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Glycoprotein CD44 expression in benign, premalignant and malignant epithelial lesions of the larynx: An immunohistochemical study including correlation with Rb, p53, Ki-67 and PCNA

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Summary. CD44 is an integral membrane glycoprotein that has diverse functions in cell-cell and cell-substrate interactions. It has been suggested that it may be a determinant of metastatic and invasive behavior in carcinomas. The immunohistochemical expression of CD44 was examined in a series of 34 squamous cell carcinomas, 13 in situ carcinomas, 35 cases with various degrees of epithelial dysplasia, 10 papillomas and 17 cases of keratosis. We used the monoclonal mouse anti-human phagocytic glycoprotein-1 CD44 (clone DF 1485), on formalin-fixed, paraffin-embedded tissue. CD44 expression was correlated with the expression of Rb and p53 proteins, with the proliferative indices Ki-67 and PCNA as well as with conventional clinico-pathological data. The mean value of CD44 expression was 78.84 in squamous cell carcinomas, 78.04 in in situ carcinomas, 54.93 in dysplasia, 26.8 in papillomas and 24.97 in keratosis. There was no significant difference of CD44 expression between in situ and invasive carcinomas. However, a strong difference of reaction between carcinomas and the other cases was observed. CD44 expression was statistically higher in dysplastic lesions than the cases of keratosis ($p<0.0001$) and papillomas ($p=0.01$). In the group of invasive carcinomas, CD44 expression was statistically correlated with pRb ($p=0.011$), while in preinvasive lesions it was correlated with PCNA ($p=0.016$). The relationship with the degree of dysplasia or grade of carcinoma and p53 protein expression was insignificant.

These observations suggest that CD44 expression may be involved in the multiple mechanism of the development and progression of laryngeal lesions and may help to predict the risk of transformation of the benign or precancerous lesions to cancer.

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Introduction

CD44 is a multifunctional integral cell membrane glycoprotein, expressed in epithelial, mesenchymal and hematopoietic cells (Jackson et al., 1994) and implicated in such diverse biological processes, such as lymphocyte recirculation and activation, and cell-cell and cell-matrix interactions (Bartolazzi et al., 1994). It is a widely-distributed receptor type protein which exists as a standard form (CD44H) and as various isoforms generated by alternative splicing of the products of at least 10 variant exons (Fox et al., 1994). Overexpression of whole CD44 or some splice variants has been found in various types of human tumours and is supposed to be implicated in tumour progression (Wielenga et al., 1993; Fox et al., 1994). Elevated CD44 expression has also been observed in metastatic deposits (Matsumura and Tarin, 1992) and data from animal experiments have suggested that overexpression of isoforms containing exon 11 (v6) could be involved in the metastatic process (Gunthert et al., 1991; Fox et al., 1994).

CD44 has been associated with proliferation, suggesting that it has a role in tumour cell growth (Alho and Underhill, 1989; Abbasi et al., 1993) and has been recently associated with expression of mutant p53 protein in colorectal tumourigenesis (Mulder et al., 1995). To our knowledge, there are a few studies of CD44 expression in laryngeal lesions, and those there are have controversial results (Spafford et al., 1996; Ostwald et al., 1997; Sugar et al., 1997). Therefore, the role of CD44 is currently unknown.

In the present study, we evaluated the immunohistochemical expression of the CD44 molecule in a series of hyperplastic, premalignant and malignant lesions of the

larynx in order to determine its role during larynx-tumour development and to assess its relationship to other markers, such as Rb and p53 proteins as well as the proliferative indices Ki-67 and PCNA.

Materials and methods

Formalin-fixed and paraffin-embedded tissues from 34 cases of squamous cell carcinomas, 13 in situ carcinomas, 35 cases of dysplasia (as the only lesion or adjacent to the carcinomas), 10 papillomas and 17 cases of keratosis (material from our Pathology Laboratory) were examined. Paraffin sections adjacent and far from the tumour were used as controls. Histological slides from squamous cell carcinomas were reviewed and classified according to the standard criteria into well-moderately and poorly-differentiated carcinomas (grade I, II, III) with or without keratinization (Ferlito and Friedmann, 1993). Keratosis of the larynx is nearly synonymous with the process known as epithelial or squamous cell hyperplasia. Microscopically, keratotic lesions are characterized by hyperkeratotic epithelium (often with a granular layer) and acanthosis. Dysplasia refers to a microscopic change present in some cases of keratosis or papilloma (and sometimes independently of them), that is characterized by cellular atypia, loss of normal maturation and loss of stratification. According to the WHO Collaborating Center for the Histological classification of Upper Respiratory Tract Tumours, dysplasia was graded as mild, moderate or severe on the basis of the degree of nuclear abnormalities (including changes in polarity) and the level of the epithelium showing loss of stratification (Shanmugaratnam and Sobin, 1991). Carcinoma in situ, like dysplasia, may be present as the only lesion or at the peripheral margins of an invasive carcinoma. The standard microscopic criteria for diagnosis are essentially the same as for its more common counterpart in the uterine cervix; that is, the presence of atypical changes throughout the epithelium without evidence of surface maturation (Shanmugaratnam and Sobin, 1991). However, some authors accept the diagnosis of in situ laryngeal carcinoma in the presence of surface maturation, in the form of keratinization, if nuclear atypia is prominent enough (Crissman et al., 1987).

Immediately after excision, specimens were fixed in 10 per cent neutral formalin, embedded in paraffin, cut at 4 μm and stained with haematoxylin and eosin for routine light microscopy.

For immunohistochemical staining, additional 4 μm thick sections were cut from paraffin blocks. After blockage of endogenous peroxidase with H_2O_2 in methanol for 30 minutes, sections were immersed in citrate buffer (pH 6.0) in a microwave-resistant container. The antibody sources and dilutions are shown in Table 1. Immunoperoxidase detection was employed using the ABC method (Dako) and diaminobenzidine substrate. Counter-staining was performed with haematoxylin.

Appropriate positive and negative controls were used.

Immunohistochemical evaluation

The evaluation of immunostaining (membrane cytoplasmic for CD44 or nuclear for Rb, p53, Ki-67 and PCNA) was calculated as the percentage of positive epithelial cells in relation to the total number of cells encountered in 20 representative high power fields. Every stained nucleus was considered positive, irrespective of intensity. Each sample was first scanned with a low magnification and at least 5 to 10 fields were assessed with a high power magnification. All slides were reviewed and scored in a blind test by two pathologists.

Statistical analysis

The association of continuous variables was confirmed using nonparametric test for two or several independent samples or Spearman bivariable correlation. P-values smaller than 0.05 were considered statistically significant.

Results

CD44 immunostaining was detected on the cell surface (Fig. 1). In the «normal» appearing adjacent epithelium the cell surface CD44 immunoreactivity was confined to the basal part and was expressed in less than 10% of cells in most cases. CD44 expression was detected in some lymphocytes and macrophages which were used as internal controls (Fig. 2). Nerves in the lamina propria also showed a strong staining of the myelin sheath (Schwann cells). For Rb, p53, Ki-67 and PCNA, labeling of a variable proportion of epithelial cell nuclei was observed in each case. In benign and premalignant lesions the immunostaining was usually

Table 1. Antibodies used.

ANTIBODIES	SUPPLIER	DILUTION	INCUBATION TIME
CD44 (Clone DF 1485)	Dako	1:40	overnight*
DO-7 (IgG2b)	Ylem	1: 200	overnight*
Anti-Rb (AB-5)	Oncogene	1: 80	overnight*
Ki-67	Dako	1: 10	30 min
PC-10	Dako	1: 50	One hour

*: with microwave oven antigen retrieval

Table 2. CD44 expression in benign, premalignant and malignant epithelial lesions of the larynx.

	Ca	IN SITU	DYSPLASIA	PAPILLOMA	KERATOSIS
<10	5		11	6	14
>10	28	13	24	4	3

detected at the basal portion of the laryngeal epithelium.

The mean value of CD44 expression was 78.84 in squamous cell carcinomas, 78.04 in in situ carcinomas, 54.93 in epithelial dysplasias, 26.8 in papillomas and 24.97 in keratosis. CD44 protein expression was detected in 81.8% (>10% of positive neoplastic cells) of the squamous cell carcinomas, Rb protein in 69.7% (>5% of positive neoplastic cells) and p53 in 40% (>5% of positive neoplastic cells). CD44 expression was gradually increased from keratosis, papillomas and dysplasias through carcinomas (Table 2). No statistically significant correlation of CD44 expression between in situ and invasive carcinomas was found. However, a strong difference of reaction between carcinomas and the other studied groups was observed. In the cases of epithelial dysplasia, CD44 levels were quite higher than in cases of keratosis ($p<0.0001$) or papillomas ($p=0.01$). We did not find any statistically significant difference of CD44 expression between papillomas and keratosis. In the group of invasive carcinomas, CD44 expression was statistically correlated with pRb ($p=0.011$), while in preinvasive in situ lesions it was correlated with PCNA ($p=0.016$) (Table 3). Both pRb and PCNA as well as Ki-67 expression increased statistically on progression to hyperplastic to premalignant and malignant lesions. The

Table 3. CD44 glycoprotein expression in correlation with RB, p53, Ki-67 and PCNA in squamous cell carcinomas.

	CD44 EXPRESSION		
	<10%	>10%	p value
Rb			
<5	3	7	p=0.011
>5	3	20	
p53			
<5	3	18	NS
>5	3	8	
Ki-67			
<5	3	8	NS
>5	3	18	
PCNA			
<50	3	8	NS
>50	3	19	

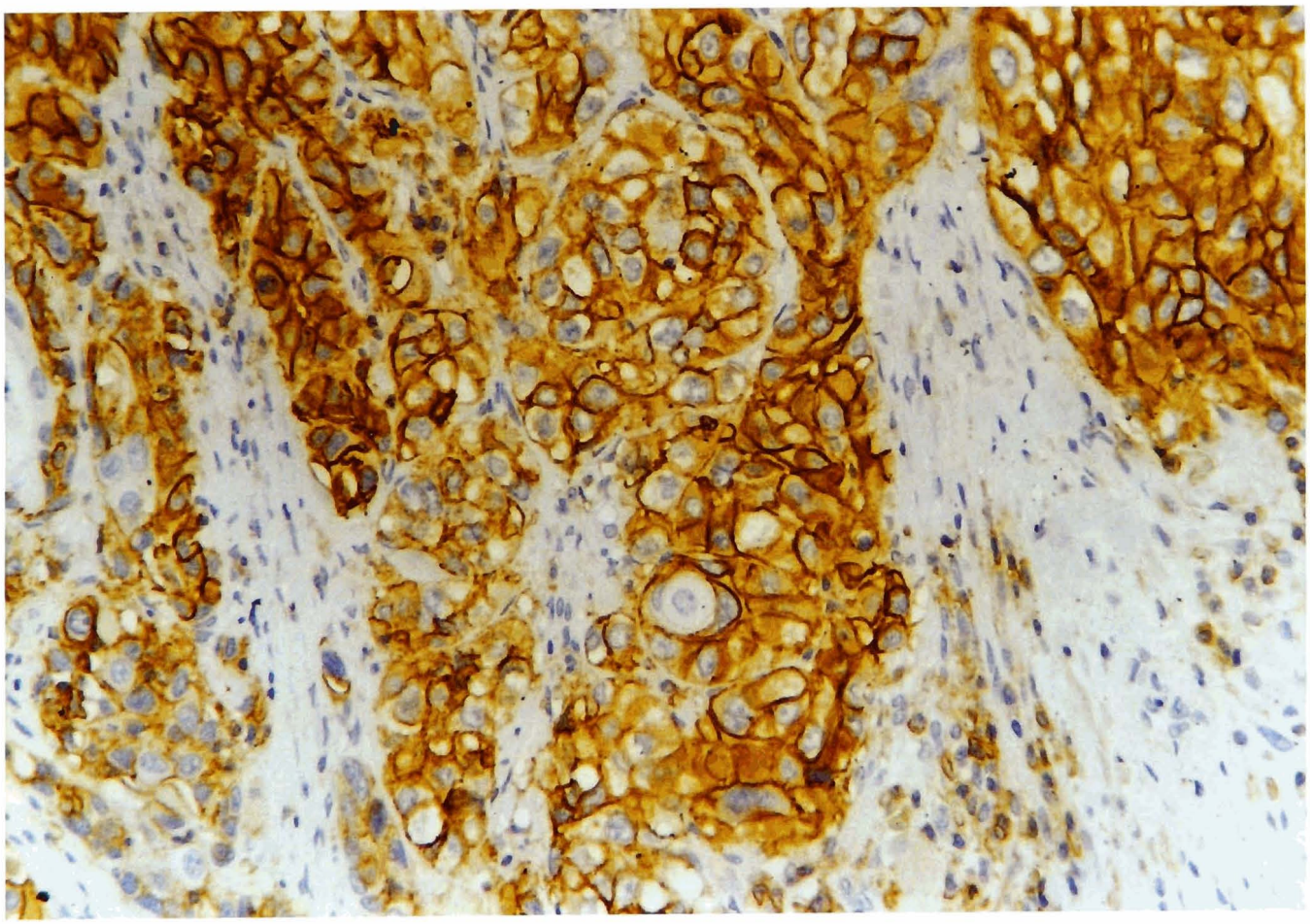

Fig. 1. Intense cell membrane immunoreactivity for CD44 in the majority of tumour cells in a case of moderate differentiated squamous cell carcinoma. ABC, x 200

relationship of CD44 immunoreactivity with the degree of dysplasia, the grade of carcinoma and p53 protein expression was insignificant.

Discussion

Knowledge of molecular mechanisms in cell-cell and cell-matrix adhesion has increased dramatically during the past decade. Adhesive and dishesive forces between tumour cells and neighbouring elements, such as matrix and endothelium, determine invasion and metastasis. Since adhesion molecules actively participate in the arrest and transmigration of inflammatory and tumour cells, they constitute a possible target for therapeutic intervention.

The traditional prognostic factors, including stage of disease and tumor grade, have shown a limited prognostic significance and an inability to predict clinical response to specific treatment in patients with laryngeal squamous-cell carcinoma. Recent basic discoveries about the biological significance of nuclear and cell-surface marker proteins have opened new areas of research into head and neck cancer. However, the clinical significance of these markers is not yet clearly understood.

CD44 is a cell membrane glycoprotein, the function of which has been hypothesized to be related to its action as a cell hyaluronate receptor (Hallen et al., 1996). Overexpression of CD44 has been found in various types of human tumours and is supposed to be implicated in tumour progression (Wielenga et al., 1993; Fox et al., 1994).

In laryngeal squamous cell carcinomas the role of CD44 expression is not clearly known and is relatively controversial. It has been reported that loss of cell adhesion implied by decreased expression of CD44, correlates with an increase in metastasis and a shorter patient survival (Spafford et al., 1996). There are also studies in which CD44 expression does not appear to be a strong connection to the metastatic behavior (Ostwald et al., 1997).

In this work, we studied the immunohistochemical

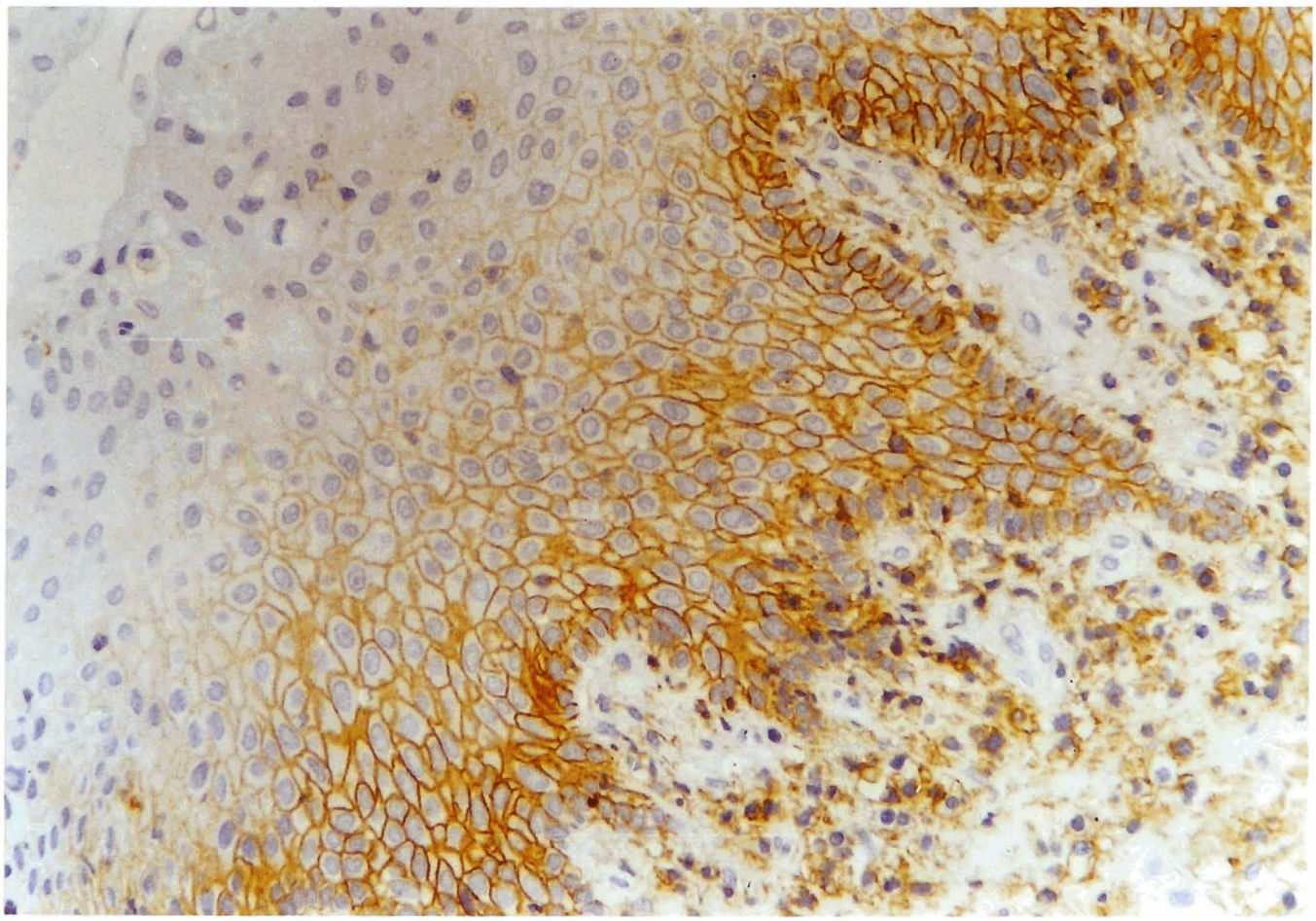

Fig. 2. A case of moderate dysplasia exhibiting CD44 protein expression at the lower part of the epithelium. Normal lymphocytes are also positive in the substrate. ABC, x 100

expression of CD44 in a series of hyperplastic, premalignant and malignant lesions of the larynx, in an attempt to elucidate the role of this molecule in the development and progression of laryngeal carcinogenesis.

CD44 was recognized in all lesions and its expression was gradually statistically increased from keratosis, papillomas and dysplastic lesions to in situ or invasive carcinomas. A strong statistical difference of CD44 expression between carcinomas (in situ and invasive) and the other studied groups was observed. In addition, in dysplastic lesions, CD44 expression was higher than in hyperplastic lesions without dysplasia. The observation of this progressive expression suggests that CD44 may be involved at the developmental stage of laryngeal carcinogenesis. However, CD44 expression was not correlated with the histological grade of both benign and malignant epithelial lesions.

Rb is a tumour suppressor gene and loss of its functioning protein suggests that it plays a growth regulatory role in many, if not all, cell types. In addition, this loss may contribute to the formation of many types of tumours. To our knowledge, the role of Rb in laryngeal lesions has not been studied extensively and in the already existing reports there is controversial information. It has been shown that inactivation of Rb protein is involved in the carcinogenesis of head and neck lesions at all levels of the process of protein expression: DNA, mRNA and protein (Yokoyama et al., 1996). On the other hand, there are findings which suggest that the expression of Rb protein than the loss of its expression correlates with the presence of metastasis in patients with laryngeal carcinoma (Takes et al., 1997). In the current study, we found a high expression of Rb protein in malignant lesions (in situ and invasive carcinomas), a lower one in lesions with various degrees of dysplasia and enough lower in the other examined lesions without dysplasia the difference being statistically significant. This unexpected finding probably mean that Rb protein expression may be related to the progression of the stage of laryngeal lesions. Furthermore, we observed a statistically positive correlation between Rb and CD44 expression in the series of invasive carcinomas. These results indicate that the simultaneous positivity of these two markers may provide preliminary information about the selection advantage of the tumour cells.

It is known that p53 gene is a tumour suppressor gene negatively regulating the cell cycle and that mutation of this gene is the most common genetic alteration in human tumours (Levine et al., 1994; Soussi et al., 1994). With regard to laryngeal squamous cell cancer, there are studies which report that abnormalities of p53 gene occur more frequently than in adenocarcinomas (Sakai et al., 1992). In addition, an increased expression of p53 and decreased patient survival have been reported in the late stages of squamous cell carcinomas (Spafford et al., 1996). Other investigations indicated that positive p53 immunostaining could be demonstrated early in the development of carcinomas. In fact, p53 expression has been reported to be present in normal mucosa and in dysplastic mucosa adjacent to the tumours (Dolcetti et al., 1992), as well as in premalignant lesions of the larynx (Shin et al., 1994). In our study we found that p53 was expressed in all studied cases suggesting that changes in the p53 gene and its protein product may play a role from the early phase of laryngeal tumourogenesis. While an inverse relationship has been reported between p53 and CD44 expression (Spafford et al., 1996), we did not find any correlation between these markers.

Both PCNA and Ki-67 antigens are nuclear proteins that function in the active phases of the cell cycle. Their expression is considered a measure of tumour proliferative activity. High PCNA and Ki-67 indices have correlated with aggressive behavior in tumours of various sites (Hall et al.,1990; Kearsley et al., 1990). In patients with laryngeal squamous cell carcinomas there are data which indicate that cell proliferative indices may be considered as reliable and reproducible indicators of biological aggressiveness (Munck-Wikland et al., 1994). In addition, studies analyzing the proliferative activity of epithelial cells in benign epithelial hyperplastic lesions conclude that the proliferative fraction progressively increases with the degree of epithelial hyperplasia, suggesting that the degree of proliferative dysregulation might be used as a prognostic marker for revealing the highest risk of progress to overt carcinoma (Coltera et al., 1992; Zidar et al., 1996). In our study, we found a strong statistically significant difference for Ki-67 and PCNA between malignant, premalignant and hyperplastic lesions, reaching the highest value in the in situ and invasive carcinomas. In addition, we observed a statistically significant correlation between CD44 and PCNA only in the cases of in situ carcinomas. We suggest that the enhanced level of CD44 in laryngeal lesions is asynchronous with cell proliferation and does not directly link with it.

In conclusion, CD44 expression may be involved in the complex mechanism of the development and progression of laryngeal lesions. With the application of this observation in the clinical practice, we might obtain preliminary information and predict the biological behavior of these lesions. Determination of both CD44 and Rb expressions may prove useful in assessing which patients, especially with premalignant lesions, are at greatest risk of progressing to cancer and who would therefore benefit from intensive surveillance.

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