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Expression of Invasion Markers CD44v6/v3, NM23 and MMP2 in Laryngeal and Hypopharyngeal Carcinoma

Gábor RÉPÁSSY,[1] Csaba FORSTER-HORVÁTH,[2,3] Attila JUHÁSZ,[4] Róza ÁDÁNY,[5] Anna TAMÁSSY,[2,3] József TÍMÁR[2,3]

[1]Department of Oto-Rhino-Laryngology, [2]1st Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine; [3]Department of Molecular Pathology, Joint Research Organization of the Hungarian Academy of Sciences and the Semmelweis University of Medicine, Departments of [4]Oto-Rhino-Laryngology, [5]Hygieny and Epidemiology, Debrecen Medical University, Debrecen, Hungary

Twelve laryngeal squamous cell carcinoma cases (7 laryngeal and 5 hypopharyngeal cancer; 15 samples) were analysed by immunohistochemistry for the expression of invasion markers CD44v6/v3, NM23 and matrix metalloproteinase, MMP2. The laryngeal epithelium showed $CD44v6^+/v3^+/NM23^-/MMP2^-$ phenotype. When tumors were grouped into TNM categories the phenotype of the T2 and T3 tumors was similar, characterised by decreased $CD44v3^+$ and lack of MMP2 expressions. Meanwhile the NM23 expression was more frequent in T3 tumors. In T4 stage the frequency of NM23 and MMP2 positive cases increased (5/6 and 4/6, respectively) but there was no correlation with the appearence of lymph node metastasis. Comparison of the phenotype of laryngeal and hypopharyngeal tumors, irrespective of the TNM stages, revealed characteristic differences: T2 stage laryngeal tumors showed decreased CD44v3 and occasional NM23 and MMP2 positivity, while in T3 stage these tumors were characterised by increased frequency of NM23 positivity. The phenotype of the hypopharyngeal tumors was significantly different with a high frequency of MMP2 positive cases (5/6) and $NM23^+$/low $CD44v3^+$ phenotype. The sharp differences in the phenotypes of laryngeal and hypopharyngeal carcinomas were connected to the differences in their invasive capacity unlike to the size of the tumors, since the T4 stage hypopharyngeal tumors had a significantly smaller size than laryngeal ones, even at lower stages. (Pathology Oncology Research Vol 4, No 1, 14–21, 1998)

Key words: laryngeal carcinoma, CD44v3, nm23, MMP2, immunohistochemistry

Introduction

Head and neck cancer falls behind the more frequent malignancies such as lung, breast, prostate and GI tract cancers, however the frequency of the disease is much higher in particular subpopulations (alcohol abusers and heavy smokers). The interest of clinicians and researchers can partially be explained by the fact that though the most frequent histological type of head and neck cancer is squamous cell carcinoma the biological behaviour of the tumor in various anatomical locations is diverse. This fact may suggest that beside the diversity of the local microenvironment, squamous cell carcinoma may have a diverse malignant phenotype as well. Laryngeal and hypopharyngeal cancers provide a model for studying diversity of the invasive phenotype of tumors with similar histology. Unlike laryngeal cancers, hypopharyngeal counterparts are more aggressive, regional lymph node metastases develop earlier and distant metastases are more prevalent. This can only partially be explained by the differences between the microanatomy of the two regions, therefore comparison of the tumor invasive phenotype is validated.

Invasion and metastasis are the ultimate characteristics of malignancy, however, tumors with similar morphology may express different invasive/metastatic phenotype. Invasiveness is regulated by various cellular and host factors. As far as the tumor cells are concerned, the invasive

Received: Nov 15, 1997; *accepted:* Jan 10, 1998

Correspondence: Joseph TÍMÁR, M.D., Ph.D., DSc., 1st Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine, Üllői út 26., H-1085 Budapest, Hungary. Tel: (36) (1) 117 1070, fax: (36) (1) 117 1074

This work was supported by the Hungarian Ministry of Welfare: ETT No: T-01-100/93

 1219-4956/98/010014+08 $ 12.00/0

Table 1. **Clinical data**

	sex	*localisation*	*histology*	*TNM status*	*recidive*	*metastasis*	*survival (month) outcome*
G1	f	GL	SCCC	T2N0M0			50-alive
G2	m	GL	SCCC	T2N0M0			46-alive
G3,G8	m	SGL	SCCC	T3N2M0		lnd	32-alive
G4	f	SGL	SCCC	T2N0M0			42-alive
G5	m	GL	SCCC	T2N0M0			22-alive
G6,G7	m	GL	SCCC	T3N0M0			24-alive
G9	f	GL	SCCC	T4N0M0	local		5 – exit
H1	m	HPH	SCCC	T3N2M0		lnd	30-alive
H2	m	HPH	SCCC	T4N2M0	local		41-exit
H3	m	HPH	SCCC	T4N0M0	local		9-exit
H4, H5	m	HPH	SCCC	T4N0M0	local		8-exit
H6	m	HPH	SCCC	T4N3M1		lung	2-exit

GL = laryngeal; SGL = supralaryngeal; HPH = hypopharynx
SCCC = squamous cell carcinoma; lnd = lymph node

potential is characterised by an aggressive interaction with the extracellular matrix (adhesion, digestion and ultimate cross-migration),[1] resistance to local immune effector mechanisms[2] and a sustained growth potential[3] during the entire process. Studies indicate that the expression of certain genes is responsible for the development of a more aggressive phenotype therefore these have been termed metastasis genes. The first of these genes discovered was the CD44 adhesion molecule which could be expressed in various splice variants; among them the v6 is considered to be responsible for metastatic phenotype.[4] Since the majority of tumors – especially those of the epithelial origin – have to cross basement membranes, the expression of enzymes specific for collagen type IV degradation is of special importance. Matrix metalloproteases are expressed by normal cells as MMP9 while the other variant, MMP2, is more frequently found in tumor cells and is also considered to be a metastasis promoting factor.[5] On the other hand, a gene with metastasis suppressing potential was also found, namely the NM23,[6] which is an NDP kinase with a loosely characterised function. Several metastatic tumor types downregulate NM23 gene expression, indicating the invasion inhibiting potential of the gene product.[7] However, contrary to these data the expression of NM23H1 in head and neck squamous carcinomas was found to be upregulated with the progression of the disease.[8]

Prognostic factors for laryngeal cancer have been studied extensively, and can be grouped into proliferation factors and invasion markers. Among the proliferation markers Ki67 and bcl2 expressions were not correlated to prognosis[9] unlike the c-erbB1/EGFr,[10] p53 overexpressions[11] and DNA ploidy.[9] On the other hand, several invasion/metastasis factors were also studied, where a strange phenotype was identified; the more invasive laryngeal cancers tend to down-regulate expression of the CD44v6 metastasis gene.[11] Furthermore, in laryngeal cancers with less favourable prognosis the expression of various cathepsins was found to be upregulated[12] while the adhesion molecule syndecan-1 was downregulated.[13]

The aim of the present study was to analyse the expression of certain invasion markers in laryngeal and hypopharyngeal squamous cell carcinomas by immunohistochemistry. The unique splice variant of the CD44, the heparan sulphate proteoglycan, v3,[14] was chosen because it has not been studied in epithelial tumors though it is the only variant of CD44 with growth factor-binding potential. Also, the matrix metalloproteinase MMP2 expression has not been studied in this tumor type before. Finally, NM23 was also tested because of the contradictory results of its overexpression in squamous cell carcinomas of the head and neck.

Materials and Methods

Patients

Twelve patients with laryngeal and hypopharyngeal tumors were used in this study. In three cases repeated biopsies were performed and all the 15 tumor samples were used for pathological analysis. The most important clinical data of the patients including survival are shown on the *Table 1*. The G1-, G2-, G5-labelled tumors originated from the laryngeal free margin, with sub-laryngeal spreading. The G6-, G7-, *(Figure 1)* G9-labelled tumors were advanced tumors of laryngeal origin, showing supralaryngeal or intralaryngeal spreading. Local invasion of the G9-tumor broke through the thyroid cartilage and the tumor spread toward the prelaryngeal muscle. The G3,8- and G4-labelled tumors were supralaryngeal, start-

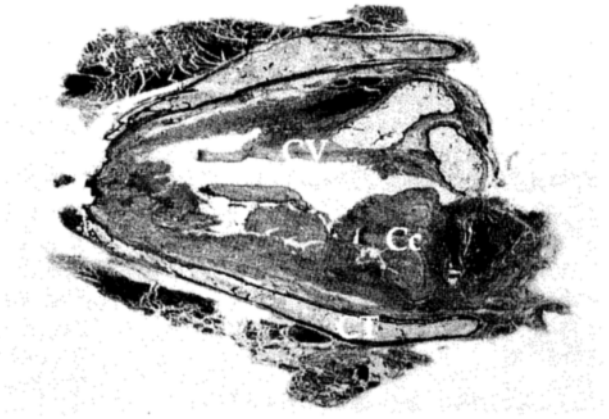

Figure 1. Whole horizontal organ section of the larynx. Carcinoma of laryngeal origin (G7) is on the left side, infiltrating the whole paralaryngeal space. Cc: tumor, E: epiglottis, CT: thyreoid cartilage. Mallory trichrom staining.

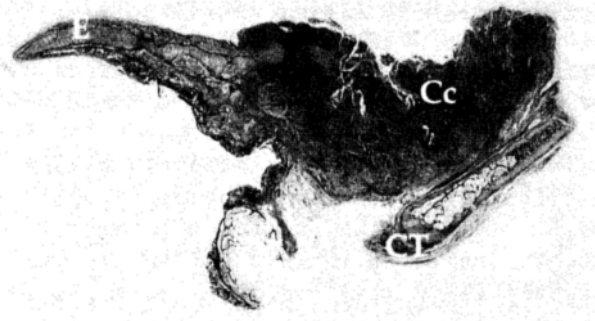

Figure 2. Parasagittal section of the larynx. The carcinoma originating from the epilaryngeal laryngeal surface (G4) is seen to invade the pre-epilaryngeal space. Cc: tumor, CT: thyreoid cartilage, CV: corda vocalis. Mallory trichrom staining.

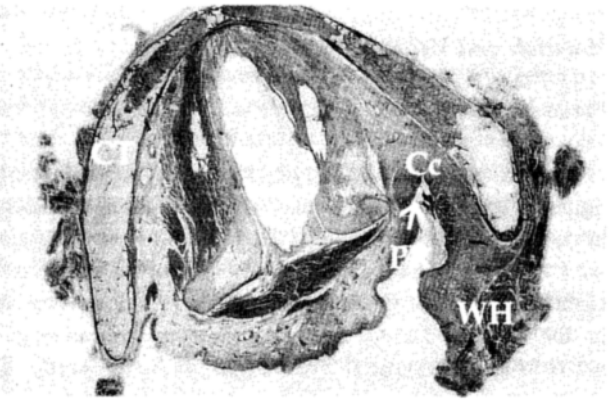

Figure 3. Whole horizontal organ section of the larynx. A small-sized hypopharynx carcinoma (H6) is observable in the right pyriform recess (PR). Details of the lateral pharynx wall (WH) are also detectable to the right of the section. Cc: tumor, CT: thyreoid cartilage. Mallory trichrom staining.

Table 2. **Sizes of the laryngeal tumors**

	Diameter (cm)	
	Vertical	*Horizontal*
G1	2,1	2,8
G2	1,7	2,6
G3,8	3,9	3,2
G4	2,3	1,8
G5	1,8	2,2
G6,7	2,7	3,3
G9	2,5	4,1
H1	0,8	2,1
H2	0,6	0,8
H3	1,0	0,8
H4,5	1,2	1,8
H6	1,0	1,5

ing from the epi-laryngeal laryngeal surface *(Figure 2)*. The course of their local invasion was the pre-epilaryngeal space and the laryngeal spreading of the G3,8 tumor disabled laryngeal movement. The tumors labelled H1, H2, H3, H4,5 and H6 *(Figure 3)* were hypopharyngeal, originating from the pyriform recess. The H2, H3 tumors started from the lateral wall of the pyriform recess and showed spreading towards the hypopharynx. The H1, H4,5 and H6 tumors enclosed the whole pyriform recess and infiltrated part of the hypopharyngeal lateral wall. The sizes of the primary tumors are demonstrated in *Table 2*, the first size showing largest vertical diameters, the second showing horizontal diameters. Tumors were treated primarily with surgery, followed by local irradiation.

Immunohistochemistry

Paraffin sections of surgically removed tumor samples were mounted onto Superfrost slides, deparaffinized and antigen retrieval technique was applied by using microwave exposure as described.[15] Endogenous peroxidase activity was inhibited by H_2O_2 preincubation while non-specific binding sites were blocked by 3% BSA/PBS (30 min). CD44v6 and CD44v3 were detected by using mouse monoclonal anti-CD44v6 and v3 antibodies (R&D, UK) diluted 1:100. NM23H1 protein was detected with mouse monoclonal anti-NM23H1 antibody (Novocastra) diluted 1:100. MMP2 metalloproteinase protein was detected by a rabbit anti-MMP2-peptide antibody (produced as described[16]) diluted 1:50. Sections were incubated with the primary antibodies for 60 min at 37°C. After washings with 3% BSA/PBS the bound antibodies were detected by biotin conjugated anti-mouse or anti-rabbit

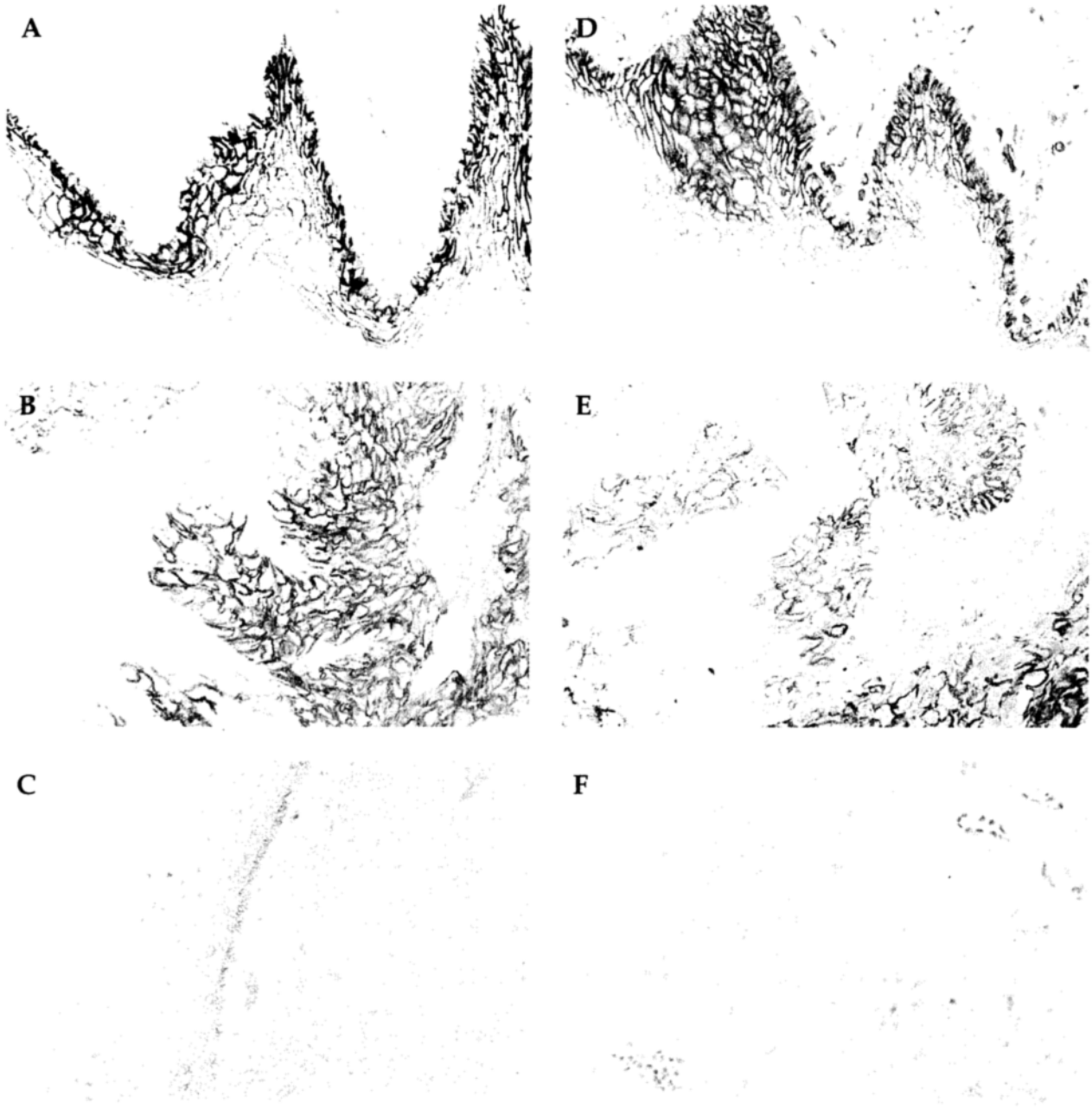

Figure 4. *Expression of CD44 variants in laryngeal and hypopharynx tumors. (a) CD44v6 in normal squamous laryngeal epithelium. Note the intense intercellular reaction in the basal-suprabasal cell layer. (b) Case G6. Note the intense intercellular reaction for CD44v6 in tumor cell nests. (c) Case H1. The tumor tissue is negative for CD44v6 unlike the associated covering epithelium. (d) CD44v3 in normal squamous laryngeal epithelium. (e) Case G8. Note the heterogenous intercellular reaction for CD44v3 in tumor cell nests. (f) Case H1. The tumor tissue is negative for CD44v3. x500*

IgG (Dako) diluted 1:200 in PBS/BSA and Streptavidin-peroxidase (1:100 dilution, Dako). Peroxidase enzyme activity was detected by AEC/H_2O_2 and sections were stained with haemalaun or methyl green. Immunologic reactions were quantitated by determining the percent of positive tumor cells. Three areas were scored by counting 100 tumor cells in each field/ tumor.

Results

Twelve cases of laryngeal and hypopharyngeal tumors were used in our study *(Table 1)*. In all cases the histological diagnosis was squamous cell carcinoma. The tumors were localised to the larynx (7 cases) and the hypopharynx (5 cases). Staging was determined according to the UICC

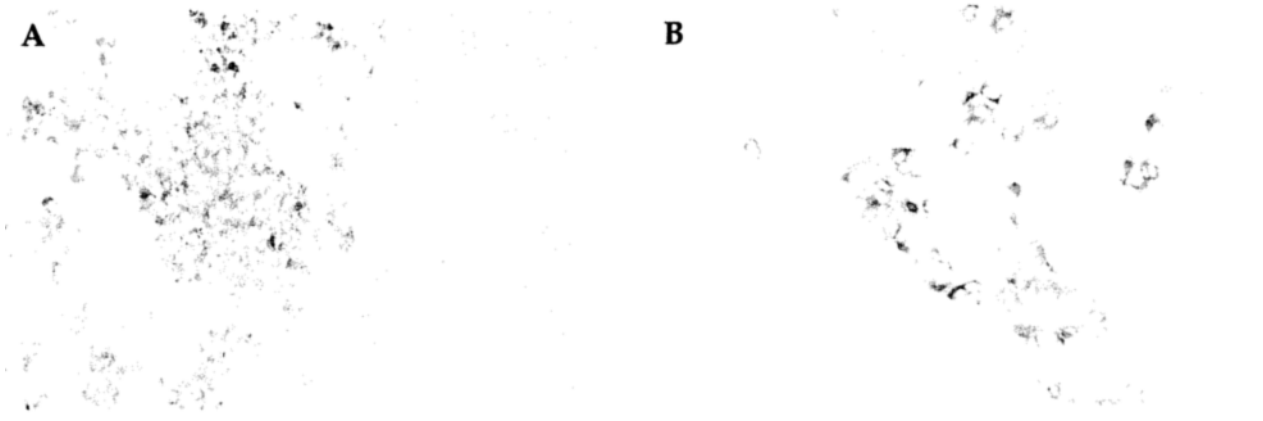

Figure 5. Detection of NM23H1 in laryngeal tumors. (a) Case G8. Note the negative reaction of tumor cell nests and the intense cytoplasmic labelling in the tumor infiltrating lymphoid cells. x500. (b) Case G6. Intense reaction for NM23H1 in tumor cells in the invasive papilla. x500

protocol.[22] There was a strong male predominance among the patients. The majority of the laryngeal tumors was diagnosed with T2 status(4/7) while the rest with T3 and one case with T4. Fatal outcome only occurred in one T4 case. It was shocking to realise however, that hypopharynx tumors were diagnosed with T4 status except one patient *(Table 1)* and from the 5 hypopharynx cases only one survived at the end of the study period.

The epithelium of the larynx was positive for CD44v6 antibody mainly in the basal and spinocellular layers and was shown in the intercellular junctions *(Figure 4a)*. The intensity of the immunoreaction for CD44v6 was variable in carcinomas; in some tumors it is persisted in the junctional areas *(Figure 4b)* but in others it was negative *(Figure 4c)*.

The normal squamous epithelium of the larynx exhibited intense labelling for CD44v3 which was localised to intercellular junctions of the basal and spinocellular cell layers. The labelling became weak or negative with the keratinization of the cells *(Figure 4d)*. Both NM23H1 and MMP2 immunoreactions were negative in the normal epithelium (data not shown).

CD44v3 expression was heterogenous in tumor cell nests, mainly confined to the baso-spinocellular areas where the stratified centers were mostly negative. The percent of positive cells as well as the intensity of the reaction were found to be heterogenous *(Figure 4e* and *f)* in the individual tumors.

Tumors could be divided into two categories in respect to the NM23H1 expression; negative *(Figure 5a)* and positive *(Figure 5b)*. The NM23H1 protein was localised in the cytoplasm of epithelial cells where inflammatory lymphocytes served as inner positive controls *(Figure 5a)*. Among the positive tumors low and high expressors could be found, where >50% positivity characterised the latter.

The MMP2 protein was rarely detected in the tumor cells of tissues studied, and it was localised to the cytoplasm mostly of the keratinizing cells in the squamous cell nests *(Figure 6)*. On the other hand, stromal cells (fibroblasts and histiocytes) regularly exhibited intense reaction in the tumors (data not shown).

Seven out of 15 tumor tissue samples exhibited a phenotype similar to the normal epithelium

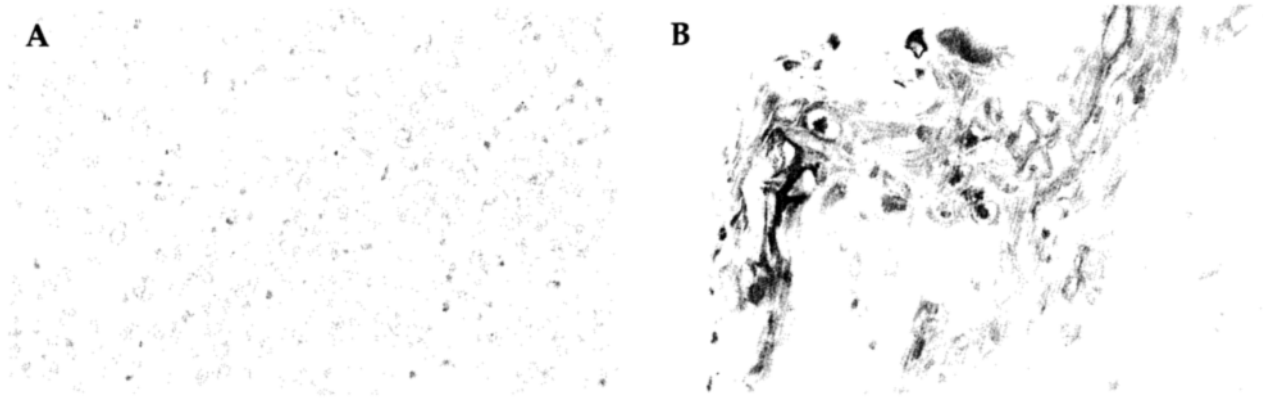

Figure 6. Detection of MMP2 in laryngeal and hypopharynx tumors. (a) Case G8. Note the negative cytoplasmic reaction of tumor cells for MMP2. x500. (b) Case H1. Note the intense cytoplasmic reaction of tumor cells for MMP2. x500

Table 3. **Expression of invasion markers in laryngeal and hypopharyngeal cancers**

Stage *(normal epithel*	*CD44v3* *77*	*NM23* *–*	*MMP2* *–)*
T2N0M0	46	–	–
T2N0M0	28	–	–
T2N0M0	79	48	+
T2N0M0	32	–	–
	3/4 decr	**1/4 incr**	**1/4 incr**
T3N0M0	24	–	–
T3N0M0	97	38	–
T3N0M0	38	–	–
T3N2M0	–	93	+
T3N2M0	62	–	–
	3/5 decr	**2/5 incr**	**1/5 incr**
T4N0M0	46	19	–
T4N0M0	55	65	+
T4N0M0	41	39	–
T4N0M0	89	58	+
T4N2M0	68	–	+
T4N3M1	41	50	+
	3/6 decr	**5/6 incr**	**4/6 incr**

numerical = % of positive tumor cells; incr = increased; decr = decreased (>25%); + = occurrence of positive cells (MMP2)

(CD44v3$^+$/NM23H1 /MMP2) *(Table 3)* where only one out of the seven specimens exhibited focal MMP2 positivity. In nine out of 15 samples CD44v3 expression was lowered compared to the normal epithelium whereas in 8 out of 15 samples the NM23H1 protein appeared in the tumors. Only a minority of tumor samples (6 out of 15) contained scattered MMP2 positive tumor cells.

When the tumors were grouped into TNM categories *(Table 3)* the phenotype of the T2 and T3 tumors was very similar, characterised by decreased, but significant CD44v3 expression with only occasional MMP2 positive tumors. In the T4 stage the frequency of the decreased CD44v3 expressing tumors was similar to other stages, but both the NM23H1 and MMP2 positive tumors were more prevalent (5/6 and 4/6 respectively). There was no correlation between the node positive cases and the expression of a certain marker phenotype.

When the tumors were grouped into categories according to their anatomical location irrespective of their stage *(Table 4)* the pattern of marker expression in the individual group became more characteristic. Laryngeal tumors of T2 stage were characterised by decreased CD44v3 expression and only occasional NM23H1 and MMP2 expression.

Among the laryngeal tumors of T3 stage, beside decreased CD44v3, the NM23H1 positive tumors were more frequent. In the hypopharyngeal tumors decrease in CD44v3 expression was less prevalent but apart from the high frequency of NM23H1 expression, MMP2 positivity was the hallmark.

Discussion

This is the first report on the universal expression of the CD44v3 splice variant in laryngeal cancers which parallels the expression of the v6 one. It is interesting from this point of view, that the normal laryngeal epithelium strongly expresses the CD44v3 variant in the basal/suprabasal cell layer – again similarly to the v6. The expression of the v3 splice variant in tumors is heterogenous and does not seem to correlate with tumor type, stage or lymph node status.

Table 4. **Expression of invasion markers in laryngeal and hypopharyngeal cancers**

Localisation *(normal epithel*	*volume (cm^3)*	*CD44v3* *77*	*NM23* *–*	*MMP2* *–)*
G1–T2	6.5	46	–	–
G2–T2	3.9	28	–	–
G4–T2	3.9	79	48	+
G5–T2	3.7	32	–	–
T2	4.5±0.7	3/4 decr	1/4 incr	1/4 incr
G6–T3		97	38	–
G7–T3	12.6	24	–	–
G3–T3		38	–	–
G8–T3	17.2	62	–	–
G9–T4	26.3	46	19	–
T3/4	18.7±4.0	3/5 decr	2/5 incr	0/5 incr
total laryngeal		**6/9 decr**	**3/9 incr**	**1/9 incr**
H1–T3	0.70	–	93	+
H2–T4	1.50	68	–	+
H3–T4	3.34	41	39	–
H4–T4		89	58	+
H5–T4	1.36	55	65	+
H6–T4	0.79	41	50	+
total hypopharyngeal	1.54±0.48	**3/6 decr**	**5/6 incr**	**5/6 incr**

G = glottis; H = hypopharynx; numerical = % of positive tumor cells; incr = increased; decr = decreased (>25%); + = occurrence of positive cells (MMP2). Tumor volume data are expressed as means ± S.E.M. and were calculated by the formula of $(a^2 x b x \pi)/6$
where **a** and **b** are the measured diameters (a<b) *(see Table 2).*

Our study further supports those previous observations[8,11] that head and neck cancers, especially laryngeal cancer, acquire a unique phenotype during tumor progression characterised by the downregulation of CD44v6 and v3 splice variants and the upregulation of NM23H1 genes. By definition, the invasive potential in various other tumor types (colon) is correlated to the upregulation of CD44v6[17] metastasis promoting genes and the downregulation of NM23H1 metastasis suppressor genes.[7] This indicates that the same molecules identified as metastasis factors in one tumor type may have opposite functions in other tumor types. Since the normal epithelium of the larynx is characterised by CD44v3/v6 expression and negativity for NM23H1, this "normal" phenotype will change during carcinogenesis and tumor progression. However, among the 12 cases, 5 tumors preserved the original phenotype of $CD44^+/NM23H1^-$ – though these tumors were occasional in the T4 group. Loss of this phenotype does not correlate with the nodal status of these tumors.

Our study indicated that in laryngeal cancer – similar to other types of head and neck cancer[8] – the advancing T status parallels the upregulation of NM23H1 expression and we have provided new data that hypopharyngeal tumors have similar phenotype in this respect to the laryngeal tumors.

Comparison of laryngeal and hypopharyngeal carcinomas indicated that beside the similar metastasis marker phenotype (CD44/NM23H1) there is a difference in the expression of a matrix metalloproteinase MMP2, namely the hypopharyngeal tumors are characterised by the expression of this enzyme. Since T4 tumors were more frequent in this limited study among hypopharyngeal tumors, we can not exclude the possibility that the appearance of MMP2 expression reflects the higher T status. However, the MMP2 expression could well be the hallmark of hypopharyngeal cancer phenotype since the size of these tumors are much smaller at T4 stage than laryngeal ones at a lower stage. This suggests that MMP2 expression in hypopharyngeal cancer is acquisited at an earlier stage of tumor progression than in laryngeal cancer. In other anatomical locations, squamous cell carcinoma cells exhibited increased MMP2 or 9 expressions with the more advanced stage of the disease.[18] As the clinical course of hypopharyngeal tumors is much less favourable than that of laryngeal tumors it is suggested that MMP2 expression could well be one of those invasive phenotype characteristics which may explain this difference, since the histological type or even other phenotypic characteristics – including CD44v6/v3 and NM23H1 expressions – are highly similar.

In case of head and neck cancers including laryngeal ones several biomarkers have been suggested, the expression of which either correlates to the transformed phenotype or to tumor progression. Among them three categories can be identified, expression of genes which regulate cell proliferation or differentiation and those which might have function in invasion and metastasis.[19]

The proliferation rate of laryngeal cancer has been shown to correlate to tumor progression detected by Ki-67,[9] MIB1[20] and PCNA labelling.[9] It is also important to note that the expression of EGFR in laryngeal cancer is considered to be associated with the progression of this tumor type.[10] On the contrary, data on the role of tumor suppressors as p53 and mdm2 are controversial[9,20] in the determination of laryngeal tumor progression.

On the other hand, it is accepted that tumor progression is regulated by several genes which are not related to cell proliferation, rather than to the metastatic process. Laryngeal cancer as shown in this report provides an example that biological dogmas do not exist. Genes which have been shown to be metastasis promoters (CD44v6,v3) or metastasis inhibitors (NM23) have opposite roles in laryngeal and hyopopharyngeal cancers. The expression of metastasis genes, CD44v6 and v3, is downregulated while the expression of NM23 metastasis suppressor is upregulated in this tumor type with progression to a more advanced disease (with a higher T status) though the expression is not associated to lymph node metastasis. It is tempting to speculate that the expression of CD44(v6/v3) may be conversely linked to the expression of NM23 in normal cells as seen in the squamous epithelium. This balanced expression may be changed during tumor progression where down- or upregulation of one of the gene may occur independently. However, the imbalance between the expression of the metastasis-related genes CD44 and NM23 may be insufficient for tumor invasion in case of laryngeal cancer. The acquisition of matrix degrading potential seems to be equally important in this tumor type. We have provided evidence that induction of the expression of MMP2 type IV collagenase in hypopharyngeal cancer leads to a much more aggressive phenotype, when the CD44/NM23 imbalance is very similar. Previous reports have indicated that in other types of head and neck cancers (all squamous cell carcinomas) the expression of MMP2 collagenase[21] as well as the expression of some cathepsins[12] are closely related to a more aggressive biological behaviour. These enzymes are capable of degrading the basement membranes around the epithelial tumor nests, the vascular subendothelial matrices as well as the interstitial matrix, thereby providing a powerful universal weapon for tumor cells ready to migrate.

Collectively, these data shed some light on the differences between the biological behaviour of laryngeal and hypopharyngeal cancers. Though these tumors have a similar phenotype they are characterised by different MMP2 expression which might explain the more aggressive behaviour of hypopharyngeal cancers. The data also indicate that in certain tumors the proliferative characteristics

are not necessarily related to the invasive/metastatic phenotype, since hypopharyngeal tumors are characterised by an early invasion at a much smaller primary size than laryngeal cancers. All these information suggest that a different therapeutic strategy may be required in the management of hypopharyngeal cancers where matrix degrading enzymes are considered as primary targets.

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