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ARTICLE

MUC1 Mucin and Carbohydrate Associated Antigens as Tumor Markers in Head and Neck Squamous Cell Carcinoma*

MariaV CROCE,[1] Martin E RABASSA,[1] Mike R PRICE,[†2] Amada SEGAL-EIRAS[1]

[1]Centro de Investigaciones Inmunológicas Básicas y Aplicadas (CINIBA), Facultad de Ciencias Médicas, UNLP, La Plata, Argentina; [2]School of Pharmaceutical Sciences, University of Nottingham, UK

An immunological analysis to study MUC1 mucin core protein and carbohydrate associated antigens as tissue tumor markers in head and neck carcinoma was performed. Twenty nine patients with the following tumor localizations were included: tongue (n=10), larynx (n=8), oral cavity (n=4), maxillary sinus (n=3), tonsillar ring (n=3) and pharynx (n=1); seven samples of epithelium obtained from normal organs at the same localizations were studied as controls. Immunohistochemical analysis was performed following standard procedures and reaction was graded according to staining intensity and distribution. From each tissue section, membrane, cytoplasmic and nuclear moieties were obtained by differential centrifugation with subsequent fractionation by density gradient centrifugation (6M guanidium chloride-CsCl); subcellular moieties and CsCl derived fractions were analyzed by immunoblotting. Monoclonal antibodies (MAbs) reacting with the core protein of MUCI (C595) and associated carbohydrate antigens were: Tn, 83D4 MAb; Lewis y antigen (Le y), C14 MAb; Lewis x antigen (Le x), KM380 MAb and sialyl Lewis x (sLe x), KM93 MAb. Statistical analysis was undertaken by Spearman rank correlation. In tumor samples, the immunohistochemical identification of MUCI core protein and associated antigens was extended; differences were found in the pattern and intensity of expression; results were corroborated by immunoblotting although in a few samples there was not coincidence between both methods. Localization, tumor mass or node involvement did not show significant differences for any of the antigens studied. Conclusions: 1) head and neck carcinoma expressed MUCI and associated carbohydrate antigens in high levels; 2) no relationship between antigenic expression and tumor status was found. (Pathology Oncology Research Vol 7, No 4, 284–291, 2001)

Keywords: Head and Neck Squamous Cell Carcinoma (HNSCC); MUCI mucin; carbohydrate associated antigens

Introduction

Head and neck squamous cell carcinoma (HNSCC) involves malignant tumors which prognosis depends on the dissemination of the disease at diagnosis, when two thirds of patients present already an advanced stage of disease.[36] Despite improvement in the fields of surgery and radiotherapy, the 5-year survival rates of HNSCC patients did not increase substantially during the last 20 years.[1] It is probable that, even with different therapeutical strategies, tumor cells remain in patients resulting in local and/or distant relapse.

Furthermore, as a consequence of an inadequate tumor staging often a failure in treatment is obtained; for instance, about 50% of patients showing tumor-free margins by conventional histopathological examination, after surgery develop either local or distant recurrence;[2] therefore, current methods have some limitations in regard to their sensitivity suggesting that new strategies should be developed.

In this context, the identification of tumor markers is crucial and constitutes a field which continues to expand progressively; in last years, many glycoproteins and glycolipids have been described as tumor associated antigens since they may be overexpressed or either be different to normal as a consequence in their synthesis alteration; they may also

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Correspondence: Prof Amada SEGAL-EIRAS, CINIBA, Facultad de Ciencias Médicas, Calle 60 y 120, 1900, La PLata, Argentina. Fax: 0054 221 4871481; E-mail: as-eiras@netverk.com.ar.

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show a different cellular distribution and probably, they may provide an easier accessibility compared with normals; for instance, carbohydrate structures present in glycoproteins can be attached to the peptide sequence either through O-glycosidic linkage, in the case of O-glycans or through aspartylglycosylamine linkage, in the case of N-glycans.[8,10,14]

On the other hand, some glycocarbonate moieties identified as a part of these glycoproteins and also glycolipids have been associated with cell migration and invasion, cell differentiation, apoptosis and cell proliferation;[24,33] in cancer patients, several studies reported the induction of an immune response against the peptidic fraction of glycoproteins such as mucins as well as against their carbohydrate moiety.[5,18,21,23]

In this study, we have investigated the immunological expression of tumor-associated antigens; in tumor sections we have detected and identified MUC1 mucin and carbohydrate associated antigens.

This research was undertaken to identify new tumor markers in HNSCC, which may be useful for diagnosis and treatment; it may also improve our knowledge on biological and immunological aspects of this type of malignant tumors.

Materials and Methods

Tumor samples

Twenty nine patients with head and neck tumors were included: 8 localized in the larynx, 10 in tongue, 4 in oral epithelium, 3 were excised from maxillary sinus, 3 from tonsils while one was localized in the pharynx. Patients age range was 44-77 years (23 men and 6 women) and were clinically categorized and staged according to the TNM classification system[17] (***Table 1***); prior to biopsy or surgery, no patient had undergone chemotherapy or radiotherapy. Tumor specimens and/or regional node samples were obtained; seven normal tissue samples from the same localization were included as controls; they were obtained at the time of surgery from patients without a history of HNSCC or local inflammation.

Table 1. **Clinical and pathological characteristics of patients**

Patient number	*Age*	*Sex*	*Localization*	*TNM*	*Immunohistochemical analysis Monoclonal Antibodies against*				
					MUC1	*Le y*	*Tn*	*SLe x*	*Le x*
1	53	M	Tonsillar ring	T2N0M0	+	+	–	–	––
2	54	M	Tonsillar ring	T4N1M0	+	+	+	+	+
3	57	F	Tonsillar ring	ND	–	–	–	–	–
4	65	M	Oral Cavity	T3N0M0	+	+	+	+	+
5	61	M	Oral Cavity	T4N0M0	+	+	+	+	+
6	62	M	Oral Cavity	T3N0M0					
7	44	M	Oral Cavity	T4N1M1	+	+	–	+	+
8	55	M	Larynx	T3N1M0	–	–	–	–	–
9	55	M	Larynx	T3N1M0	+	+	+	+	+
10	64	M	Larynx	T3N3M0	+	+	+	+	+
11	46	M	Larynx	T4N3M0	+	+	+	+	+
12	56	M	Larynx	T4N3M1	+	+	+	+	+
13	60	M	Larynx	T4N3M0					
14	62	M	Larynx	ND	+	–	–	–	–
15	ND	M	Larynx	T3N0M0	+	+	–	+	+
16	77	F	Tongue	T1N1M0	+	+	–	–	–
17	66	M	Tongue	T2N0M0	–	+	+	+	+
18	63	M	Tongue	T2N0M0	–	+	+	+	+
19	54	F	Tongue	T2NIM0	+	–	–	–	–
20	54	F	Tongue	T2N1M0	+	+	+	–	+
21	59	M	Tongue	T2N2M0	+	+	+	+	+
22	55	M	Tongue	T3N2M0	+	–	–	+	+
23	55	M	Tongue	T4N3M0	+	+	–	–	–
24	46	M	Tongue	T3N3M0					
25	62	M	Tongue	T2N2M0					
26	54	F	Maxillary Sinus	T3N0M0	+	–	–	–	–
27	56	M	Maxillary Sinus	T4N0M0	–	+	+	+	+
28	ND	F	Maxillary Sinus	T4N0M0	+	+	–	–	–
29	74	M	Pharynx	T4N2M0	+	–	–	–	–

A piece of tissue was fixed in phormaldehyde for histopathological diagnosis and immunohistochemical analysis while another tissue section was rinsed with fresh sterile Hank's balanced salt solution and subsequent processed for preparation of subcellular fractions.

Monoclonal antibodies (MAbs)

The following monoclonal antibodies (MAbs) were employed: anti MUCI MAb C-595 (IgG3) which defines the tetrameric epitope Arg-Pro-Ala-Pro in the MUC 1 protein core;[27] C-14 MAb, an IgM anti-Lewis y hapten against the difucosylated Type-2 blood group chains;[3] MAb 83D4 (IgM) against Tn determinant;[26] MAb KM93 (IgM), an anti syalil-Lewis x and KM380 (IgM), an anti-Lewis x .[13]

Immunohistochemical analysis

The technique was developed according to previous reports.[6] All specimens were fixed in phosphate buffered formalin, embedded in paraffin and cut into 5 µm sections. Deparaffinized sections were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes;[31] then, they were incubated overnight at 4°C with mouse monoclonal antibodies. Negative controls were incubated with saline phosphate buffer instead of monoclonal antibodies.

Sections were examined by light microscope, and the antibody staining patterns were scored in a semi-quantitative manner. The whole area of each sample was observed by sequentially examining lower power (x10) optical fields; the staining of cytoplasm, plasma and nuclear membranes were also evaluated. Cells were considered positive when at least one of these components was stained; staining was graded according to positive reaction, intensity and distribution and the pattern of reaction was classified as sparse, heterogeneous and uniform.[8] Staining intensity was graded as negative, low, moderate and strong.[9]

Preparation of extranuclear membrane fractions

Fractions were prepared from human tumor tissues.[27] Briefly, tissues were homogenised in 0.01M TRIS, pH 7.2 and 0.01M polymethylsulphonylfluoride; homogenates were centrifuged at 600xg and at 105000xg at 4°C and precipitates (extranuclear membrane fraction) were resuspended in 1.41M PBS, lyophilised and stored at –20°C for subsequent density gradient centrifugation.

Density gradient centrifugation

Samples from subcellular fractions in 4M guanidinium chloride 1% NP40 were centrifuged in 6M guanidinium chloride/Cs CI (initial density 1.48 g/ml; gradient from 1.35 to 1.60 g/ml) at 40000 rpm for 66 h at 10°C in a Beckman Ti 70.1 fixed-angle rotor; 500 µl fractions were collected and the density of each fraction determined using a Hamilton syringe (Merck, Dagenham, Essex, L1K) as a pycnometer.

SDS PAGE and Immunoblotting

Fractions were collected and dialysed against 1.41M PBS at 4°C for 48h and lyophilised, resuspended in SDS-PAGE sample buffer at reducing conditions and run following standard procedures[22] in a discontinuous buffer system. After electrophoresis gels were either stained with Coomassie blue or they were transferred electrophoretically to nitrocellulose membranes,[35] which were incubated with different monoclonal antibodies.

Statistical analysis

Statistical analysis was undertaken by Spearman rank correlation. Differences were considered significant at values of $P<0,05$.

Results

Immunohistochemical expression of tumor associated antigens

Table 1 summarizes the results obtained by indirect immunohistochemistry of HNSCC tumor samples with an anti MUC 1 core protein MAb and four MAbs, which reacted with, associated carbohydrate antigens; as it is shown, an extended staining was detected.

Most malignant tumors localized in the larynx stained positively with the panel of MAbs employed; reaction with C595 anti MUC1 core protein showed a homogeneous cytoplasmic staining with perinuclear localization; several cellular membranes also revealed a positive reaction while nuclei remained negative. Lewis x showed a strong cytoplasmic expression in most tumors; Lewis y antigen was expressed with a strong reaction restricted to the cytoplasm in some cells while Tn hapten stained moderately with an uniform pattern; sialyl-Lewis x reacted in a few cells in some tumor specimens. Only one tumor sample excised from larynx was negative with the panel of MAbs here assayed.

Regarding tongue malignant tumors, MUC1 mucin reaction presented a low and sparse expression, although some cells showed a strong reaction; immune staining was found mainly at cytoplasmic level while the entire cellular membrane in a few cells reacted intensely. These tumors stained with anti carbohydrate associated antigens following a similar pattern of expression; in neoplastic tissues, the general features comprised an expression mainly at the superficial layer, intermediate strata presented some positive cells while the basal layer was mostly negative, although in some tumors a few cells showed a predominant reaction at

cytoplasmic level with a microgranular pattern; at the subepithelial stroma, malignant infiltrating cells were reactive mainly in tumor nests and cords. Differences were found in relation to the percentage of cell staining as well as to the intensity of reaction; Lewis y antigen showed a significant immune reaction in extension and intensity, sialyl Lewis x a moderate expression, while Tn antigen as well as Lewis x were expressed in a low degree.

Oral cavity tumors reacted with C595 anti MUC1 core protein with a homogeneous pattern not restricted to any of the cellular layers although at superficial stratum the reaction was mainly at plasmatic membrane; intermediate and basal layers showed a cytoplasmic diffuse and low reaction; subepithelial cells were stained in neoplastic nests (***Figure 1***). Lewis y was expressed at cellular membranes with strong intensity, some cells also showed a moderate staining at cytoplasmic level; Tn antigen, sialyl Lewis x and Lewis x presented a low expression restricted to the cytoplasm of some cells with a diffuse pattern; in only one sample, a few cellular membranes expressed Lewis x.

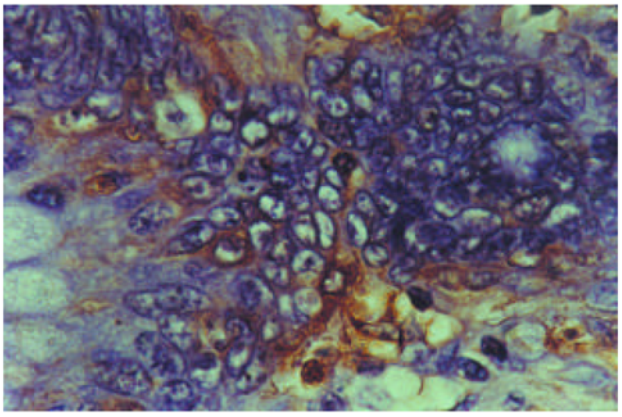

Figure 1. *Squamous cell carcinoma excised from oral cavity incubated with anti-MUC1 MAb (C595); on the left, a strong immune reaction located at peripheral cells of a nest is observed. In some cells, a diffuse granular reaction at cytoplasm and membrane is found (x40).*

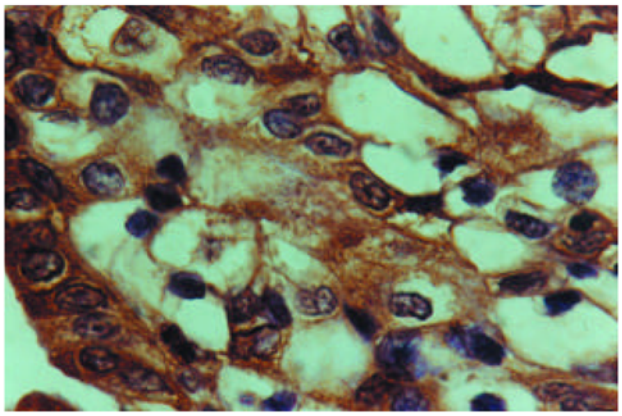

Figure 2. *Reaction of a tonsillar carcinoma incubated with anti-Lewis y MAb (C14). A reaction is observed in cytoplasm and cell membranes. On the left, the surface cell layer of the tumor tissue shows a remarkable staining (x63).*

Antigenic expression in maxillary sinus tumors showed a homogeneous perinuclear cytoplasmic pattern being Lewis y the most extensively expressed with plasmatic membrane reaction; one sample also expressed the other carbohydrate antigens, showing decreasing intensity of staining in the following order: Lewis x, sialyl Lewis x and Tn antigen; this sample did not express MUCI mucin.

Finally, two tumors localized in the tonsillar ring showed a low MUC1 membrane expression restricted to a few cells. On the contrary, C14 MAb (against Lewis y antigen) showed a strong reaction mainly at cytoplasmic level with a granular pattern, some cellular membranes were also reactive; this staining involved all neoplastic cells (***Figure 2***). One sample reacted with the other three anti carbohydrate associated antigens MAbs showing a decreased staining in the following order: 83D4 (Tn hapten), KM93 (sialyl Lewis x) and KM380 (Lewis x); the cellular pattern of expression was similar to C 14 but the intensity as well as the number of reactive cells were lower than C 14 MAb changing from a diffuse to a sparse distribution.

The tumor excised from pharynx expressed Tn hapten while no reaction was found with anti MUC1, Lewis y, sialyl Lewis x and Lewis x MAbs.

Considering the results obtained by immunohistochemistry, some general features can be pointed out: 1) Lewis y was the antigen expressed by the majority of samples studied (19/25); 2) Tn hapten was expressed with the lowest frequency (12/25); 3) sialyl Lewis x and Lewis x were coexpressed (14/25) while Tn hapten was expressed in all the samples which were also positive with anti Lewis y; 4) MUC 1 was expressed at varying levels, usually in keratinizing foci and 5) four samples did not express MUC1 but reacted with at least one of the anti-carbohydrate MAbs studied.

From several patients we obtained a sample of the primary tumor and also from a regional lymphatic node; malignant cells belonging to one of these patients expressed MUC1 core protein, sialyl Lewis x, Lewis x, Lewis y and Tn hapten mainly at cytoplasmic level; the other tumor sample was reactive with anti MUC I , Lewis y and Tn hapten while the lymphatic metastases only expressed MUC1 mucin. Three other samples from regional lymphatic nodes also showed invasion with malignant cells; these cells reacted with anti MIJC 1 core protein in two specimens; in one of them MUC1 positive reaction coincided with Tn hapten expression while in the other, expression of sialyl Lewis x and Lewis x (***Figure 3***) was found; the third lymphatic node sample was negative for all the MAbs assayed.

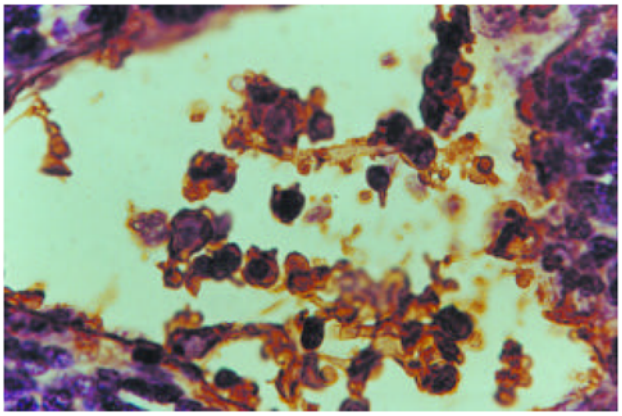

Figure 3. Ganglionar metastases of a laryngeal squamous cell carcinoma incubated with anti-Lewis x MAb (KM380): a positive staining at cytoplasm and cell surface is observed (x40).

On the other hand, samples from normal squamous epithelium of the same localization were included as controls; MUC 1 mucin was expressed weakly at the cytoplasm of several basal cells in some samples besides some superficial cells showed a weak reactivity. Carbohydrate associated antigens showed a more extended expression than MUC1 although lower than their tumor counterparts; staining was usually restricted to a few cells belonging to the superficial layer although in some cases basal and intermediate strata also showed reaction. The pattern of distribution was homogeneous, mostly a moderate cytoplasmic reaction; Lewis x showed the strongest reaction followed by sialyl Lewis x and Lewis y and finally, Tn hapten.

Antigenic expression of subcellular fraction detected by Western blot

A set of fourteen tumor samples were also subjected to homogenization and subsequent ultracentrifugation to obtain subcellular fractions, which were identified by Western blot. All MAbs assayed showed some reaction with neoplastic subcellular fractions although there were differences in the intensity as well as in the pattern of the reaction.

MUCI mucin was detected in most tumor cell fractions (membrane, cytoplasmic and nuclear) from the tumors under this study; a smear pattern was usually obtained at more than 200 kD to approximately 50 kD; in some samples a characteristic double band at 200 kD was observed. An interesting feature was the presence of bands at 70-80 kD mainly in nuclear and cytoplasmic fractions. Only subcellular fractions from two tumors were negative with C595 by Western blot, one obtained from oral epithelium and the other from the tongue.

Lewis x antigen was detected in all fractions; nuclear, membrane and cytoplasmic moieties showed a smear reactivity from high to low molecular weight; membrane and nuclear fractions showed bands at 180 kD; in some samples a strong band at 80-70 kD (***Figure 4***) was observed. Anti Tn hapten MAb reacted with fractions derived from most tumors showing a similar pattern as described for MUCI while some fractions derived from an oral cavity tumor showed a lower intensity than the previous ones with bands between 180 and 116 kD. An interesting feature was the presence of bands of low MW (70-60 kD). Most samples reacted with anti-sialyl Lewis x showing several bands with a similar pattern as the reaction obtained with anti-MUC1, although two samples from maxillary sinus tumors also showed a strong band of 70 kD; finally, some samples reacted with anti-Lewis y showing several bands around 60 kD and between 48-38 while in some others a reaction at about 180 kD was obtained.

Isolation of tumor associated antigens

Solubilized subcellular fractions obtained from four samples (two from larynx tumors, one of tongue and the other one localized in the tonsillar ring) were subjected to

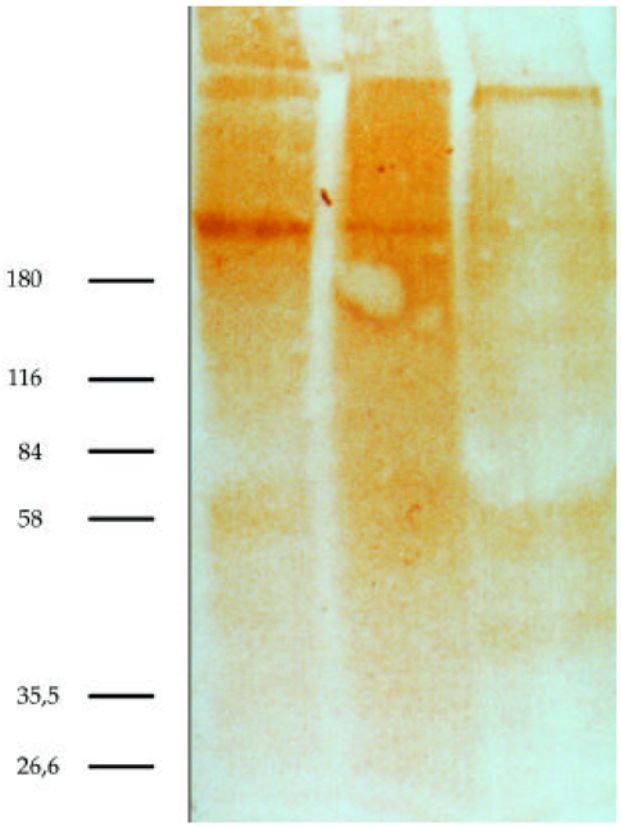

Figure 4. SDS-PAGE and Western blot of subcellular fractions obtained from a pharynx carcinoma incubated with anti Lewis x Mab (KM380). From left to right: in nuclear and cytoplasmic fractions a band at about 200kD is observed, being strong the first one; the membrane fraction (third band) shows a smear reaction.

density gradient centrifugation in guanidium CsCI; different fractions were obtained according to density which varied from 1.35 g/ml to 1.60 g/ml; when fractions were subjected to Western blot analysis with the panel of MAbs, they showed reaction with at least one of them; the density of the reactive fractions varied in a range between 1.50 to 1.43 g/ml CsCI.

CsCI fraction reactions with anti-MUC1, anti Lewis y, sialyl Lewis x, Lewis x and Tn hapten showed a similar pattern; they looked like a smear from more than 400 kD to less than 30 kD while in one fraction two bands of approximately 200kD were identified. In fractions derived from a larynx tumor, two bands at approximately 200kD were found after incubation with anti-MUCI and anti-sialyl Lewis x.

Correlation studies among tumor antigenic expression, tumor localization and clinical stage were performed; there was no relationship between the various tumor antigenic expressions, the tumor localization and tumor or nodal burden.

Discussion

Many cancer-associated markers are protein or carbohydrate antigens derived from mucins, which suffer alterations during the process of malignant transformation. MUCl mucin is one of the best known; it is a transmembrane molecule with an extracellular domain and a cytoplasmic tail; it is expressed by normal cells of different epithelia such as breast, pancreas, ovary, urothelium, digestive tract and uterus[11,34] where MUC1 has lubrication and protection roles. In malignant tumors, MUCl mucin is often overexpressed and presents an aberrant glycosylation with the result of exposing the core protein and consequently the appearance of new antigens either of peptidic or carbohydrated nature.

In the present research, we have studied the usefulness of MUC1 mucin and carbohydrate associated antigens as tumor markers in HNSCC by means of immunohistochemical and Western blot analysis. The current selection of MAbs has proved to be useful since we found that MUC1 and carbohydrate antigens were widely expressed in this tumor localization. Using immunohistochemical staining performed on sections from the same paraffin block in each tumor, we have examined the pattern of reactivity that appears to be extended and simultaneous such we have already described in larynx carcinoma;[7] in the present report we found a high frequency of carbohydrate antigenic co expression.

Although MUC1 mucin and associated carbohydrate antigens have been previously studied in other neoplastic tissues, only a few detailed reports concerning their expression in HNSCC can be found. Nitta et al[25] have suggested that MUC1 mucin may be a useful indicator of malignant potential in oral squamous cell carcinoma; Itoh et al[19] and Ikeda et al[16] also reported that DF3 anti MUC1 MAb may be an effective tumor marker for pharyngeal, laryngeal and esophageal carcinomas while Jeannon et al[20] found that MUC1 and MUC2 expression is altered in laryngeal cancer.

According to other authors,[25] we have found that both normal and malignant cells were reactive but in a different quantitative grade of expression and also with a remarkable different pattern.

By Western blot, we have examined the pattern of reactivity with the panel of selected MAbs; in most cases, they reacted with the same MW components and showed a similar pattern of reaction. The mobility of high-molecular-weight recognized by anti-MUC1 antibodies varied among samples; these variations were previously found by different authors which attributed them to genetic polymorphism;[28,32] furthermore, we have detected bands at 80-70 kD which were also previously recognized with HMFG2 anti MUCI by Burchell et al.[4] and Griffiths et al[12] in some breast cancer cell lines such as T47D. Usually, we detected these low MW bands in cytoplasmic fractions while membrane and nuclear extracts showed high MW mobility; furthermore, by immunohistochemistry, we have found a cytoplasmic staining with C595 anti MUC1 which has proved to detect both intra and extracellular MUC1. Similar results have been previously found by Griffiths et al[12] in breast cancer cell lines; employing HMFG-2 MAb, they detected intracellular localization of MUC1 by electron microscopic studies using gold-labeled antibodies and also low molecular weight components by Western blot.

In a previous report,[7] we have employed CsCl gradients to isolate MUC1 from laryngeal carcinoma specimens; in the present study, we have confirmed that the methodology is useful for similar purposes. The isolation and identification of tumor-associated antigens is a critical step for further studies on the immune response against HNSCC, which can sustain new therapeutic approaches, mainly in disseminated disease.

The feature that anti carbohydrate antigens showed similar bands to MUC1 suggest that this mucin should carry these antigenic moieties which we have probably detected at different glycosylation stages; the immunohistochemical reaction mainly at cytoplasmic level indicates this possibility and makes unlikely the hypothesis that low MW bands are breakdown products. Some samples were not reactive with C595 and did express carbohydrate antigens; one of the reasons could be that either the technique here applied was not able to detect MUC1 in these samples and/or another glycolipid or glycoprotein could be their carrier. It has been largely described that these carbohydrate antigens may be carried by diverse glycoproteins and glycolipids[10,14] and were

described linked to the protein core of mucins,[34] they have been already involved in invasive and metastatic processes[15,24,29,33] according to this fact, it is of remarkable interest that most tumors under this study were disseminated and also, their cells were reactive with the panel of MAbs in lymph nodes.

Carcinoma cells are phenotypically different from their normal tissue counterparts; in the present report, normal samples expressed MUC1 and carbohydrate-associated antigens lower than tumor tissues. In coincidence with Rubin et al,[30] MUC1 expression was less prominent in non-neoplastic specimens and usually at epithelial level early in this tissue differentiation process. Immunohistochemistry and Western blot proved to be complementary studies since non-reactive samples by immunohistochemistry were positive by Western blot; the opposite was also found showing negative samples by Western blot being positive by immunohistochemistry.

The antigenic changes in malignant epithelium of the head and neck offer possibilities for the search of targets which may sustain new treatments in disseminated stages of disease; since invasive malignant cells express different antigens it is attractive that strategies designed to use antibodies against these antigens might be of clinical usefulness.

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References

1. *Brakenhoff RH, Stroomer JGW den Brink C, et al:* Sensitive detection of squamous cells in bone marrow and blood of head and neck cancer patients by E48 reverse transcriptase polymerase chain reaction. Clin Cancer Res 5:725-732, 1999.
2. *Brennan JA, Mao L, Hruban RH, et al:* Molecular assessment of histopathological staging in squamous cell-carcinoma of the head and neck. N Engl J Med 332:429-435, 1995.
3. *Brown A, Feizi T, Gooi HC:* A monoclonal antibody against human colonic adenoma recognises a difucosylated type-2 blood group chains. Biosci Rep 3:163-169, 1983.
4. *Burchell J, Durbin H, Taylor-Papadimitriou J:* Complexity of expression of antigenic determinants recognised by monoclonal antibodies HMFG-1 and HMFG-2 in normal and malignant human mammary epithelial cells. J Immunol 131:508-513, 1983.
5. *Croce MV, Price M R, Segal-Eiras A:* Expression of monoclonal antibody-defined antigens in fractions isolated from human breast carcinoma and patients' serum. Cancer Immunol Immunother 40:132-137, 1995.
6. *Croce MV, Colussi AG, Price MR, et al:* Immunohistopathological characterization of spontaneous metastases in a human lung mucoepidermoid adecarcinoma (HLMC) xenograft. Pathol Oncol Res 4:259-266, 1998.
7. *Croce MV, Price MR, Segal-Eiras A:* Detection and isolation of MUC 1 mucin from larynx squamous cell carcinoma. Pathol Oncol Res 2:93-99, 2000.
8. *Federici MF, Kudryashov V, Saigo P, et al:* Selection of carbohydrte antigens in human epithelial ovarian cancers as targets for immunotherapy: serous and mucinous tumors exhibit distinctive patterns of expression. Int J Cancer 81:193-198, 1999.
9. *Feickert HJ, Anger BR, Cordon-Cardo C, et al:* Cell-surface antigens of human lung tumors detected by mouse monoclonal antibodies: definition of blood-group- and non-blood-group-related antigenic systems. Int J Cancer 46: 1007-1013, 1990.
10. *Fukuda M:* Possible roles of tumor-associated carbohydrate antigens. Cancer Res 56:2237-2244, 1996.
11. *Gendler SJ, Spicer AP:* Epithelial mucin genes. Ann Rev Physiol 57:607-634, 1995.
12. *Griffiths A, Burchell J, Gendler S, et al:* Immunological analysis of mucin molecules expressed by normal and malignant epithelial cells. Int J Cancer 40:319-327, 1987.
13. *Hanai N, Shitara K, Yoshida H:* Generation of monoclonal antibodies against human lung squamous cell carcinoma and adenocarcinoma using mice rendered tolerant to normal human lung. Cancer Res., 46:4438-4443, 1986.
14. *Hakomori S:* Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res. 45:2406-2414, 1985.
15. *Hilkens J, Ligtenberg JL, Vos HL, Litvinov S:* Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem Sci 1992; 17:359-63, 1992.
16. *Ikeda Y, Kuwano H, Ikebe M, et al:* Immunohistochemical detection of CEA, CA19-9 and DF3 in esophageal carcinoma limited to the submucosal layer. J Sur Oncol 56:7-12, 1994.
17. International Union Against cancer (UICC) Classification of Malignant tumors. ED.L.H. Sobin and Ch. Wittekind, 5th edition, New York, 1997.
18. *Ioannides CG, Fisk B, Jerome KR, Irimura T:* Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J Immunol 151:3693-3703, 1993.
19. *Itoh T, Yonezawa S, Nemoto M et al:* expression of mucin antigen and Lewis x-related antigens in carcinoma s and dysplasia of the pharynx and larynx. Pathol Int 46: 646-655, 1996.
20. *Jeannon JP, Stafford FW, Soames JV, Wilson JA:* Altered MUC1 and MUC2 glycoprotein expression in laryngeal cancer. Otolaryngol Head and Neck Surg 124:199-202, 2001.
21. *Jerome KR, Barnd DL, Bendt KM:* Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Cancer Res 5:2908-2916, 1991.
22. *Laemmli UK:* Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680-681, 1980.
23. *Nakagoe T, Sawai T, Tsuji T, et al:* Preoperative serum levels of sialyl Lewis (a), sialyl Lewis(x) and sialyl Tn antigens as prognostic markers after curative resection for colorectal cancer. Cancer Detect Prev 25:299-308, 2001.
24. *Nakamori S, Kameyama M, Imaoka S, et al:* Involvement of carbohydrate antigen sialyl Lewis x in colorectal cancer metastasis. Dis Colon Rectum 40:420-431, 1997.
25. *Nitta T, Sugihara K, Tsuyama S et al:* Immunohistochemical study of MUC1 mucin in premalignant oral lesions and oral squamous cell carcinoma. Cancer 88:245-254, 2000.
26. *Pancíno GF, Osinaga E, Vorauher W, et al:* Production of a monoclonal antibody as immunohistochemical marker on paraffin embedded tissues using a new immunization method. Hybridoma 9:389, 1990.

27. *Price MR, Edwards S, Owainati A, et al:* Multiple epitopes on a human breast carcinoma associated antigen. Int J Cancer 36:567, 1985.
28. *Price MR, Pugh J, Hudecz F, O'Sullivan, C et al:* Immunological and structural features of the protein core of human polymorphic epithelial mucin. Mol Immunol 27:795-801, 1990.
29. *Regimbald LH, Pilarski LM, Longenecker BM:* The breast mucin MUC1 as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. Cancer Res 56:4244-4249, 1996.
30. *Rubin JS, Bloor BK, Hart IR, Morgan PR:* Muo-1 gene expression in head and neck squamous cell carcinoma. J Laryngol Otol 114:772-776, 2000.
31. *Svitacheva N, Davies JR, Lessuileur T:* Characterization of mucins produced by human HT29-MTX cell line. 5`h International workshop on Carcinoma Associated Mucins, Cambridge, LJK 1998.
32. *Swallow DM, Griffiths B, Bramwell ME, et al:* Detection of the urinary „pum" polymorphism by the tumour binding monoclonal antibodies Cal, Ca2, HMFG-I and HMFG-2. Dis Markers 4:247-254, 1986.
33. *Takada A, Ohmori K, Yoneda T, et al:* Contribution of carbohydrate antigens sialyl Lewis a and sialyl Lewis x to adhesion of human cancer cells to vascular endothelium. Cancer Res. 53:354-361, 1993.
34. *Taylor-Papadimitriou J, Finn O:* Biology, biochemistry and immunology of carcinoma associated mucins. Immunol Today 18:105-107, I 997.
35. *Towbin H, Staehelin T, Gordon J:* Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76:4350-4354, 1979.
36. *Vernham, GA, Crowther JA:* Head and neck carcinoma: stage at presentation. Clin Otolaryngol 19:120-124, 1994.