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Simple mucin-type carbohydrate antigens (Tn, sialosyl-Tn, T and sialosyl-T) and gp 230 mucin-like glycoprotein are candidate markers for neoplastic transformation of the human cervix

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Abstract Mucins and simple mucin-type carbohydrates are cancer-associated antigens in several human tumors. Expression of Tn, sialosyl-Tn, Thomsen-Friedenreich (T), sialosyl-T and of a recently identified mucin-like glycoprotein (gp230) has not yet been thoroughly investigated in human cervix carcinogenesis. In the present study sections from normal cervix (n=10), CIN III lesions (n=10), and invasive carcinomas (n=47) were evaluated immunohistochemically using monoclonal antibodies. In normal cervix there was: cytoplasmatic expression of Tn in 1 case (10%); membranous expression of STn in 8 cases (80%); no expression of T and cytoplasmatic expression of ST in 1 case (10%); gp 230 was expressed in all cases with a membranous pattern. In CIN III lesions there was cytoplasmatic and membranous expression of Tn in 3 cases (30%) and of STn in 9 cases (90%); T and ST were not expressed; gp 230 was expressed in 5 cases (50%) both in the cytoplasm and at the cell membrane. In invasive carcinomas we observed Tn expression in 30 cases (63.8%) and STn in 31 cases (66%); T antigen was not expressed; expression of both ST and gp 230 in 24 cases (51.1%); all antigens showed membranous and cytoplasmatic staining. Our results show that Tn and ST are good markers of invasive carcinomas of the human cervix. We have also shown that loss of expression of the mucin-like glycoprotein gp 230 is associated with malignant transformation at a preinvasive stage.

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Introduction

Aberrant glycosylation is frequently associated with neoplastic transformation and includes the incomplete synthesis of carbohydrate chains and accumulation of precursor structures, namely Tn, sialosyl-Tn (STn) and Thomsen-Friedenreich (T) antigens [7, 8]. These simple mucin-type carbohydrates, *O*-linked to proteins, are accumulated and expressed in several human tumors and are rarely expressed in normal adult cells [1, 5, 6, 11–14, 16, 17, 21, 23, 25, 26, 28]. In normal cells, simple mucin-type carbohydrates are masked by sialylation and/or chain elongation or ramification by the addition of other sugar residues or ABH blood group determinants [2, 25]. The production of specific monoclonal antibodies allowed the study of glycosylation patterns in normal and pathologic conditions and led to the conclusion that aberrant expression of simple mucin-type carbohydrates can be useful in the diagnosis and evaluation of prognosis of several human carcinomas [5, 6, 9, 11, 17, 24, 25]. Few studies have addressed the expression of simple mucin-type carbohydrates in cancer of the human cervix and the putative relationship between such expression and the invasive and metastatic ability of the neoplastic cells. Yonezawa et al. [30] observed that in 1 of 17 cases there was a weak expression of STn in normal cervical squamous epithelium, whereas Ogawa et al. [20] pointed out STn as a marker of squamous cell carcinoma of the cervix. In 1993, Hirao et al. [9] demonstrated a significant association between the expression of Tn and the metastatic potential of cervix cancer. Terasawa et al. [28] showed that the expression of Tn and STn is associated with neoplastic progression from carcinoma in situ to invasive carcinoma and from a normal condition to dysplasia, respectively.

Recently, a high-molecular-weight mucin-like epithelial glycoprotein has been identified (gp 230) as a major carrier of *O*-glycans in the oral and the exocervical epi-

Table 1 Structure of carbohydrate epitopes and monoclonal antibodies used

Antigen	Structure	Antibody (isotype)	Dilution	Reference
Tn	GalNacα1→O-Ser/Thr	HB-Tn (IgM)	1:15	H. Clausen, S. Hakomori, unpublished work
Sialosyl-Tn	NeuAcα2→6GalNAcα1→O-Ser/Thr	HB-STn (IgG1)	1:8	[16]
T	Galβ1→3GalNAcα1→O-Ser/Thr	HB-T (IgM)	1:10	[3]
gp 230	Membrane mucin-type glycoprotein	PANH4 (IgM)	1:10	[19]

thelium [19]. In neoplastic lesions of the oral cavity and exocervix, gp 230 expression was significantly decreased or absent [19], suggesting that gp 230 is a candidate marker for neoplastic transformation in these epithelia.

The aim of the present study was to define the immunohistochemical expression of simple mucin-type carbohydrate antigens: Tn, STn, T and ST, and of the gp 230 mucin-like glycoprotein in the normal cervix, severe dysplasia/carcinoma in situ and invasive carcinoma, in an attempt to evaluate the putative usefulness of these markers for the evaluation of neoplastic development and progression of human cervix carcinomas.

Materials and methods

Tissue

Cervical specimens from 57 patients with cervix cancer diagnosed at the Department of Pathology of the Maputo Central Hospital in Mozambique were studied. All samples were fixed in 10% formalin and embedded in paraffin and cut into 4-µm sections. The cases examined were 47 of invasive carcinoma and 10 of cervical intraepithelial neoplasms grade III (CIN III). In 51 cases (44 invasive carcinomas and 7 CIN III) the study was based on biopsies and in 6 cases (3 invasive carcinomas and 3 CIN III) the material was obtained from surgical specimens. We further studied 10 samples from normal cervix obtained from hysterectomy specimens with leiomyomas.

Since most of the neoplasms were considered nonresectable and no reliable information could be obtained on the type of treatment no attempt was made to study the outcome of the patients.

Hematoxylin–eosin-stained sections were used to classify the tumors according to the WHO recommendations [29]. The 47 invasive carcinomas included 44 squamous carcinomas (19 keratinizing, 22 large-cell nonkeratinizing and 3 small-cell nonkeratinizing), 2 adenosquamous carcinomas and 1 adenocarcinoma.

Antibodies

Mouse monoclonal antibodies, their isotype, specificities and source are given in Table 1. All antibodies were used as hybridoma supernatant in dilutions specified in Table 1. The detection of ST antigen was performed using antibodies directed to the T antigen, after treating the sections with neuraminidase.

Immunohistochemistry

Four-micrometer serial sections from 10% formalin-fixed paraffin-embedded material were used for immunostaining. The immunohistochemistry method was carried out according to the avidin–biotin peroxidase complex (ABC) method [10] after dewaxing. Sections designed for neuraminidase treatment were preincubated with neuraminidase from *Clostridium perfringens* type VI (Sigma) diluted in a 0.2 M sodium acetate buffer, pH 5.5, to the final concentration of 0.1 U/ml. Incubation at 37°C was followed by three washings in ice-cold water. In all sections blockage of endogenous peroxidase was performed using 0.3% hydrogen peroxide (H_2O_2) in methanol for 10 min. 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 overnight with the primary antibodies (dilutions specified in Table 1) at 4° C followed by incubation with a 1:200 dilution of biotin-labeled anti-mouse secondary antibody (Dako, Copenhagen, Denmark) for 30 min and ABC for 30 min. Careful rinses were done with TBS between each step of the procedure. The slides were then treated with 3,3'-diaminobenzidine tetrachloride, counterstained with Mayer hematoxylin, 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 antibody, and staining with conjugate alone. The presence of sialosyl-T antigen in red blood cells was used as an internal positive control for the sections subjected to neuraminidase.

Scoring

All cases with stained cells were considered positive. A semiquantitative approach was used to score the staining: + less than 5% of immunoreactive cells; ++ between 5% and 50% of immunoreactive cells; +++ between 50% and 75% of immunoreactive cells; ++++ more than 75% of immunoreactive cells. Membranous and/or cytoplasmatic staining (diffuse or granular) was also evaluated and recorded.

Statistical analysis

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

Expression of simple mucin-type carbohydrates and gp 230 glycoprotein in normal cervix epithelia, severe dysplasia/carcinoma in situ (CIN III) and invasive carcinomas

Tn antigen expression was rarely observed in normal cervix (10%), and was detected in 30% of CIN III cases and in 63.8% of invasive carcinomas (Table 2). The overall difference of Tn expression among the three groups of lesions is statistically significant (P=0.003; Table 2); the same holds true for the comparison of normal cervix with invasive carcinoma (P=0.003; Table 3). Tn expression was suggestively higher in invasive carcinomas than in CIN III lesions (P=0.07; Table 3). STn was expressed in most cases of normal cervix and neoplastic lesions, with

Table 2 Expression of simple mucin-type carbohydrates and gp 230 glycoprotein in normal cervix epithelia, severe dysplasia/carcinoma in situ (CIN III) and invasive carcinomas (*ns* not significant)

Histology	Tn	STn	T	ST	Gp 230
	Positive cases (%)				
Normal cervix (n=10)	1 (10.0%)	8 (80.0%)	0 (0.0%)	1 (10.0%)	10 (100.0%)
CIN III (n=10)	3 (30.0%)	9 (90.0%)	0 (0.0%)	0 (0.0%)	5 (50.0%)
Invasive carcinoma (n=47)	30 (63.8%)	31 (66.0%)	0 (0.0%)	24 (51.1%)	24 (51.1%)
P-value	0.003	ns	ns	0.001	0.01

Table 3 Comparison between the expression of simple mucin-type carbohydrates and gp 230 in normal cervix epithelia, CIN III and invasive carcinomas

	Tn	STn	T	ST	Gp 230
	P-value				
Normal cervix epithelia Vs CIN III	ns	ns	ns	ns	0.03
Normal cervix epithelia *Vs* invasive carcinoma	0.003	ns	ns	0.03	0.003
CIN III *Vs* invasive carcinoma	0.07	ns	ns	0.03	ns

slight variations that did not reach the threshold of statistical significance (Tables 2, 3). In contrast, T antigen was never expressed in any of the samples (Tables 2, 3). ST antigen was expressed in one case of normal cervix (10%), was not expressed at all in the CIN III lesions and was positive in 51.1% of invasive carcinomas (P=0.001; Table 2). ST antigen expression was significantly higher in invasive carcinomas then in normal cervix (P=0.03) and in CIN III lesions (P=0.03; Table 3).

The gp 230 glycoprotein was always expressed in the normal cervix (Table 2), and its expression was significantly lower in the group of neoplastic lesions (CIN III and invasive carcinoma: P=0.03 and P=0.003, respectively; Tables 2, 3).

Patterns of expression of simple mucin-type carbohydrates and gp 230 glycoprotein

Normal cervix epithelium

Tn antigen was expressed in 1 case in the cytoplasm of few cells of the intermediate and superficial layers. STn antigen was expressed focally in the majority of cases (4 cases scored + and 4 cases scored ++), in intermediate and superficial cells with a membranous staining pattern (Fig. 1A). In 1 case there was expression of STn in few parabasal cells. ST antigen was detected in the membrane of few intermediate cells in 1 case (10%). The glycoprotein gp 230 was strongly and diffusely expressed (score ++++) in all cases. The expression displayed a membranous pattern in all intermediate and superficial cells (Fig. 1B).

Severe dysplasia/carcinoma in situ

Tn antigen was expressed focally in 3 cases (score ++). The positivity was observed in cells dispersed in all layers with a cytoplasmatic pattern (predominantly diffuse and occasionally granular paranuclear; Fig. 1C). STn antigen was focally expressed in 9 cases (score ++) with cytoplasmatic and membranous pattern, predominantly in intermediate and superficial layers. Expression in parabasal and basal cells was occasionally observed. Gp 230 glycoprotein was focally expressed in 5 cases (score ++) in intermediate and superficial layers with a cytoplasmatic and membranous pattern.

Invasive carcinomas

All antigens (excluding the T antigen) were expressed in more than 50% of the cases (Table 2). The Tn antigen was expressed in 30 cases (63.8%), STn in 31 cases (66.0%) and ST and gp 230 in 24 cases (51.1%).

Tn antigen was focally expressed (score ++) in the central and peripheral areas of the neoplastic cell nests, with diffuse and strong cytoplasmatic pattern (Fig. 1D) and occasionally membranous. STn antigen was distributed diffusely in the neoplastic cell nest predominantly in the central areas. The positivity was membranous and cytoplasmatic (Fig. 1E). In most cases the expression was focal (score ++), in 8 cases it was extensive (score +++) and in 4 cases it was throughout (score ++++). T antigen was not expressed in any case. ST antigen was identified in 24 cases (51.1%), with a similar distribution regarding cell and tissue localization as was observed for STn. Gp 230 glycoprotein showed total loss of positivity in 23 cases, while 24 cases exhibited focal positivity (score ++) in well-differentiated areas in the center of the neoplastic cell nests (Fig. 1F), including keratin pearls. The expression was membranous and cytoplasmatic.

In the single adenocarcinoma case Tn, STn and ST antigens were diffusely expressed in the apical membrane and, occasionally, in the cytoplasm. The 2 adenosquamous carcinomas expressed Tn and ST diffusely in

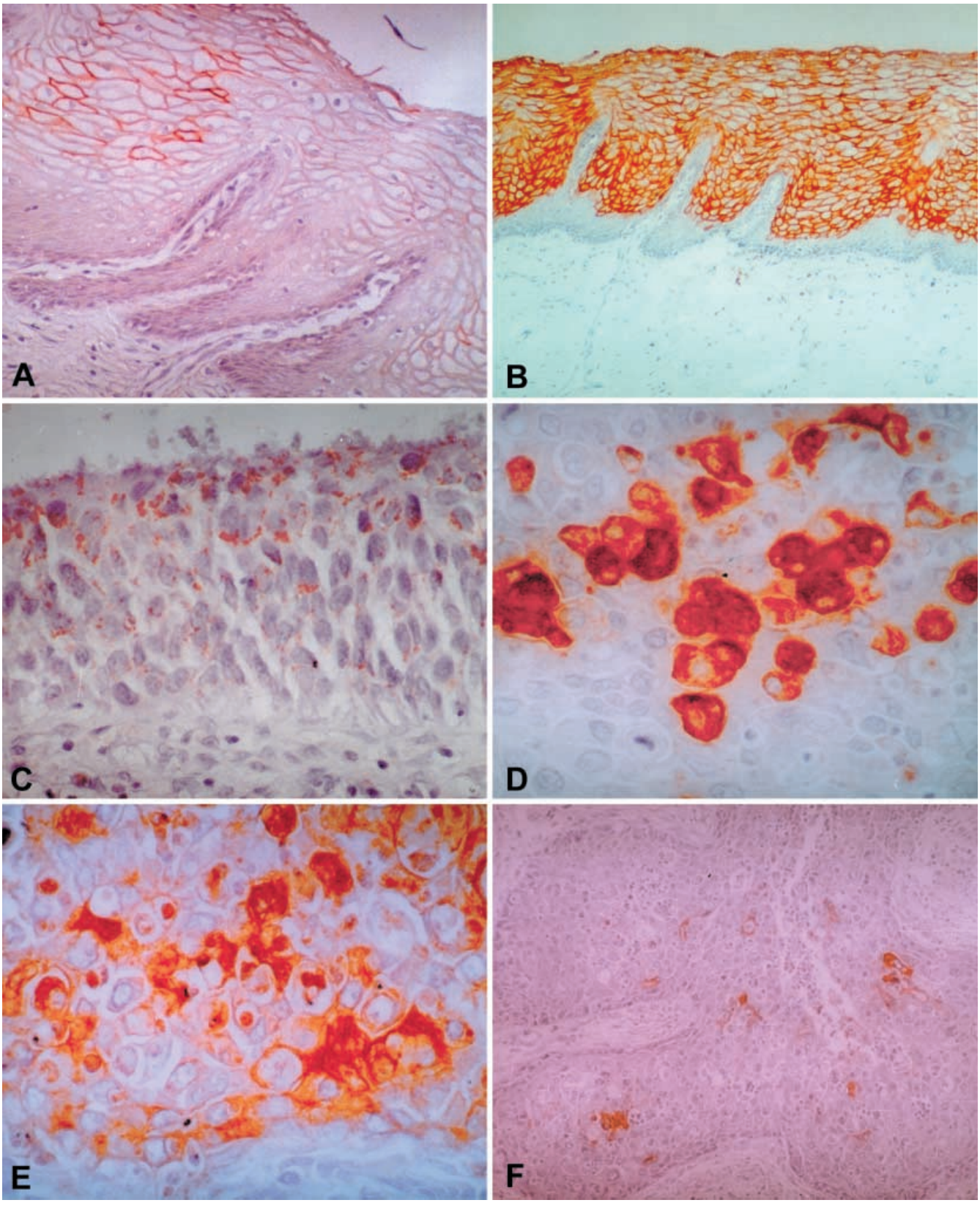
A
B
C
D
E
F

Table 4 Specificity and sensitivity of simple mucin-type carbohydrates and gp 230 for diagnostic purposes in human cervix neoplasia

	"Meaning"	Specificity	Sensitivity
Tn as a marker of Invasive carcinoma	Absent in normal tissue and expressed in invasive carcinomas	0.96	0.34
ST as a marker of invasive carcinoma	Absent in CIN III and expressed in invasive carcinomas	1	0.30
Loss of gp 230 as a marker of malignancy	Loss of expression in neoplastic lesions and expression in normal exocervix	1	0.26
Loss of gp 230 as a marker of pre-invasive malignancy	Loss of expression in CIN III and expression in normal exocervix	1	0.66
Gp 230 as a marker of differentiation of squamous cell carcinoma	Expression in well-differentiated carcinomas	0.43	0.95

the apical membrane of the cells of glandular areas and focally in squamous areas; only 1 of the adenosquamous carcinoma expressed STn. None of these 3 carcinomas with glandular differentiation expressed gp 230, even in the squamous areas of adenosquamous carcinomas.

Taking all the invasive carcinomas together there was coexpression of Tn and STn in 24 cases (51.1%); 10 cases (21.2%) did not express STn or Tn; 7 cases expressed only STn; and 6 cases only expressed Tn. Co-expression of T and ST was never observed. The expression of the different mucin-type carbohydrates (Tn, STn and ST) was independent of the expression of gp 230.

The comparison of the expression of simple mucin-type carbohydrates and gp 230 with the histological subtype of squamous cell carcinomas revealed an association between the loss of expression of gp 230 and lower levels of keratinization: gp 230 positivity was identified in 17 of 19 (89.5%) keratinizing squamous carcinoma, 7 of 22 (31.8%) large cell nonkeratinizing squamous carcinoma and 0/3 (0.0%) small cell nonkeratinizing squamous carcinoma (P=0.0001).

Specificity and sensitivity of simple mucin-type carbohydrates and gp 230 glycoprotein for the diagnosis of CIN III and invasive carcinomas

The results are summarized in Table 4: Tn and ST antigens appear to be specific markers of invasive carcinoma; the lower expression of gp 230 is observed in preinvasive and invasive carcinomas; Gp 230 is a marker of differentiation in squamous cell carcinomas.

◀ **Fig. 1A-F** Immunohistochemical staining of **A, B** normal cervix epithelium, **C** severe dysplasia and **D, E, F** invasive squamous carcinoma. **A** Focal membrane staining of intermediate and superficial cells with antibody HB-STn to Sialosyl-Tn. ×170 **B** diffuse cell membrane staining of suprabasal cells with antibody PANH4 to Gp230 mucin-like glycoprotein. ×85 **C** Granular cytoplasmic staining of suprabasal cells with antibody HB-Tn to Tn antigen. ×340 **D** Diffuse cytoplasmic staining of neoplastic cells with antibody HB-Tn to Tn antigen. ×340 **E C**ytoplasmic and membranous staining of neoplastic cells with antibody HB-STn to sialosyl-Tn antigen. ×340 **F** Focal cytoplasmic staining in neoplastic cells with antibody PANH4 to Gp 230. Note loss of expression in comparison to the normal diffuse expression pattern of normal cervix documented in B. ×85

Discussion

In the present study we demonstrated that Tn and ST are good markers of invasive carcinoma of the human exocervix.

Both Tn and ST were rarely expressed on the normal exocervix (10% of the cases), which is in agreement with previous observations by Terasawa et al. [28], who showed absence of expression of Tn antigen in the human exocervix and by Nielsen et al. [19], who showed occasional expression of ST. Similarly, absent expression or low levels of expression of Tn antigen have been reported in other squamous epithelia, namely the oral cavity [18] and the epidermis [4], and occasional expression of ST was described in the gastric mucosa [1, 6], colon [14], bladder [17] and larynx [26]. As compared with normal exocervix, we found that Tn expression was increased in CIN III lesions, although not significantly so. Previous studies did not demonstrate increased expression of Tn antigen in cervical preinvasive lesions of the cervix [28] and larynx [26].

Our results show that both Tn and ST have a significantly higher expression in invasive carcinomas of the human cervix than in normal mucosa and CIN III lesions. Both antigens, Tn and ST, show a high specificity (0.96 and 1, respectively) despite a relatively low sensitivity (0.34 and 0.30, respectively) for the detection of invasive carcinomas of the cervix. High specificity of Tn as a marker of malignancy in the human cervix has previously been described [9, 28]. To the best of our knowledge, increased expression of ST has not been previously evaluated in human cervical carcinomas. In agreement with our results, increased expression of ST was described in invasive squamous carcinomas of the larynx [26]. Available data from human adenocarcinomas are conflicting: frequent expression of ST was observed in invasive carcinomas of the stomach [6], whereas decreased expression was observed in breast carcinomas [23].

The expression of Tn and ST was identified both in the cytoplasm and at the cell membranes of carcinoma

cells, in contrast to normal exocervix where Tn is found exclusively in the cytoplasm and ST exclusively at the cell membrane. This finding suggests that Tn and ST are de novo expressed and aberrantly processed in tumor cells.

In our hands STn antigen has little value as a marker for malignancy in the human cervix. STn antigen was frequently expressed in normal cervical epithelium, CIN III lesions and invasive carcinomas (80%, 90% and 66% of cases, respectively). Our results contradict previous reports showing that STn expression in the normal exocervix epithelium is weak or absent [9, 28, 30]. Frequent expression of STn was previously described in other normal squamous epithelia such as the oral cavity [18] and larynx [26], and absent expression of STn was described in squamous epithelia of esophagus [11]. These discrepancies on STn expression probably stem from the different antibodies used in each study as well as from differences in the glycosylation process between different squamous epithelia [15]. STn was expressed in most neoplastic lesions (90% pre-invasive carcinomas and 66% invasive carcinomas), in agreement with the results previously described by Terasawa et al. [28]. At variance with Terasawa et al. [28], however, we did not observe statistically significant differences between carcinomas and normal cervix.

In normal cervix, as well as in the oral cavity, STn expression is localized in the intermediate and superficial layers [18, 27], suggesting that STn expression is related to epithelial cell maturation and terminal differentiation.

In invasive carcinomas, we observed expression of STn in the central and peripheral areas of the neoplastic nests, as previously reported by Terasawa et al. [28].

In contrast to its localization at the cell membrane in normal cervix, STn was expressed both in the cell membrane and in the cytoplasm of neoplastic cells, indicating that the glycolysation process, namely the transport to the cell membrane, may be modified in tumor cells.

T antigen expression was never identified in our cases, suggesting that T antigen could be masked by addition of other sugars or blood group determinant antigens [22, 27]. Similarly, absent expression of T antigen has been reported in human larynx [26].

The mucin-like glycoprotein gp 230 was expressed in 50% of the CIN III lesions and in 51.1% of the invasive carcinomas in contrast with its expression in the parabasal layers of every case of normal exocervix, thus supporting the assumption that loss of expression of gp 230 is a relatively early phenomenon in cervix carcinogenesis. A previous report by Nielsen et al. [19] described total loss of gp 230 in severe oral and exocervical dysplasia and in the majority of invasive carcinomas.

The loss of expression of gp 230 in carcinomas appears to be associated with differentiation, since we observed that gp 230 is expressed in well-differentiated carcinomatous areas in the center of neoplastic cell nests. We also found expression of gp 230 in keratin pearls of well-differentiated carcinomas. This finding contrasts with the absence of gp 230 reported by Nielsen et al. [19] in the keratin layer of epidermis and palate, but fits with gp 230 expression in normal Hassal's corpuscles in the thymus (unpublished data). The expression of gp 230 in the keratin pearls of keratinizing carcinomas of our series indicate that these cervical neoplasms may have an aberrant keratinizing pattern different from that produced in the skin. In contrast to its consistent membranous pattern in the normal exocervix, the gp 230 expression was also observed in the cytoplasm of the neoplastic cells, thus pointing to a putative alteration of the transport to the cell membrane, as also seen with STn.

The gp 230 glycoprotein was co-expressed with Tn in 31.9% of the cases of invasive carcinomas, and with STn and ST in 38.3% and in 29.8% of cases, respectively. We found no association between the expression of gp 230 and any of the simple mucin-type antigens, suggesting that Tn, STn, T and ST antigens are linked to other mucin-type glycoproteins present in cervix. This hypothesis is supported by the simultaneous expression of CD44 and ST in the basal layer of normal exocervix, described by Nielsen et al. [19].

Summing up and with regard to diagnosis, the loss of gp 230 appears to be a specific and relatively sensitive marker for CIN III lesions (malignant transformation of the exocervix at a preinvasive stage); this result should, however, be confirmed in a larger series. On the other hand, the usefulness of Tn and ST, which we have shown to be good markers of cancer invasion, in routine diagnosis is limited by their low sensitivity.

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