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Detection and Isolation of MUC1 Mucin from Larynx Squamous Cell Carcinoma

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The progression from uncontrolled cell proliferation to invasion and metastasis of epithelial tumors is partially understood. Alteration of epithelial mucin expression have been described in different malignant localizations but only few attempts have been made to identify mucin expression in malignant laryngeal tumors. In the present report, results are shown of studies on the expression of mucins and carbohydrate related antigens in laryngeal cancer and on the isolation of MUC1 mucin from this tumor tissue. Malignant laryngeal specimens were processed for immunohistochemical analysis and for extranuclear membrane fractions (ENM) which were obtained by ultracentrifugation. Subsequently, ENM samples were centrifuged in density-gradient; the analysis of fractions was performed by means of SDS-PAGE and Western-blotting. The panel of mon-

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oclonal antibodies (MAbs) included anti MUC1 mucin, anti Lewis x, anti sialyl Lewis x, anti Lewis y, anti MUC-5B, anti oral mucin (gp230), anti Tn hapten, anti p53 and anti cytokeratins. By immunohistochemistry, it was possible to detect MUC1 mucin, Lewis x and Lewis y showing strong reactions while sialyl-Lewis x and Tn antigen only reacted weakly in a few cells; cytokeratins were detected in all samples. In ENM derived fractions obtained by CsCl centrifugation, MUC1 was demonstrated by Western blotting. Conclusions: (1) laryngeal cancer antigenic expression comprises mostly MUC1 mucin, Lewis x, Lewis y as well as Tn antigen and (2) the methodology here employed is useful to isolate MUC1 from tumor samples. (Pathology Oncology Research Vol 6, No 2, 93–99, 2000)

Introduction

Worldwide, incidence of larynx tumors are on increase,^{22,31} smoking of cigarettes and/or alcohol consumption have been etiologically associated with their carcinogenesis. Different antigenic changes occur within epithelial tumor development; among these, mucins may be altered during the neoplastic process and progression to a metastatic phenotype.¹⁶

These molecules are produced and secreted by a wide variety of normal and neoplastic epithelial cells; during the last decade, different mucins associated to carcinoma have been investigated being described at least nine human mucin genes (MUC1-4, MUC5B, MUC5AC,

MUC6-8).^{13,42} MUC1 mucins are high molecular weight transmembrane glycoproteins which extracellular domain is made up of tandem repeats of 20 aminoacids characterized by many O-linked oligosaccharides attached to the mucin core polypeptides through serine and threonine residues.¹² The complexity of carbohydrate moieties have been investigated proving the presence of truncated side chains in carcinoma as result of changes in glycosyl and sialyltransferases.⁴⁷ Mucin alterations have been related with cellular motility, and consequently, with tumor invasion and metastatic dissemination;^{41,36} on the other hand, several tumor antigens have been detected related to tumor mucin molecules which may induce cellular^{2,19,21,38} and humoral immune responses in the host.^{7,23}

Although progress has been made in the characterization of mucin structure and organ specific expression, few attempts were undertaken to identify mucin expression in malignant tumors of the larynx.

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In the present report we discuss results obtained on the expression of mucin antigens as well as the MUC1 mucin isolation from laryngeal carcinoma cells.

Materials and Methods

Tissue samples

The present study was performed in a total of 20 malignant laryngeal tumors belonging to patients treated with surgical resection and classified according to UICC criteria TNM Classification of Malignant Tumors, 5th edition.¹⁸ Clinicopathological characteristics of patients are depicted in *Table 1*.

Normal squamous epithelia from the same localization were obtained by biopsy and they were included as controls.

Tissues were processed following routine directives; specimens were sectioned to cover different programmed studies (*Figure 1*); a piece of tissue was fixed in formaldehyde for histopathological diagnosis and immunohistochemical analysis while another tissue sample was rinsed with fresh sterile Hank's balanced cell solution and subsequently processed for the preparation of subcellular fractions.

All human studies have been reviewed by the University of La Plata Ethical Committee and experiments were performed according with the Helsinki Declaration. Informed consent was obtained from all patients included in this study.

Preparation of extranuclear membrane fractions

Fractions were prepared from human tumor tissues.³² Briefly, tissues were homogenized in 1.41M PBS pH 7.2, at 4ml/g; homogenates were centrifuged at 600 x g and at 105000 x g at 4°C and precipitates (extranuclear membrane fraction) were resuspended in 1.41M PBS, liophilized and stored at -20°C for subsequent density gradient centrifugation.

Density-gradient centrifugation

Samples from extranuclear membrane fractions in 4 M guanidium chloride 1% NP40 were centrifuged in 6 M guanidium chloride/CsCl (density 1.45 g/ml) at 40000 rpm for 66 h at 10°C in a Beckman Titanium 70.1 fixed-angle rotor; 500 µl fractions were collected and the density of each fraction determined using a Hamilton syringe (Merck, Dagenham, Essex, UK) as a pycnometer.⁴⁵

SDS-PAGE and Immunoblotting

Fractions were collected and dialysed against 1.41 M PBS at 4°C for 48 h and then liophilized, resuspended in SDS-PAGE sample buffer at reducing conditions and run following standard procedures²⁴ in a discontinuous buffer

system. After electrophoresis gels were either stained with Coomassie blue or they were transferred electrophoretically to nitrocellulose membranes⁴⁶ and incubated with different monoclonal antibodies.

Immunohistochemical analysis

This technique was developed according to a previous report,⁸ with minor modifications. All specimens were fixed in phosphate buffered formalin, embedded in paraffin and cut into 5 µm serial sections. Deparaffinized sections were treated with 10mM sodium citrate buffer at 100°C for 5 minutes,⁴⁰ then, they were incubated overnight at 4°C with mouse monoclonal antibodies. Negative controls were incubated with PBS instead of monoclonal antibodies.

The whole area of each sample was observed by sequentially examining low 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; heterogeneity was graded according to positive reactions, intensity and distribution. Staining intensity was graded as negative, low, moderate and strong.¹¹

With respect to p53, and considering other reports,³⁴ a sample was coded as positive even when only a few cells were reactive.

Table 1 Clinicopathological characteristics of patients.

Characteristics	No. of patients (%)
Sex	
Male	20/20
Female	0/20
Age, years	
<65	2/20
>65	18/20
T stage	
T1	0/20
T2	2/20
T3	9/20
T4	9/20
N stage	
N0	0/20
N1	4/20
N2	16/20
Metastases	
M0	19/20
M1	1/20
Differentiation	
Well differentiated	2/20
Moderately differentiated	9/20
Poorly differentiated	9/20

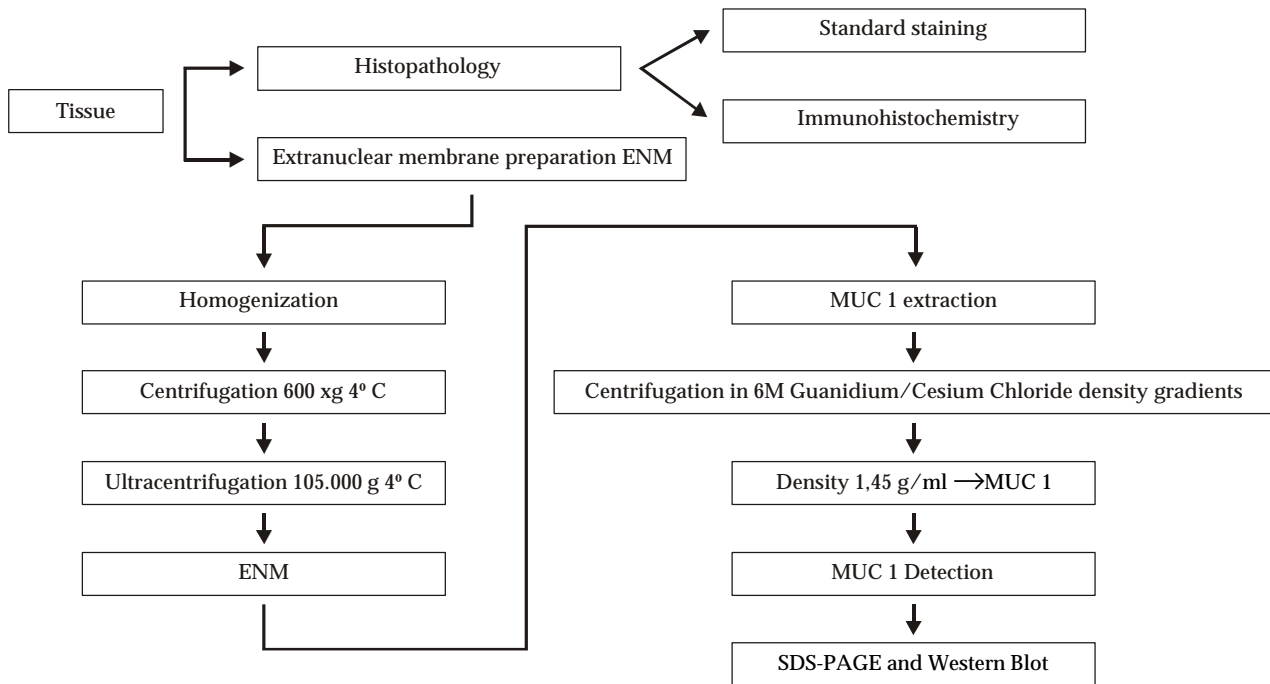


Figure 1. Summary illustration of the methodology applied in this work.

Monoclonal antibodies (Mabs)

The following monoclonal antibodies (Mabs) were assayed: C14 Mab, an IgM anti-Lewis y hapten against the difucosylated Type-2 blood group chains,⁴ Mab KM93 (IgM), an anti sialyl-Lewis x and KM380 (IgM), an anti Lewis x.¹⁵ Three anti MUC1 Mabs were employed: Mab C-595 (IgG3) defines the tetrameric epitope Arg-Pro-Ala-Pro in the MUC1 protein core,³² HMFG1 and HMFG2⁴³ define the epitopes PDTR and DTR respectively.³³ Mab 83D4 (IgM) against Tn determinant,³⁰ an anti MUC-5B (PANH2 Mab) (IgG1) raised against partially deglycosylated MG1,²⁸ PANH3, an anti MUC-7 and PANH4 Mab (IgM) an anti gp230 derived from buccal mucosa.²⁷ Also, anti-p53 Mab (mouse IgG2a isotype) (SIGMA No. P-5813) and finally, an anti-human cytokeratin (CK)/HRP against keratins 5, 6, 8, 17 and 19 (Dako EPOS, Code No. U 7022) was assayed.

Results

By routine techniques, histopathological examination was performed; all tumors were identified as invasive squamous cell carcinoma of the larynx; it was possible to observe two different types of cells: tumor large cells and small ones with intense staining and with round nuclei; prominent anaplasia, anisocytosis as well as anisocariosis were present; bizarre mitotic figures were also seen. In a group of samples, the expression of different tumor anti-

gens was studied using a panel of monoclonal antibodies; results are summarised in *Table 2*.

MUC1 mucin was detected with C595 MAb in four out of seven tumor patients' samples; in most reactive cells, a homogeneous cytoplasmic immune-staining was shown (*Figure 2a*); often, a perinuclear reaction was detected; also several cellular membranes revealed a positive staining while nuclei remained negative the other anti-MUC1 MABs did not display any reactivity.

Another feature of the distribution of antigenic expression was a strong cytoplasmic reaction observed with anti Lewis x MAb (KM380) in four out of seven samples while only a moderate reaction was detected in one sample; an example of one tumor is depicted in *Figure 2b*. C14 MAB (anti Lewis y antigen) showed a consistent staining restricted to the cytoplasm in some cells; 83D4 (antiTn hapten) stained weakly with a homogeneous pattern while KM93 MAB (anti-sialyl Lewis x) only reacted in a few cells in some tumor specimens.

All samples stained with the anticytokeratin MAB mostly in the cytoplasm while p53 was widely detected in cell nucleus; only one sample showed a positive reaction with anti oral mucin gp230 while negative results were obtained with the other MABs: anti MUC-5B as well as MUC-7.

Normal squamous epithelia belonging to the same localization were analysed; expression of oral mucin (gp230), and Lewis x was found in three out of seven samples considered; sialyl Lewis x and Lewis y in 4/7 while MUC1

Table 2. Summary of immunohistochemical analysis of tumor samples

Patient Number	Monoclonal Antibodies									
	Lewis y	Sialyl Lewis x	Lewis x	MUC1	Tn hapten	Muc-5B	Muc-7	Gp230	P53	Cytokeratins
1	☐	☐	●●	●	☐	○	○	○	☐	☐
2	☐	○	●●	○	☐	○	○	○	●	☐
3	☐	☐	●	☐	☐	○	○	○	☐	☐
4	○	○	○	●	○	○	○	☐	●	☐
5	○	○	○	○	○	○	○	○	☐	●
6	○	○	●●	○	☐	○	○	○	☐	●
7	☐	☐	●●	☐	○	○	○	○	●	●

○ = negative

☐ = mild positive reaction

● = moderate positive reaction

●● = strong positive reaction

and Tn antigen in 2/7. The outstanding feature of normal expression was its restriction to a few cells belonging to one epithelial stratum being the pattern of distribution quite homogeneous; mainly a weak cytoplasmic reaction was detected in all samples with one exception: MUC1 was exclusively expressed at cellular membranes. In one specimen, sialyl Lewis x was expressed in a few cells belonging to different epithelial strata; on the other hand, cytokeratins were detected in small amounts with a weak and homogeneous cytoplasmic pattern while in most cases, p53 was observed in a few cells from one normal specimen.

In order to isolate MUC1 mucin from laryngeal tumors, extranuclear membranes were prepared and analysed by SDS-PAGE and Western-blotting incubated with anti MUC1 Mabs. In specimen belonging to patient number 1, these studies showed a positive reaction with C595 Mab; the same positive staining was obtained with anti Lewis x and anti Tn hapten of the same tumor sample (data not

shown). Extranuclear membranes isolated from this tumor sample were subjected to density gradient centrifugation in guanidin Cs Cl; fractions obtained were subsequently analysed by SDS-PAGE and Western-blotting employing the panel of Mabs. Fraction 7 (from a density of 1.43 g/ml), reacted with anti MUC1 C595 Mab by Western-blot; the major feature of staining pattern was the presence of two bands of high-molecular-mass material of more than 180 kDa and some trace minor bands (*Figure 3*); this fraction also showed reactivity with anti Lewis x and anti Tn hapten employing the same technique.

Discussion

Neoplastic cells express a wide variety of molecules since the development and progression of cancer are regulated by the expression of different molecules encoded by a continuous enlarged list of diverse oncogenes and suppressor genes. Most immuno-biological functions as well

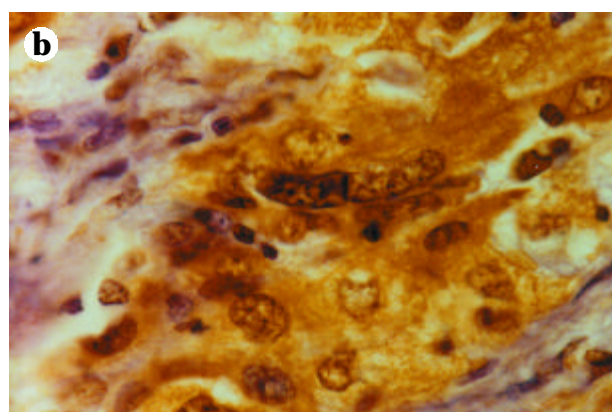
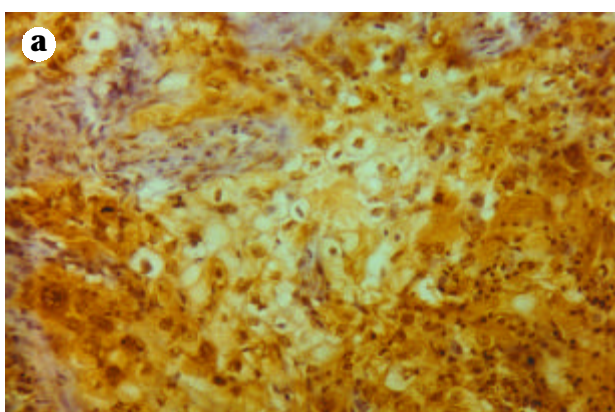


Figure 2. a) Immunohistochemistry of larynx carcinoma with anti MUC1 MAb C595. Intense staining is observed in cytoplasm and plasmatic membrane. Non-reactive cells show a clear cytoplasm. (x100). **b)** Immunoreactivity obtained by incubation with antiLewis x Mab (KM380). The positive reaction is observed in the cytoplasm with a vesicle pattern; mitosis in the centre of the picture (x630).

as clinical properties of these molecules are still unknown, although some progress has been obtained in recent years. This relationship should be useful for the identification of accurate indicators in diverse aspects of neoplastic phenomena from basic knowledge to clinical practice.

Mucin alteration occurs during tumor development, such changes include differences in mucin-associated antigenic expression between cancer cells and their normal counterparts. By immunohistochemistry, series of MAbs against epithelial mucins and carbohydrate related antigens have been evaluated. It was possible to show a high expression of mucins and/or carbohydrate related antigens such as Lewis x, Lewis y and Tn hapten being sialyl Lewis x also expressed in a small number of samples; furthermore, this expression may be related to an advanced malignant disease since most tumors included in the present study were disseminated. Tumor associated antigens here evaluated have been reported to be factors associated with increased metastatic potential;^{16,35,36} for instance, diverse mechanisms have shown to be involved in MUC1 cellular adhesion to vascular endothelium through exposing usually cryptic associated carbohydrate epitopes such as sialyl Lewis a and sialyl Lewis x expression⁴¹ as well as through the interaction with ICAM-1.³⁶ Itoh et al²⁰ have studied the expression of MUC1, MUC2 and mucin carbohydrate antigens (Tn, sialyl-Tn, T, Lewis x, Lewis y and sialyl Lewis x-i) in normal, dysplastic and carcinoma of the pharynx and larynx; they found that only MUC1 was an effective marker of dysplasia and carcinoma in these localizations being Tn antigen expressed exclusively by carcinoma specimens; T antigen expression increases according with malignant progression. In the present approach, one of the most significant finding was that only one specimen failed to react with the MAbs generated against mucin and/or carbohydrate related antigens. It is interesting to point out that these carbohydrate antigens may be carried on by a variety of carrier molecules, e.g., lipids, glycoproteins and mucins; this fact may partly explain the reaction in specimens number 2 and 6 of carbohydrate moieties without mucin expression.

The coexpression of Lewis y and sialyl Lewis x in samples that were also reactive with anti Lewis x was also a remarkable finding taking into account that they are structures based on type 2 (Gal14GlcNAc-) carbohydrate sequence; Lewis y is the result of further fucosylation of the basic Lewis x antigen being sialyl Lewis x the consequence of the addition of sialic acid to its terminal galactose.¹⁴ On the other hand, Tn hapten is the first step of O-glycosylation of mucins such as MUC1 which make reasonable their co expression in some specimens; it is possible that in two samples which were reactive with Tn but did not show expression of MUC1, other glycoproteins and glycolipids could be Tn hapten possible carriers.¹⁰

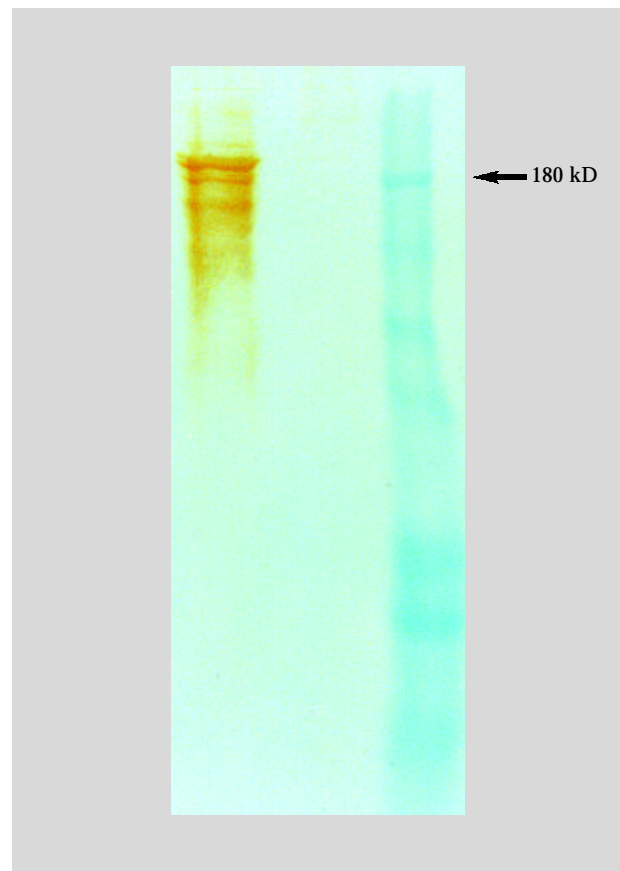


Figure 3. Example of Western blotting analysis of a fraction obtained by density gradient centrifugation in guanidine/CsCl (fraction 7 from a density of 1.43 g/ml) as described in Methods. The fraction belonged to subcellular membranes from larynx carcinoma; the reaction with anti MUC1 C595 Mab is depicted. It shows the presence of two bands of a high-molecular-mass material of more than 200 kDa and some trace minor bands.

The isolation and further investigation of tumor associated antigens should be incremented in order to design directed therapies which can be useful as anticancer strategies. Different authors have employed with success similar procedures to isolate glycoproteins from diverse materials,⁶ including mucins from tumor specimens;^{1,40} we purified MUC1 through CsCl gradients and when fractions were analysed by Western-blotting with further incubation with anti-MUC1 C595 Mab, they revealed two bands which correspond to reduced mucin subunits; similar findings have been already identified in ENM belonging to breast carcinoma specimens⁷ which were described by other authors. Furthermore, associated mucin carbohydrates have been detected such as Lewis x, Lewis y, sialyl Lewis x and Tn hapten; some of these were described linked to the protein core of mucins.⁴⁴

In recent years, among prognostic factors determining the course of different tumor localizations, genetic alter-

ations such as those affecting p53 have been the focus of considerable interest; mutations in p53 result in the synthesis of a protein with longer half-life, which is immunohistochemically detectable.¹⁷ Its routine clinical application is a promising field since p53 overexpression and tumor chemosensitivity has been emphasised by Bradford et al.³ We have included p53 which has been largely studied as a prognostic factor in laryngeal cancer with controversial significance about its reliability⁵. We found that all tumor samples expressed p53 being sporadically found in normal tissues; this would be in coincidence with data involving this molecule with the progression of malignant tumors, although its exact role has not been yet elucidated. Previous studies^{26,29} have shown associations between high levels of p53 expression and advanced disease stage as well as metastatic tumor dissemination, although p53 over expression has been identified as an early event in the development of carcinoma of the larynx.⁹ On the other hand, different efforts have been performed trying to establish p53 expression as a useful prognostic indicator but it does not appear to be of clinical value in this last aspect.³⁷

Cytokeratins 8,18 and 19 that are normally expressed in head and neck tissue only in small amounts, become overexpressed in head and neck carcinoma; furthermore, cytokeratins 5/6 and 13 have been detected more frequently in metastatic squamous cell carcinoma than in the primary tumors.^{25,39} The anticytokeratin MAb employed in the present research stains cytokeratins 5, 6, 8, 17 and 19; hence it is not surprising that all tumor specimens were reactive being normal samples sporadically positive stained; these differences may reflect neoplastic phenotypic changes involved in invasive, migrating and proliferative activities.

In the present study, we have generated information on antigenic expression of carcinoma of the larynx that could be used for active and passive immunotherapy. An additional goal of our research was to perform a routine procedure in larynx carcinoma which covered different programmed studies, favouring the detection and isolation of MUC1 previously detected by immunohistochemistry.

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