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ARTICLE

Influence of Sialic Acid Removal on MUC1 Antigenic Reactivity in Head and Neck Carcinoma

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To investigate the influence of sialic acid removal on MUC1 peptidic and carbohydrate epitope reactivity in head and neck squamous cell carcinoma (HNSCC), tumor samples belonging to 24 HNSCC patients were studied by standard immunohistochemistry (IHC) with and without desialylation with 0.1 U/ml neuraminidase. From each tumor sample, subcellular fractions were obtained and analyzed by SDS-PAGE and Western blotting (WB). Three monoclonal antibodies (MAbs) were used: C595 MAb directed to MUC1 protein core, an anti-Tn hapten MAb, and an anti-sTn hapten MAb; a comparative analysis between desialylated and sialylated samples was performed. By IHC without neuraminidase treatment, 19 of 24 samples reacted with anti-MUC1 peptidic epitope, while Tn hapten was not detected and sTn was found in 1 of 24 cases. Desialylation increased either the number of reacting cells or the intensity of the reaction with C595 and anti-Tn MAbs, and some negative samples became positive. On the other hand, sTn expression decreased with desialylation. By WB, several bands from >200 to 25 kDa were found; desialylation increased high-molecular-weight bands, diminishing the detection of low-molecular-weight ones. The use of desialylation is a suitable treatment that contributes to the exposure of MUC1-associated epitopes, which may be related to the spreading of HNSCC. (Pathology Oncology Research Vol 11, No 2, 74–81)

Key words: head and neck carcinoma, MUC1, carbohydrate antigens, sialic acid

Introduction

Head and neck squamous cell carcinomas (HNSCC) are frequently aggressive tumors in their biological behavior, and are very often detected at T3-T4 tumor stage, which contributes to the bad prognosis. Lymphatic metastasis in cervical nodes is the single most important determinant of therapy and prognosis for patients with tumor at this localization. Neoplastic spreading is a complex mechanism in which adhesion interactions between endothelial and cancer cells seem to be crucial. These interactions are modulated by different cell surface molecules that may include MUC1 mucin.[14]

MUC1 is an epithelial luminal surface glycoprotein which protects normal epithelia as a physical and biological barrier; the molecule was first identified in human milk and is expressed by most glandular epithelial cells.[40] This mucin is a high-molecular-weight transmembrane molecule with a polymorphic protein core containing a large domain of variable number of highly conserved tandem repeats composed of 20 amino acids which are normally heavily *O*-glycosylated.[13] This glycosylation occurs in a sequential step-by-step process resulting in elongated carbohydrate chains with di- or trisaccharide structures.[16] MUC1 glycosylation in carcinomas differs from that of their normal counterparts, often leading to a reduction in the length of the carbohydrate chains demasking normally cryptic peptidic and carbohydrate structures.[27] These changes generate immunogenic epitopes that may be recognized by the host immune system[4] and may potentially contribute to the elimination of carcinoma cells. The mechanisms by which these epitopes arise depend on their status of glycosylation, and may involve changes in the activity of glycosyltransferases.[16,18] Mucin-type glycan structures are influenced by both the level of expression and the Golgi localization of glycosyltransferases competing with each other for common acceptor structures.[2]

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Cancer-associated MUC1 glycoforms contain truncated precursor structures like core-GalNAc (Tn hapten) or the core 1 disaccharide Gal(β1-3)GalNAc (T hapten); additionally, α2-6 sialylation of GalNAc results in a stop of the elongation.[10] Some tumors may also express blood group antigens.[16]

In HNSCC, alterations of glycoconjugates on the cell surface have not been studied systematically, although some reports can be found in the literature.[3,7,11,21,29] Kurahara et al[25] found that the high expression of sialyl-Lewis a antigen in primary oral squamous carcinoma may be involved in nodal metastasis, and therefore predict poor prognosis in this type of carcinoma. It has also been reported that carcinomas of the pharynx and larynx express mucin-associated carbohydrate antigens such as Tn, sialyl-Tn (sTn) and T, as well as Lewis-related antigens.[19]

Removal of sialic acid has been shown to greatly increase access of anti-MUC1 tandem repeat antibodies to the peptide epitope on underglycosylated MUC1 in several cancer cell lines.[17] Therefore, we developed an immunohistochemical approach to examine the effect of desialylation on peptidic as well as carbohydrate MUC1 epitope reactivity in HNSCC. Western blot (WB) analysis was also employed in order to characterize MUC1 epitopes.

Materials and Methods

Tumor samples

The study was performed on a group of 24 patients with HNSCC, which included two females and 22 males (*Table 1*). Tumor specimens were located in the larynx (13), oral cavity (7), maxillary sinus (3) and pharynx (1). Patients' age range was 46-77 years, and they were clinically categorized and staged according to the UICC TNM classification system.

A portion of the tumors was fixed in methacarn (methanol 60%, chloroform 30%, and acetic acid 10%) for two hours for histopathological diagnosis and immunohistochemical analysis, while another portion was rinsed with fresh sterile Hank's balanced salt solution (HBSS), and processed for preparation of subcellular fractions.

Experiments were done according to the Helsinki Declaration. Informed consent was obtained from all patients included in the study. This research was approved by the Local Human Investigation Committee, Faculty of Medical Sciences, University of La Plata, Argentina.

Seven normal samples from head and neck mucosa were included as controls.

Table 1. **Clinical characteristics of patients**

	Sex	*Age*	*Localization*	*T*	*N*	*M*
1	M	59	Pharynx	T3	N2	M0
2	F	65	Larynx	T2	N0	M0
3	M	52	Larynx	T3	N0	M0
4	M	51	Larynx	T3	N0	M0
5	M	64	Larynx	T3	N3	M0
6	M	58	Larynx	T4	N0	M0
7	M	53	Larynx	T4	N0	M0
8	M	62	Larynx	T4	N0	M0
9	M	52	Larynx	T4	N0	M0
10	M	77	Larynx	T4	N0	M0
11	M	47	Larynx	T4	N2	M0
12	M	46	Larynx	T4	N3	M0
13	M	56	Larynx	T4	N3	M0
14	M	68	Larynx	T4	N3	M0
15	F	54	Maxillary sinus	T3	N0	M0
16	M	56	Maxillary sinus	T4	N0	M0
17	M	60	Maxillary sinus	T4	N0	M0
18	M	63	Oral cavity	T2	N0	M0
19	M	57	Oral cavity	T2	N0	M0
20	M	65	Oral cavity	T3	N0	M0
21	M	62	Oral cavity	T3	N0	M0
22	M	46	Oral cavity	T3	N3	M0
23	M	61	Oral cavity	T4	N0	M0
24	M	55	Oral cavity	T4	N2	M0

Monoclonal antibodies (MAbs)

The following monoclonal antibodies were employed: C595 MAb (IgG3), an anti-MUC1 antibody defining the tetrameric epitope Arg-Pro-Ala-Pro in the MUC1 protein core;[33] anti-Tn MAb (IgM) against the Gal-NAcα1-*O*-Ser/Thr determinant (DAKO, Glostrup, Denmark); and anti-sTn MAb (IgG), an anti NeuAcα2-6Gal-NAcα1-*O*-Ser/Thr determinant (DAKO).

Immunohistochemical analysis

The analysis was performed following standard procedures;[6] before immunostaining with MAbs, tissues were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes for antigenic retrieval.[37]

Dewaxed sections were placed in methanol with hydrogen peroxide (3%) for 15 minutes to block endogenous peroxidase activity. After three washes in phosphate buffered saline (PBS), sections were blocked for non-specific binding with normal horse serum diluted 1:10 in 3% bovine serum albumin (BSA) in PBS for 15 minutes and rinsed. Then, sections were incubated with MAbs overnight at 4°C; MAbs were diluted as follows: C595, 1:1000; anti-Tn and sTn, 1:100. After three washes with PBS, peroxidase-conjugated goat anti-mouse IgG or IgM (Sigma, St. Louis, MO, USA), diluted 1:400, was added for 60 min. Slides were washed in PBS and developed with 3´,3´-diaminobenzidine (DAB, Sigma), followed by counterstaining with hematoxylin. Negative controls were incubated with PBS instead of MAbs.

Specimens were examined by light microscope, and the antibody staining patterns were scored in a semi-quantitative manner.[12] Staining intensity was graded as negative (–), low

(+), moderate (++), or strong (+++). The staining of cytoplasm, plasma and nuclear membranes was evaluated; cells were considered positive when at least one of these components was stained. The pattern of reaction was classified following other authors[28,35] as membrane, cytoplasmic, or mixed pattern (cytoplasmic mixed with plasma membrane staining).

Neuraminidase treatment

After deparaffination, histopathological sections were incubated with type V *C. perfringens* neuraminidase (Sigma) in acetate buffer (0.1 U/ml, pH 5.5) for 2 hours at 37 °C. Similarly, before the addition of SDS-PAGE sample buffer, subcellular fractions were treated with 1:1 v/v type V *C. perfringens* neuraminidase (Sigma) in acetate buffer (0.1 U/ml, pH 5.5) for 2 hours at 37°C with gentle agitation.

Preparation of extranuclear membrane fractions

Fractions were prepared from human tumor tissues. Briefly, tissues were homogenized in 0.01 M TRIS, pH 7.2, containing 0.01 M phenylmethanesulfonyl fluoride (PMSF, Sigma). Homogenates were centrifuged at 600 g and at 105,000 g at 4°C. Supernatant (cytoplasmic fraction) and the second precipitate were resuspended in PBS (extranuclear membrane fraction), and stored at -20°C.

SDS-PAGE and Western blot analysis

Electrophoretic analysis was conducted following standard procedures.[26] Subcellular fractions were mixed with SDS-PAGE sample buffer to a final concentration of 2 mg of protein per ml, then samples were heated at 100°C for 5 minutes. Forty microliters of samples per well were loaded into a discontinuous 4-10% acrylamide mini-gel (Gibco-BRL, Gaithersburg, MD, USA). After electrophoresis, gels were transferred to nitrocellulose membranes[41] which were then incubated with the above mentioned MAbs.

Statistical analysis

Data were standardized and a Principal Component Analysis (PCA) with Kendall correlations was performed. Dummy variables were built corresponding to the presence of bands in the different areas of the gel. The method for analysis of immunohistochemical results was Yates-corrected c^2 test (Statistica for Windows, StatSoft, 1993).

Results

Immunohistochemical results

In general, desialylation increased either the number of reacting cells or the intensity of the reaction with C595 and anti-Tn MAbs, while some negative samples became positive (10 with anti-Tn MAb and 4 with C595 MAb) *(Table 2)*.

Nineteen out of 24 (79.2%) tumor samples showed a binding with C595 MAb without desialylation (*Figure 1a*). In most samples, expression was restricted to some focal tumor areas in the cytoplasm, although some samples showed a positive reaction at the membrane level and at the luminal content. The intensity of the staining was mainly low (+), although some were moderate (++). After neuraminidase treatment, a more extended reaction was observed; in many cases, the intensity was also stronger than without desialylation (*Figure 1b*). Only one specimen (No. 15, *Table 2*) did not show reactivity either with or without neuraminidase treatment. In concordance with other authors,[36] we generally found staining in keratinizing foci.

Using anti-Tn MAb without desialylation, all samples were negative, while 10/24 (41%) samples became positive after neuraminidase treatment; a focal reaction was observed in some areas (*Figure 1c*).

sTn was detected in only one sample without desialylation; the reaction was detected in a few cells in only one area of the specimen (*Figure 1d*). The reaction decreased after neuraminidase treatment.

A group of seven normal oral mucosa samples were assayed as controls (*Table 3*). In all reactive specimens, staining was mainly observed at cells of the basal layer, and the pattern of reaction was perinuclear and, frequently, apical in the cytoplasm. With C595, desialylated samples showed an increased reactivity compared to untreated samples. An example with C595 MAb is depicted in *Figure 1* (with neuraminidase treatment and without desialylation). With anti-sTn MAb, a few cells from only one area in three positive specimens stained positive, (*Figure 1e*) and the reaction increased with neuraminidase treatment (*Figure 1f*).

Western blot (WB) analysis

All tumors reacted with at least one of the three MAbs included. WB of subcellular fractions (membrane and cytoplasm) showed the highest percentage of positive results with C595 and anti-Tn MAbs (100%), while low levels of reactivity were found with anti-sTn MAb, since about 30% of membrane fractions and 70% of cytoplasmic fractions were positive. In general, with the three MAbs similar results were found with and without desialylation.

In most cases, both membrane and cytoplasmic subcellular fractions showed bands with MW of >200 to <70 kDa. Generally, the pattern did not change with desialylation in the case of any of the MAbs assayed, although in a few tumors some differences with and without neuraminidase treatment were observed. Employing C595 MAb after neuraminidase treatment, some membrane subcellular fractions deriving from 6 tumors showed a decreased reaction between 200 and 110 kDa, while a con-

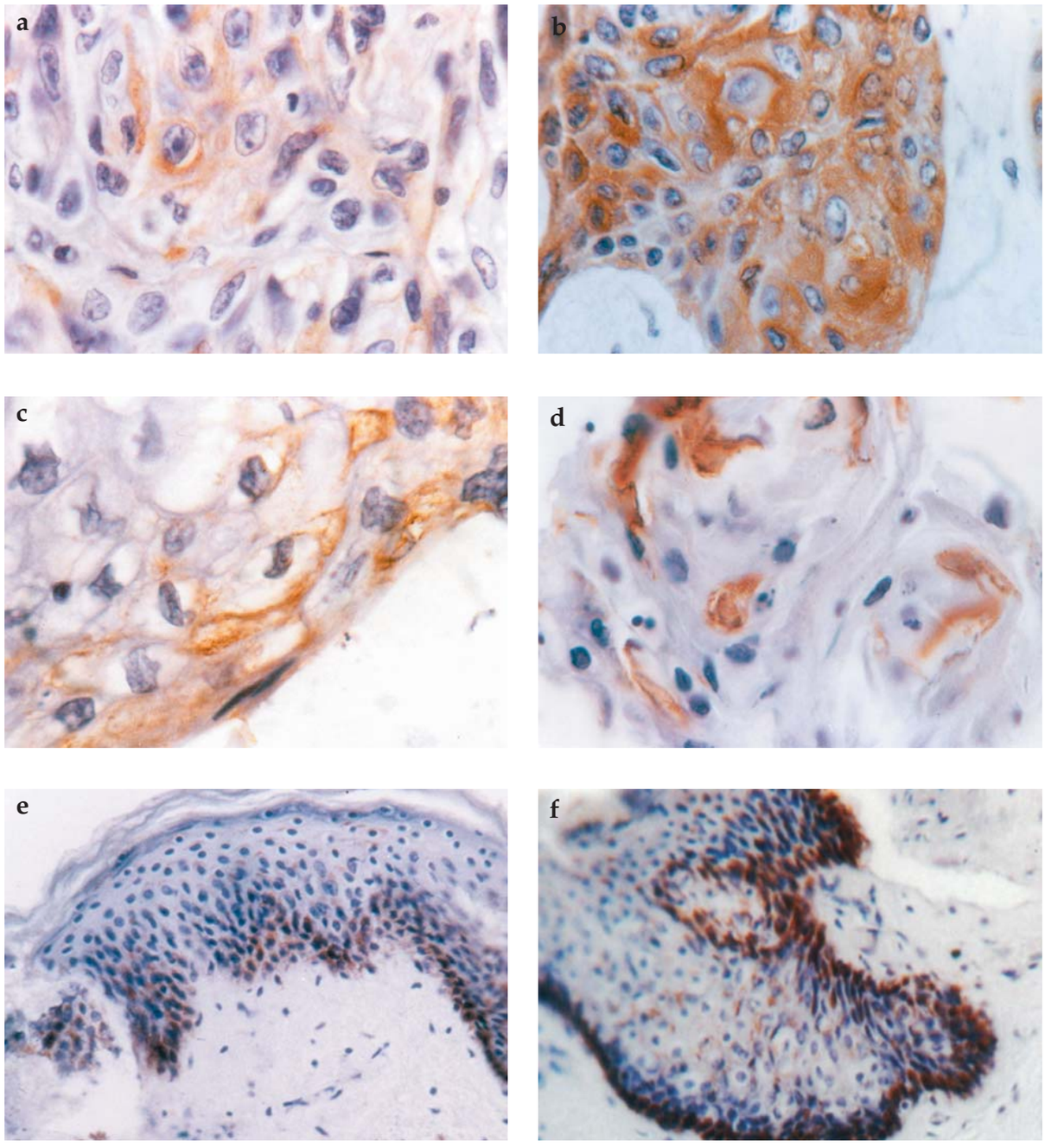

Figure 1. ***(a)*** *Immunohistochemical results obtained on a HNSCC specimen without neuraminidase treatment and incubated with C595 MAb. A positive reaction is observed restricted to focal areas where few cells present immunoreactivity at the cytoplasm, while cell membrane shows a low intensity (x100).* ***(b)*** *A section of the same tumor treated with neuraminidase and incubated with C595 MAb. An increased staining is found; most cells show a reticular reaction at the cytoplasm with different intensity. In some cells, plasma membranes are also reactive (x100).* ***(c)*** *HNSCC section showing anti-Tn MAb reaction after neuraminidase treatment; a focal pattern is depicted mainly at peripheral cytoplasm (x100).* ***(d)*** *Immunohistochemical results obtained on a HNSCC specimen without neuraminidase treatment and incubated with anti-sTn MAb; an intense positive staining is found in some cells (x100).* ***(e)*** *Normal oral mucosa without neuraminidase treatment, incubated with C595MAb. A positive immunoreaction at basal cells is depicted (x40).* ***(f)*** *A section of the same sample treated with neuraminidase and incubated with C595 MAb. An increased positive staining at basal cells is shown; some cells belonging to upper layers are also positive (x40)*

comitant increase of high-molecular-weight bands (>200 kDa) was detected in some of these samples (*Figure 2a*).

When anti-Tn MAb was assayed, an increased reactivity was detected in a cytoplasmic fraction derived from a laryngeal tumor after neuraminidase treatment, comprising low- and high-MW bands. Similarly, in a membrane fraction derived from a tumor localized in the oral cavity, reaction and the appearance of a band at 190 kDa was only observed after desialylation (*Figure 2b*).

Finally, in a membrane subcellular fraction derived from a pharyngeal tumor, reaction with anti-sTn MAb without desialylation showed bands of >170 kDa, which were not reactive after treatment.

Table 2. **Immunohistochemical results in head and neck carcinoma patients with and without neuraminidase treatment; samples are numbered according to *Table 1***

	Anti-MUC1 peptide core		*Anti-Tn*		*Anti-sTn*	
	Untreated	*Treated*	*Untreated*	*Treated*	*Untreated*	*Treated*
1	–	++	–	–	–	–
2	+	+++	–	+	–	–
3	+	+	–	–	–	–
4	+	+	–	+	–	–
5	+	++	–	–	–	–
6	–	+	–	–	–	–
7	+	++	–	+	–	–
8	–	++	–	–	–	–
9	+	+	–	–	–	–
10	+	+	–	–	–	–
11	+	+	–	–	–	–
12	++	+++	–	–	–	–
13	+	+	–	–	–	–
14	+	++	–	+	–	–
15	–	–	–	+	–	–
16	–	++	–	++	++	+
17	+	+	–	+	–	–
18	+	+	–	+	–	–
19	+	+	–	–	–	–
20	++	++	–	–	–	–
21	+	+	–	–	–	–
22	+	+	–	+	–	–
23	+	++	–	–	–	–
24	++	+	–	+	–	–

Statistical analysis

The number of positive immunohistochemical samples in relation to negative ones were analyzed, and a significant difference between neuraminidase-untreated and treated samples was found for anti-Tn MAb ($p<0.001$), while there was no statistically significant difference observed in the case of C595 and anti-sTn MAbs.

The presence of bands detected with the different MAbs was statistically analyzed by means of PCA and Kendall correlation. A positive and statistically significant correlation was found between bands of >200-170 and 170-110 kDa with three MAbs assayed being $\tau=0.6377$ with C595 MAb, $\tau=0.5580$ with anti-Tn, and $\tau=0.5272$ with anti-sTn MAb.

The relationship between tumor burden or clinical stage and the reactivity with the three MAbs included was analyzed. No correlation was detected in the case of C595 and anti-Tn MAbs without neuraminidase treatment, while after desialylation a positive correlation, although not statistically significant, was observed. In the case of anti-sTn MAb, no correlation was found either with or without desialylation.

Discussion

In HNSCC, aggressive surgery is often the only curative option for advanced as well as for recurrent cancer. In patients whose initial tumor is stage T3 or T4, the primary therapy often makes salvage even more difficult.[15] All these patients would benefit from alternative therapeutic strategies such as immunotherapy. Since most carcinoma cells can be distinguished from their normal counterparts by the presence of certain cell surface epitopes, and MUC1 is one of the most widely demonstrated tumor-associated antigens, this mucin is one of the promising targets for anti-cancer vaccine development.[24,31]

Furthermore, MUC1 expresses both peptidic and carbohydrate epitopes, and behaves as an anti-adhesive as well as an adhesive protein, since it has an extended conformation,[1] it has been involved in cell-cell[42] and cell-matrix interactions[34,22] and, on the other hand, may express carbohydrate epitopes which are ligands to endothelial cells.[44]

In this report, we present novel data for squamous cell carcinoma, in particular HNSCC. We describe the effects of the removal of sialic acid on MUC1 peptidic and carbohydrate epitope reactivity, and prove that neuraminidase treatment increased their expression in HNSCC.

Removal of sialic acid greatly increased the access of anti-MUC1 tandem repeat antibodies to the MUC1 peptide epitopes on underglycosylated MUC1 in several cell lines.[17] Our results show that neuraminidase treatment resulted in an increase in immunoreactivity with C595 MAb. It is known that this MAb reacts with RPAP sequence of MUC1 core protein (main epitope), although the carbohydrate side chain contributes to the binding.[38] Tn epitope was not detected without desialylation, while neuraminidase treatment allowed 10 samples to become positive, since anti-Tn

Table 3. **Immunohistochemical results in normal controls with and without neuraminidase treatment**

Normal controls	*Anti-MUC1 peptide core*		*Anti-Tn*		*Anti-sTn*	
	Untreated	*Treated*	*Untreated*	*Treated*	*Untreated*	*Treated*
1	–	+	+	+	++	+
2	+	++	++	++	++	+
3	+	++	–	–	–	-
4	+	++	–	–	–	-
5	–	–	–	–	–	-
6	–	–	–	+	–	-
7	+	++	+	+	+	+

MAb may detect the epitope more easily without sialic acid. On the contrary, the only sample positive with anti-sTn MAb without desialylation diminished its reactivity after treatment. The reason for the lack of staining with anti-sTn MAb could be that this epitope expressed on HNSCC may be nonclustered, since the MAb employed has specificity equivalent to that of B72.3, another anti-sTn MAb (DAKO insert) exclusively reactive with sTn clusters.[43] Interestingly though, in our studies it had a higher reactivity on normal basal cells than in malignant samples.

By WB analysis with C595 and anti-Tn MAbs, bands at >200 kDa were detected, probably corresponding to the full-length peptidic chain with short lateral carbohydrate chains.[5] A series of low molecular mass bands, likely derived from the tandem repeat region of MUC1, was also found. These results suggest that these peptides are poorly glycosylated, since they reacted with both C595 and anti-Tn MAbs and, also, not strongly sialylated since the sTn epitope was much less reactive. Reactivity of anti-MUC1 tandem repeat and anti-Tn MAbs with desialylation may vary since it is possible that different glycoforms may be present in a sample. Müller and Hanisch[30] pointed out that a complex biosynthetic process produces the pool of *O*-glycan structures present in a single cell. Also, sample treatment could increase access to MAbs or, on the contrary, diminish MAb reactivity.

Differences between sialylated and desialylated samples were not found very frequently, although with neuraminidase treatment, in some cases, an increase in high-MW bands was observed along with a decrease in low-MW ones. These bands were also reactive with both C595 and anti-Tn MAbs, which further supports the idea that the fragments are glycosylated, although probably not fully glycosylated. Another explanation would be that the sialyl epitope is not easily accessible to neuraminidase treatment; possibly, an increase in antigenic detection may be achieved employing more than one anti sTn MAb. Zhang et al[34] found that the use of different MAbs may result in the recognition of different configurations at the tumor cell surface.

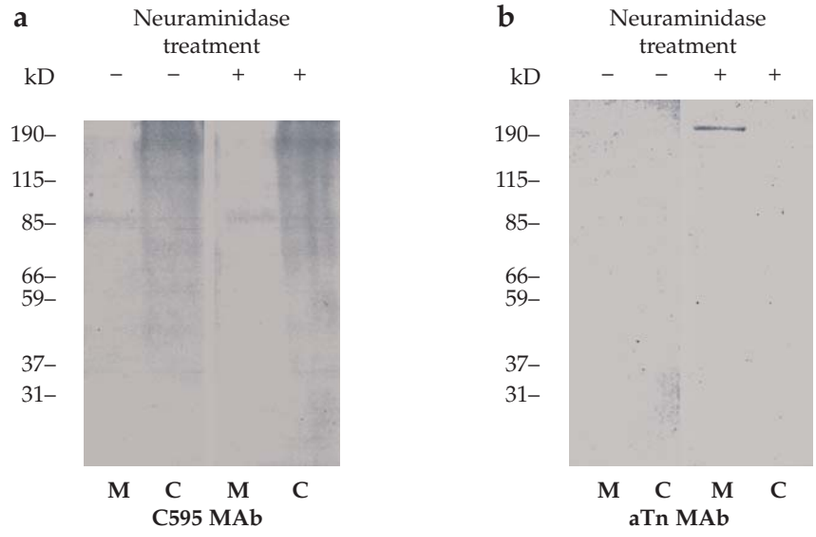

Figure 2. (a) *Western blot analysis of subcellular fractions (membrane and cytoplasm) of HNSCC with and without neuraminidase treatment; the reaction with C595 MAb is depicted. With desialylation, membrane subcellular fraction shows the appearance of high-MW bands, while in the cytoplasmic fraction an increased reaction is found.* ***(b)*** *An example of Western blot analysis of neuraminidase-treated and untreated HNSCC subcellular fractions; the reaction with anti-Tn MAb is shown. Desialylation enhanced Tn epitope recognition with the appearance of bands at 190 kD in the membrane fraction. M=membrane subcellular fraction, C= cytoplasmic subcellular fraction*

As we have previously pointed out in the case of MUC1 associated with breast cancer,[8] the reactivity depends on epitope exposition and also on the step of cell metabolic processing, which may show expected differences between IHC and WB, since both techniques have different targets. In the case of IHC, epitopes are detected at tissue and cellular levels, while in WB assays sample preparation remains an important variable.

Tn as well as sTn are cancer-associated carbohydrate antigens overexpressed in several malignant tumors including breast, gastric and colon cancer,[20,23] which have been associated with more aggressive disease and poor prognosis.[32,44] Dabelsteen et al[9] pointed out that in normal oral epithelium short carbohydrates are found on basal cells, and these carbohydrate structures are elongated parallel to terminal differentiation. They also observed mosaicism in the expression of carbohydrate antigens in tumors and, furthermore, found that the expression of a specific carbohydrate in the deep invasive parts of the tumor correlates with tumor prognosis. In agreement with other authors,[3,9] we detected sTn antigen expressed on basal cells in a number of normal oral mucosa samples employed as controls. Bryne et al[3] argued that sTn may behave as a marker for non-malignant conditions with altered basal cell activity and for highly differentiated verrucous carcinomas. Actually, sTn epitope has already been found on mucin glycoproteins in some normal mucosa localizations such as the colon.[23]

Moreover, Stenersen et al[39] studied biopsies from normal, hyperplastic and carcinomatous laryngeal epithelium with six MAbs related to the ABO- and the T-Tn blood group systems, which were used for demonstration of carbohydrate antigens by an indirect immunofluorescent staining method. They found that benign lesions had a normal sequence of glycosylation while dysplasias showed accumulation of shorter chains in superficial layers, usually focused (patchy dispersion). They assumed that it was possible by carbohydrate distribution to objectively establish the diagnosis of high-grade dysplasias/carcinoma in situ, and on the basis of carbohydrate distribution they could divide the lesions into subgroups, which had prognostic relevance.

In conclusion, we have demonstrated that neuraminidase treatment contributes to an increase in both peptidic and carbohydrate MUC1-associated epitopes; a different immunohistochemical pattern between normal and carcinoma specimens was found.

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