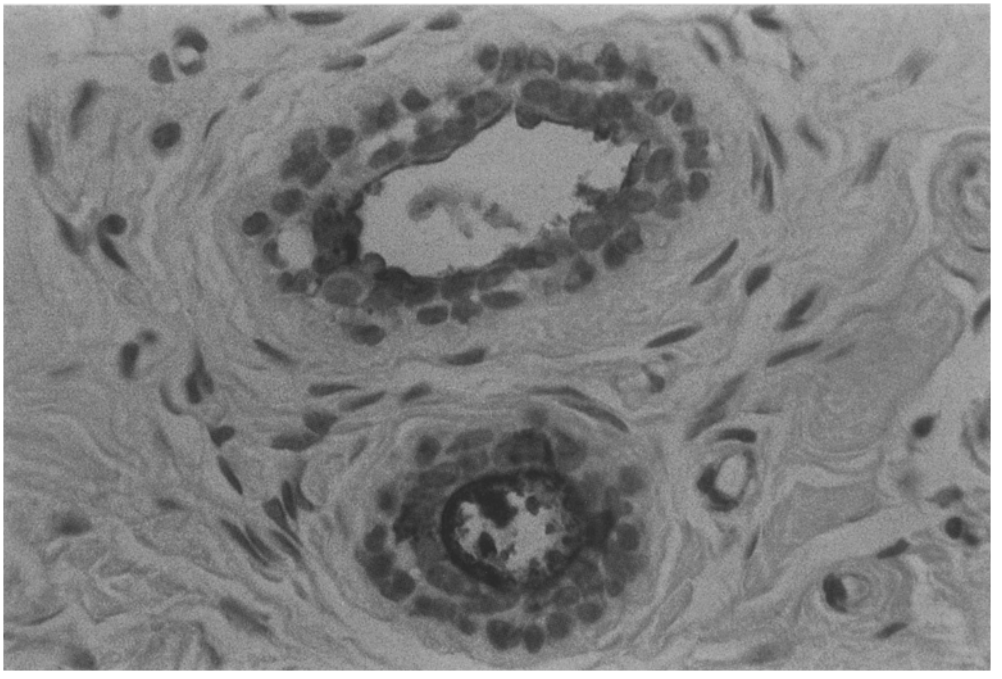

Fig. 1 Normal breast ducts. T immunostaining was present in 13.8% of the cases, mainly in the apical membrane and focally in the cytoplasm. ×320

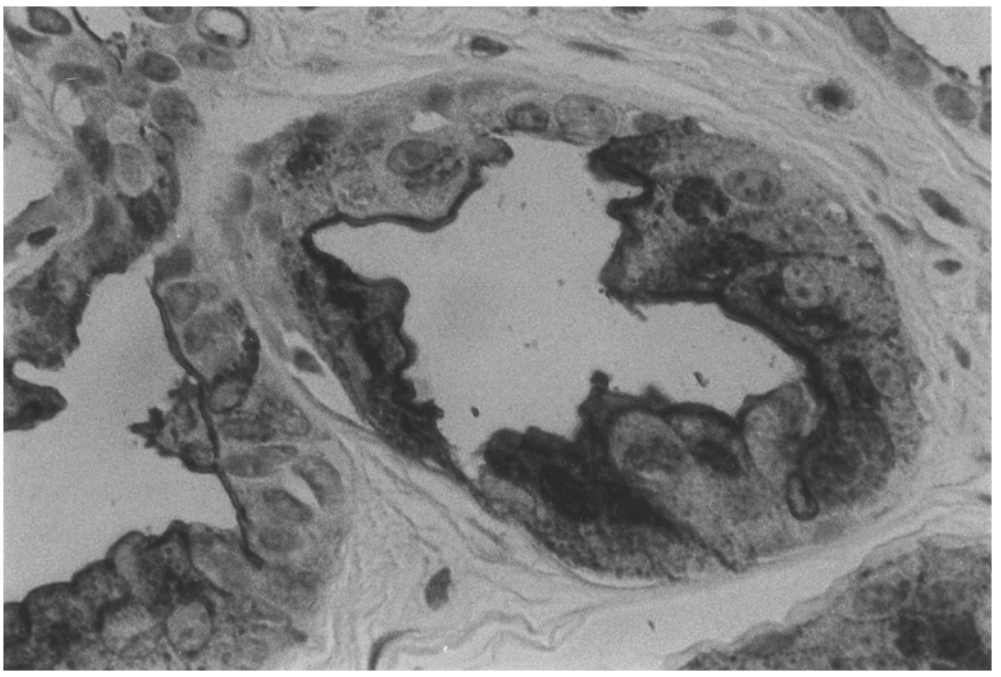

Fig. 2 Apocrine metaplasia. Tn antigen was intensely expressed in the apical membrane in 64.3% of cases. ×320

Table 3 Simple mucin-type carbohydrate antigen distribution pattern in breast lesions ns – not significant

Histological diagnosis	Tn	Sialyl-Tn	T antigen	T antigen after neuraminidase
Normal breast	25.0%	22.2%	13.8%	100%
Ductal hyperplasia	34.6%	41.7%	21.7%	98.0%
Ductal carcinoma in situ	70.3%	48.2%	31.4%	98.2%
Invasive carcinoma	69.2%	30.7%	46.2%	84.6%
Normal vs hyperplasia	ns	P=0.04	ns	ns
Normal vs DCIS	P<0.0001	P=0.01	P=0.05	
Normal vs invasive Ca	P=0.0005	ns	P=0.005	
Hyperplasia vs DCIS	P=0.0005	ns	ns	ns
Hyperplasia vs invasive Ca	P=0.005	ns	P=0.04	ns
DCIS vs invasive Ca	ns	ns	ns	ns

tic epithelium (48.2% for in situ carcinoma and 30.7% for invasive carcinoma).

T antigen was expressed in 35.7% of the cases, and after treatment with neuraminidase in all the cases. The staining pattern was mainly apical and in the supranuclear region and the percentage of positive cells were similar in sections without and with previous neuraminidase treatment. The frequency of expression did not differ from those observed in normal breast, hyperplasias and carcinomas.

Table 4 Distribution of mucin-type carbohydrate antigens in ductal carcinoma in situ (DCIS) of the breast according to histological subtype

Antigens	DCIS		
	Non-comedo type	Comedo type	P value
Tn	13/22 (59.0%)	25/32 (78.1%)	0.13
Sialyl-Tn	8/22 (36.3.%)	18/32 (56.3%)	0.15
T antigen	10/22 (45.4%)	7/32 (21.8%)	0.06
T antigen after neuraminidase	21/22 (95.4%)	32/32 (100%)	0.22

Ductal hyperplasia (without and with atypia)

For all antigens, there were more stained cells in ductal hyperplasia than in normal epithelium (data not shown). Tn staining showed a predominantly apical membranous pattern and was expressed in a similar frequency to those observed in normal breast and less often than in malignant breast epithelium: ductal carcinoma in situ (34.6% vs 70.3%; P=0.0005) and invasive carcinoma (34.6% vs 69.2%; P=0.0005; Table 3).

Sialyl-Tn was expressed in a predominantly apical membranous pattern, but cytoplasmic staining was also observed. The prevalence of expression of sialyl-Tn was higher than those observed in normal epithelium (41.7% vs 22.2%; P=0.04) and was not significantly different

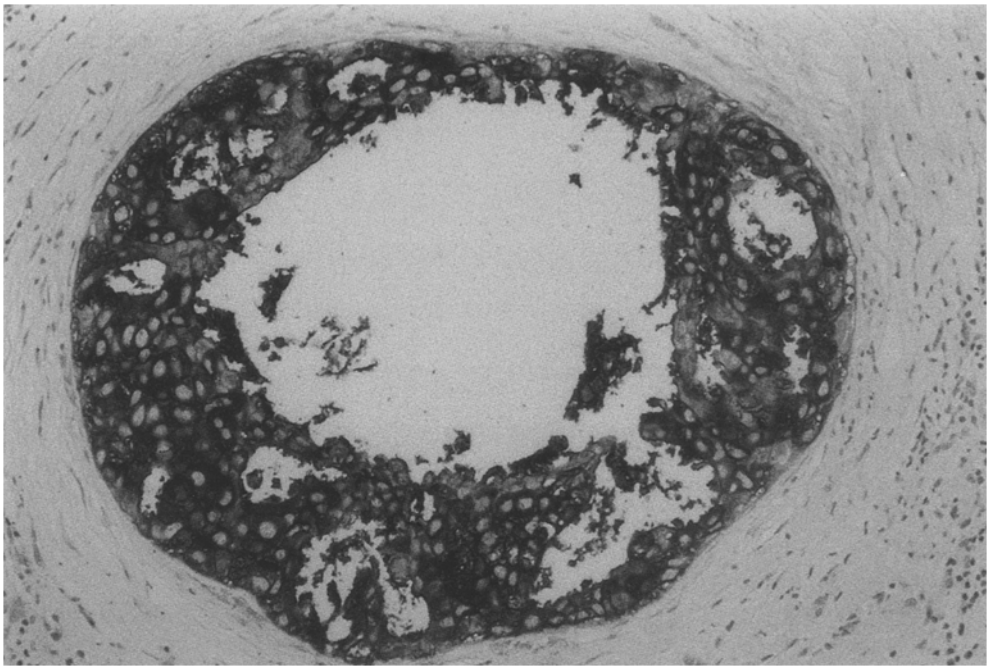

Fig. 3 Ductal carcinoma in situ. Sialyl-Tn was expressed diffusely in the cytoplasm and cell membrane of the neoplastic cells. ×160

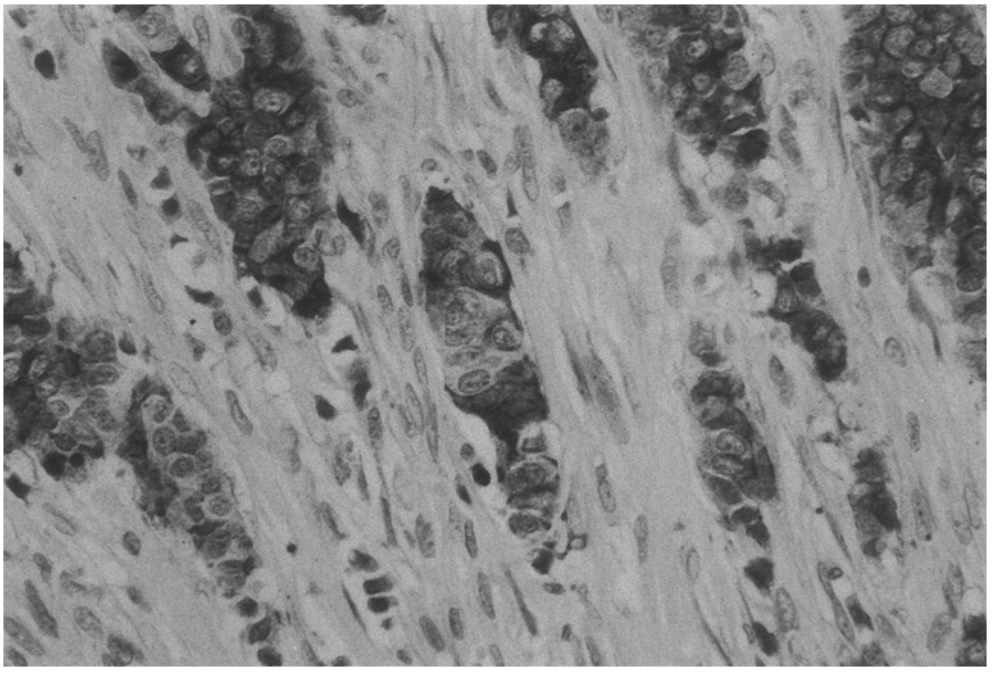

Fig. 4 Invasive ductal carcinoma. Sialyl-Tn was expressed in the cytoplasm of the neoplastic cells in 30.7% of the cases and was related with nodal metastases. ×320

from those observed in DCIS (48.2%) and in invasive carcinoma (30.7%).

T antigen was expressed in the luminal membranes and, in some cases, also in the cytoplasm with a supranuclear (Golgi area) pattern of staining. The prevalence of expression of T antigen was significantly lower in ductal hyperplasia than in invasive carcinoma (21.7% vs 46.2%;P=0.04).

When we divided the ductal hyperplasias in "without atypia" and "with atypia" we were not able to detect any significant difference in the expression of the antigens studied.

Ductal carcinoma in situ (DCIS)

Tn was usually expressed in more than 75% of the cells, mainly in a diffuse cytoplasmic pattern. We observed a significantly higher prevalence of expression of Tn antigen in DCIS than in normal (70.3% vs 25.0%; P<0.0001)

Table 5 Percentage expression of simple mucin-type carbohidrate antigens and pathological features in invasive breast carcinomas

Pathological features	Tn	Sialyl-Tn	T antigen	T antigen after neuraminidase
Tumour size(cm)				
<2.0	5/7 (71.4%)	2/7 (28.5%)	4/7 (57.1%)	6/7 (85.7%)
>2.0	13/19 (68.4%)	6/19 (31.5%)	8/19(42.1%)	16/19 (84.2%)
Histological grade				
G I	2/3 (66.6%)	1/3 (33.3%)	2/3 (66.6%)	2/3 (66.6%)
G II	6/10 (60.0%)	1/10 (10%)	4/10 (40.0%)	8/10 (80.0%)
G III	10/13 (76.9%)	6/13 (46.1%)	6/13 (46.1%)	12/13 (92.3%)
Nodal status				
Node negative	9/13 (69.2%)	1/13 (7.6%)	6/13 (46.1%)	11/13 (84.6%)
Node positive	9/13 (69.2%)	7/13 (53.8%)[a]	6/13 (46.1%)	11/13 (84.6%)
ER content				
Positive	11/18 (61.1%)	5/18 (27.7%)	9/18 (50.0%)	15/18 (83.3%)
Negative	7/8 (87.5%)	3/8 (37.5%)	3/8 (37.5%)	7/8 (87.5%)
DNA content				
Diploid	3/6 (50.0%)	2/6 (33.3%)	3/6 (50.0%)	4/6 (66.6%)
Aneuploid	15/20 (75.0%)	6/20 (30.0%)	9/20 (45.0%)	18/20 (90.0%)

[a] $\chi 2=7.9$; $P=0.004$

and hyperplastic breast epithelium (70.3% vs 34.6%; $P=0.0005$; Table 3). The expression was higher, though not significant in comedo type DCIS than in the non-comedo type (Table 4). The prevalence of Tn expression was similar in DCIS and invasive carcinomas (Table 3).

Immunoreactivity for sialyl-Tn was cytoplasmatic (diffuse) and, in some cases, it was also seen at the cell membrane (Fig. 3). The prevalence of expression was 48.2%, similar to that of ductal hyperplasias and invasive carcinomas and higher than the observed in normal breast (48.2% vs 22.2%; $P=0.01$). There was also more expression of sialyl-Tn in the DCIS of comedo type than in the non-comedo type, again without reaching the threshold of statistical significance (Table 4).

T antigen expression (before and after neuraminidase) was observed in the cytoplasm and in the luminal membrane. The expression of T antigen was suggestively more frequent ($P=0.06$) in DCIS of the non-comedo type than in comedo type DCIS (Table 4).

Invasive carcinomas

All the cases studied were ductal carcinomas. In most of them, all of the antigens were expressed in well-defined areas and this expression was heterogeneous regarding the distribution of positive cells (randomly distributed isolated positive cells or focal clusters of positive cells). Tn and sialyl-Tn had a predominantly diffuse cytoplasmatic staining pattern (Fig. 4). Tn antigen was expressed in 69.2% of the cases; this percentage is similar to that of DCIS and higher than normal ($P=0.0005$) and than that in hyperplastic breast epithelium ($P=0.005$; Table 3).

Sialyl-Tn was detected in 30.7% of the cases, which is more than in normal epithelium and less than in hyperplasia and DCIS. None of the differences attain the threshold of statistical significance.

The immunostaining pattern was similar for T antigen before and after treatment with neuraminidase, mainly at the apical cytoplasm and apical membranes and in the luminal content of the tumours with glandular differentiation. T antigen was expressed in 46.2% of the cases, and after treatment with neuraminidase in 84.6%; this percentage is smaller than those observed in all other groups of the study, but statistically different only from the prevalence of T expression in normal breast epithelium (100%).

Table 5 summarizes the correlations between Tn, sialosyl-Tn and T (before and after treatment with neuraminidase) antigens expression and different pathological features in invasive breast cancer. The only statistically significant association was found between sialyl-Tn expression and nodal metastases: the percentage of tumours with lymph node metastases expressing sialyl-Tn (53.8%) is higher than that of tumours without lymph node metastases (7.6%; $P=0.004$).

Discussion

We have analysed the expression of simple mucin-type T, Tn and sialyl-Tn carbohydrate structures in different histological types of breast lesions using a well-defined panel of monoclonal antibodies. The special interest in these structures stems from the fact that marked changes in cell membrane glycoconjugates due to abnormal expression or depression of DNA encoding glycosyl transferase are known to be often associated with malignant transformation [2, 6, 8, 20].

Our results, in agreement with others [3, 17, 23], show that the expression of simple mucin-type antigens is not restricted to malignant breast epithelium. The normal breast epithelium, like the metaplastic and hyper-

plastic breast epithelium, also expresses simple mucin-type antigens, although at different rates from malignant lesions of the breast.

We found that among the simple mucin-type antigens, the most immature structures, Tn and sialyl-Tn, are expressed more frequently in malignant than in benign breast lesions. The expression of Tn, although not limited to malignant lesions, is significantly higher in DCIS and invasive carcinomas of the breast than in benign breast epithelium. Studies about expression of sialyl-Tn in breast lesions are somewhat controversial [3, 11, 17, 23, 27]. Using the monoclonal antibody B72.3, Tavassoli et al. [23] found positivity only in carcinomas and atypical hyperplasias. However, Reed et al. [17], using the same antibody as we used (HB-STn), found positivity in 19% of all benign lesions. The reported positivity of sialyl-Tn in the normal breast are quite variable because some studies do not separate the apocrine metaplasia and in these lesions there is a strong positivity for sialyl-Tn [3, 23]. Using the antibody HB-STn, we showed positivity for sialyl-Tn in normal non-metaplastic breast epithelium and in ductal hyperplasia, which is at variance with the results of Yonezawa et al. [27]. In invasive carcinomas, our finding of 30.7% positivity fits within the spectrum of 16–80% reported in the literature [11, 17, 23].

The demonstration by our group and by Tavassoli et al. [23] that the frequency of sialyl-Tn expression is higher in DCIS than in invasive carcinomas may be related to a failure of progression of some in situ tumours to clinically detectable invasive carcinoma. Indeed, a review of an epidemiological survey of intraductal carcinomas showed that the development of ipsilateral invasive carcinoma in patients after diagnosis of intraductal carcinoma, when just a biopsy is performed, occurs in only 25% of cases after an average of 7 years [24]. However, we cannot rule out the alternative hypothesis that sialyl-Tn expression is lost when some carcinomas become invasive, because we did not study a sufficient number of tumours with both components.

The close relationship between sialyl-Tn expression and presence of lymph node metastases in invasive carcinomas suggests that the presence of sialyl-Tn may influence the invasiveness of the tumours, as previously described in other models [4].

The demonstration of T (Thomsen-Friedenreich) antigen in breast epithelium is variable from series to series [12, 17, 25, 27]. This discrepancy probably reflects the different sensitivities of antibodies and lectins used in most studies [12, 17, 25, 27]. We found T antigen expression not only in morphologically normal epithelial cells adjacent to carcinomas as shown by Reed et al .[17] but, at variance with the results of these authors, also in normal breast epithelium not adjacent to carcinomas. T antigen has already been demonstrated in normal epithelium not associated with carcinomas in other organs [1, 13], so that its expression in proliferative breast lesions should not be interpreted as a marker of early malignant transformation. However, the frequency of T antigen expression in malignant epithelium is significantly higher than that observed in normal epithelium, indicating, at least, a quantitatively abnormal expression. The T antigen masked by sialic acid was very prevalent in all cases, with a lower frequency in invasive carcinomas. We did not find a significant loss of expression of this antigen in hyperplasias or even in DCIS compared with normal epithelium, and therefore we think that the loss of immunoreactivity for masked T antigen in some benign lesions does not represent a premalignant event, an opinion in which we are at variance with Reed et al. [17].

We demonstrated also that the apocrine metaplasia has a pattern of simple mucin-type glycosylation, which is specific and distinct from that of the normal breast epithelium. There is a high frequency of marked expression of Tn and sialyl-Tn in apocrine metaplasia, which is similar to that observed in malignant epithelium. This finding probably reflects an abnormal differentiation more than a premalignant event and, therefore, does not mean that apocrine metaplasia is a precursor lesion of breast carcinoma. Indeed, other studies reinforce the view that apocrine metaplasia does not represent a risk factor for breast cancer [16].

In conclusion, although we documented an increasing percentage of positivity for T, Tn and sialyl-Tn antigens from normal to invasive carcinomas, the findings are of little or no value for the diagnosis of a given case. This has already been stated by Reed et al. [17]; we also concur with the claim that the detection of simple mucin antigens does not allow the separation of atypical hyperplasias from DCIS. Our negative diagnostic findings do not, however, undermine the biological importance of this type of study. Our finding of an overexpression of Tn and sialyl-Tn in putative precursor lesions of breast cancer and in invasive breast carcinomas supports the assumption that we may be dealing with an incomplete glycosylation phenomenon, known to occur in many neoplastic processes [1, 2, 6, 8, 20].

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