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Expression of the extracellular matrix protein tenascin in laryngeal epithelial lesions: correlation with fibronectin, CD44, cathepsin D and proliferation indices

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Abstract Tenascin (TN) is an extracellular matrix glycoprotein expressed in areas of epithelial–mesenchymal interactions during embryogenesis and in neoplasia. We studied the expression of TN in a series of 35 squamous cell invasive carcinomas of the larynx, 13 in situ carcinomas, 41 cases of dysplasia, 10 papillomas and 18 cases of keratosis using the monoclonal antibody TN2 on paraffin-embedded tissue. TN expression was correlated with the expression of fibronectin, CD44 and cathepsin D (CD) proteins, with the proliferation indices Ki-67 and proliferating cell nuclear antigen (PCNA) as well as with conventional clinicopathological variables. Malignant tumours showed a significantly greater stromal TN staining than benign lesions. In invasive carcinomas, the immunoreactivity was statistically higher than that in situ (P=0.01), dysplastic lesions (P<0.0001), papillomas (P=0.004) and keratosis (P<0.0001). A statistically significant difference of TN expression between in situ and dysplastic lesions was observed (P=0.001). In invasive lesions, TN expression was statistically correlated with CD44 expression (P=0.02) and a trend for correlation with CD of tumour cells and fibronectin expression was found (P=0.06 and P=0.09, respectively). The relationship of TN expression with the histological grade and the proliferative activity was insignificant. In conclusion, stromal TN expression may be involved in the complex mechanism of development of laryngeal lesions and may help to predict the risk of progression of pre-cancerous lesions to cancer.

Key words Tenascin · Fibronectin · CD44 · Cathepsin D · Laryngeal lesions

Introduction

The extracellular matrix (ECM) is a dynamic assemblage of interacting molecules that reorganise and regulate cell functions in response to exogenous and endogenous stimuli [31]. In particular, the ECM modulates cell attachment, proliferation, differentiation and invasion, and aberrant regulation of the adhesion of cells to the ECM is often associated with disease. The major constituents of the ECM are collagens, proteoglycans and glycoproteins. Notable among the latter is tenascin (TN).

TN is a large glycoprotein with a six-armed disulfide-bonded macromolecular structure, consisting of three isoforms of the molecule [5]. It has considerable structural homology with fibronectin, epidermal growth factor and fibrinogen [30]. Initial studies have suggested that TN expression was restricted during embryogenesis and oncogenesis [1, 4], but subsequently it was detected in various normal adult tissues as well as in reparative-hyperplastic process and in the stroma of a wide range of neoplasms of epithelial, mesenchymal and glial differentiation [18]. It is believed to have active functions in epithelial–mesenchymal interactions, and cell culture studies suggest that it has growth-promoting and anti-adhesive functions [35].

TN is produced by fibroblasts and/or myofibroblasts [8, 35] and also by epithelial cells of normal and malignant tissues [21, 37]. Strong TN expression has been reported in a variety of human malignant tumours, such as breast, lung, gastric, colon, cervical, ovarian and bladder carcinomas [12, 16, 24, 29, 34, 36, 38].

To our knowledge, there are at present very few studies concerning TN expression in human laryngeal lesions

After the acceptance of our manuscript, we found a paper by Yoshida et al. (1999) *Involvement of tenascin-C in proliferation and migration of laryngeal carcinoma cells* (Virch Arch 435: 496–500). This paper demonstrated quite similar results to ours, especially concerning the immunohistochemical distribution of tenascin in laryngeal lesions.

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[3, 23]. In squamous cell carcinomas of the larynx, TN expression has been detected in the tumour stroma and not in the histologically normal tissue adjacent to the tumour. In this study, we assessed the expression of TN in a series of hyperplastic, pre-malignant and cancerous lesions of the larynx and evaluated its relationship to clinicopathological parameters such as tumour grade and degree of dysplasia. TN expression was also correlated with the immunoreactivity of fibronectin, CD44 and cathepsin D (CD) as well as with the proliferation associated indices Ki-67 and proliferating cell nuclear antigen (PCNA).

Materials and methods

Formalin-fixed and paraffin-embedded tissue from a total of 117 laryngeal lesions [35 squamous cell invasive carcinomas, 13 in situ carcinomas, 41 cases of dysplasia (as the only lesion or adjacent to the carcinomas), 10 papillomas and 18 cases of keratosis] were examined. Histological slides from squamous cell carcinomas were reviewed and classified according to the standard criteria into well, moderately and poorly differentiated carcinomas (grades I, II, III) with or without keratinization. Keratosis of the larynx is nearly synonymous with the process known as epithelial or squamous cell hyperplasia. Microscopically, keratotic lesions are characterised by hyperkeratotic epithelium (often with a granular layer) and acanthosis. Dysplasia refers to a microscopic change present in some cases of keratosis (and sometimes independently from it) that is characterised by cellular atypia, loss of normal maturation and loss of stratification. According to the World Health Organization (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 [28]. Carcinoma in situ, like dysplasia, may be present as the only lesion or at the peripheral margin 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 presence of atypical changes throughout the epithelium without evidence of surface maturation [28]. However, some authors accept the diagnosis of laryngeal carcinoma in situ in the presence of surface maturation in the form of keratinization if nuclear atypia is prominent enough.

Immunohistochemical analysis was performed on 4-µm tissue sections from formalin-fixed, paraffin-embedded tissue placed on poly-L-lysine-coated glass slides. In brief, sections were deparaffinised in xylene and dehydrated. For the detection of TN and CD, slides were pre-treated with 0.1% pronase (Dako) for 10 min at room temperature. Sections staining for CD44, fibronectin, Ki-67 and PCNA were immersed in citrate buffer (0.1 M, pH 6.0) in plastic Coplin jars and subjected to microwave irradiation twice for 6 min. The heat-mediated antigen-retrieval method was not used for PCNA staining. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. We used the method involving the avidin–biotin–peroxidase complex (ABC) and developed the chromogen with immersion of the slides in a diaminobenzidine-H_2O_2 substrate for 5 min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction, in all cases a negative (no primary antibody) control was used. The sources and dilutions of the antibodies used are shown in Table 1.

Table 1 Antibodies used

Antibodies	Supplier	Dilution	Incubation time (h)
TN2	Dako	1:25	1
CD44 (DF1485)	Dako	1:40	1*
CD (D13 A)	Dako	1:300	1*
Fibronectin	Dako	1:50	1*
Ki-67	Dako	1:10	1*
PC-10	Dako	1:50	1

* With microwave oven antigen retrieval

The immunoreactivity of TN was interpreted by means of light-microscopic examination and evaluated independently by two observers. The staining was evaluated only in the areas with well-preserved tissue morphology and away from necrosis or artefacts. Analysis of staining was mainly restricted to stromal cell reactions. The extent and intensity of TN expression was scored semi-quantitatively as –/+, ++ and +++, corresponding to negative/weak, moderate and strong immunoreactivity.

Only intense membrane cytoplasmic (CD44) or nuclear (Ki-67, PCNA) immunostaining was considered to represent the expression of these proteins. The immunoreactivity of CD in both epithelial and stromal cells was considered as positive when a brown, fine-to-coarse granular cytoplasmic staining was seen. The immunoreaction was calculated as the percentage of positive epithelial cells in relation to the total number of cells encountered in ten representative high-power fields (at least 1000 cells). The results were evaluated quantitatively and separated into several groups (Table 3). The staining for fibronectin and CD of stromal cells was evaluated semi-quantitatively graded from –/+ to +++ in each section.

Statistically significant differences were analysed using either a non-parametric test for two or several independent samples or a Spearman bivariable correlation. The level of significance was set at $P<0.05$.

Results

In benign lesions, a weak and focally distributed TN immunoreactivity band was noted at the epithelial–stromal interface. In cases of dysplasia and in in situ carcinomas, a more intense TN immunoreaction was observed at the epithelial–stromal interface with predominance at the latter (Fig. 1). In invasive carcinomas, TN expression was markedly increased compared with the other studied groups (Table 2). The entire extracellular space of the tissue surrounding the tumour cells was stained, whereas the malignant cells themselves were reactive in some cases. In 57.2% of the tumours examined, a strong intense and diffuse TN staining was noted around and within the tumour cell nests in a fine fibrillary pattern (Fig. 2). Of the cases, 34.3% showed a moderate staining and 8.5% a weak or negative immunoreactivity. Statistically, stromal TN staining of invasive lesions was higher than in in situ ($P=0.01$), dysplastic lesions ($P<0.0001$), papillomas ($P=0.004$) and keratosis ($P<0.0001$). A statis-

Table 2 Tenascin expression in laryngeal lesions

Tenascin	Invasive Ca	In situ	Dysplasia	Papilloma	Keratosis
–/+	3	5	34	7	14
++	12	5	6	2	4
+++	20	3	1	1	0

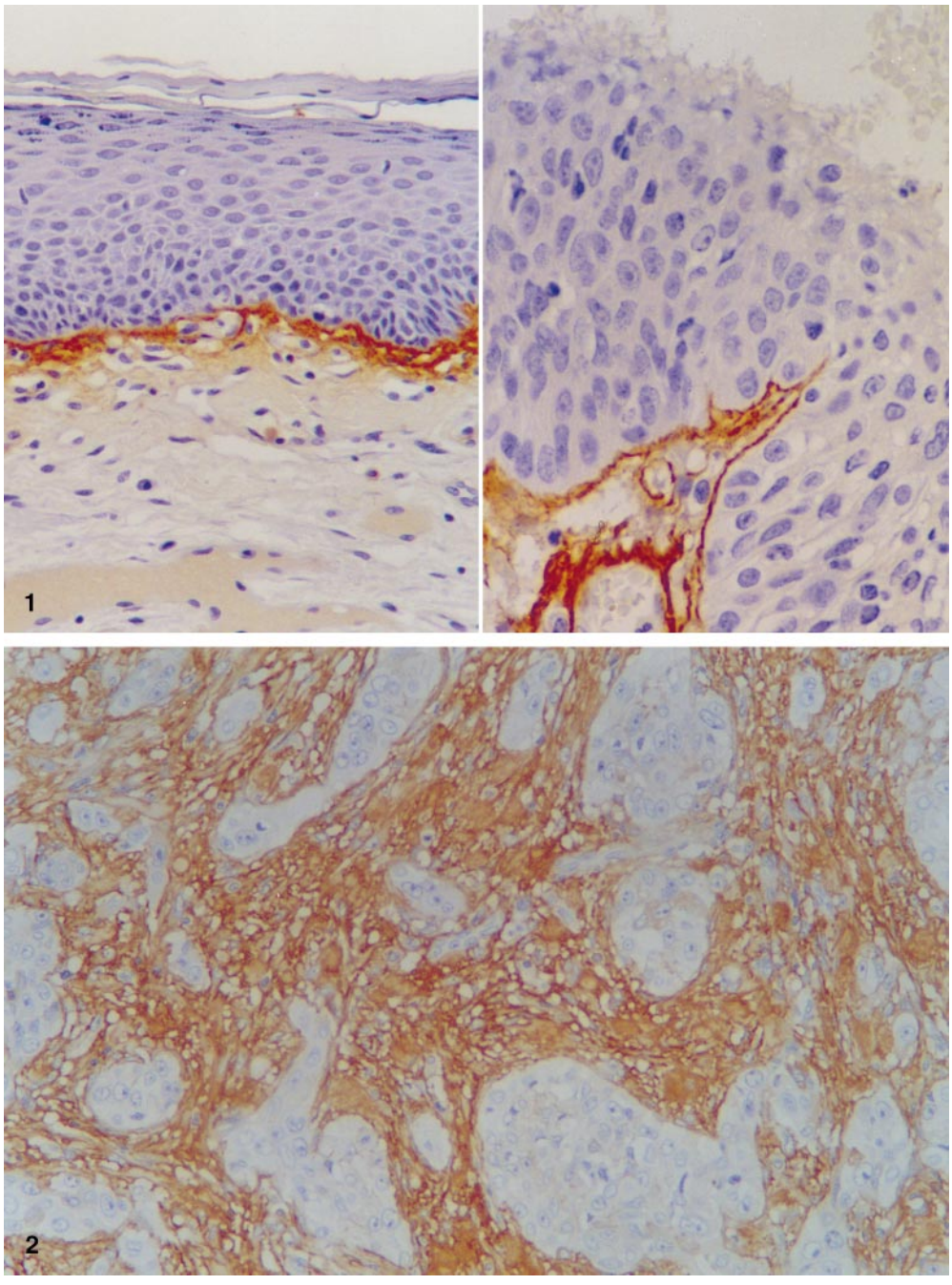

Fig. 1 Mildy dysplastic laryngeal epithelium (*left*). Tenascin (TN) immunoreactivity appears as a band at the epithelial–stromal interface (×200). In situ carcinoma (*right*) displaying more extensive TN staining underneath the epithelium. Note brightly stained vessels in the mesenchyme (×400)

Fig. 2 Invasive laryngeal carcinoma displaying extensive tenascin (TN) immunoreactivity in the extracellular space (×200)

tically significant difference of TN expression between in situ and dysplastic cases (P=0.001) was also detected. In addition, in infiltrating carcinomas, a statistically significant correlation between TN and CD44 expression was observed (P=0.02) (Table 3). Although a trend for correlation of TN with CD expression of cancer cells and fibronectin expression was noted, it was not statistically significant (P=0.06 and P=0.09, respectively). The relationship with the degree of dysplasia or grade of carcinoma as well as with stromal CD expression and the proliferation indices was insignificant.

Discussion

Interactions between mesenchymal and epithelial cells have been considered to have an important role in the normal embryogenesis of numerous tissues and organs [19]. These interactions are also significant for the maintenance of normal adult structure and function and for abnormal development and oncogenesis. It has been suggested that the process of tumour invasion and metastasis requires complex changes in the normal cell–cell and cell–matrix interactions [2], which, in turn, are reflected in variable up- and downregulation of significant mole-

Table 3 Tenascin expression in invasive laryngeal carcinomas. *NS* non-significant

	Tenascin expression			
	–/+	++	+++	*P* value
CD44				
<10	1	3		0.02
>10	2	7	15	
CD (epithelial)				
<50	2	3	2	0.06
>50	1	9	16	
CD (stromal)				
–/+	1	2	2	NS
++	2	6	6	
+++		4	10	
Fibronectin				
–/+	2	2	2	0.09
++	1	6	6	
+++		4	11	
Ki-67				
<5	2	3	5	NS
>5	1	9	13	
Proliferating cell nuclear antigen				
<50	2	4	8	NS
>50	1	8	11	

cules. One of these molecules is TN, the expression of which may suggest an altered cell matrix interaction that may facilitate epithelial tumour cell invasion during carcinogenesis and tumour progression [20].

It has been reported that TN is expressed in the tissue stroma of various human malignancies. To our knowledge, there are very few studies concerning TN expression in laryngeal cancer [3, 23]. In squamous cell carcinomas of the larynx, TN deposit has been found in the tumour stroma and in association with blood vessels within the tumour cell nodules [3]. In addition, TN has not been detected in the histologically normal tissue adjacent to tumour [3]. The present study demonstrates that TN is highly expressed in the desmoplastic fibrous stroma of invasive laryngeal carcinomas. Interestingly, in some cases, TN immunoreactivity was often more evident in the invasion front of tumour cells. The intensity of TN staining was not related to the degree of tumour differentiation. Given that, in the stroma of epithelial cells, fibroblasts or myofibroblasts may be responsible for most TN synthesis, it can be suggested that extensive TN staining accompanies abundant fibroblastic proliferation, but is not necessarily related to the degree of architecture distortion or to tumour grade. In other studied lesions, TN immunoreactivity was limited to the epithelial–stromal interface. A statistically significant difference of this reaction between invasive and non-invasive tumours as well as between invasive and benign and pre-malignant lesions was found. In addition, in in situ cancer cases, TN staining was more extensive and intense than in dysplastic lesions. In the latter, TN expression was not correlated with the degree of dysplasia.

The distribution of TN in other benign, pre-malignant and malignant epithelial lesions has been also reported. In colon adenomas, TN expression is increased in the basal lamina compared with the normal mucosa. Furthermore, in invasive colon adenocarcinomas, TN is detected in the basal lamina and also in the stroma [25]. Similar results have been reported in the urinary bladder [34], in salivary gland tumours [32] and in the endometrium [26]. However, in a recent study concerning TN expression in cervical lesions, it has been shown that TN was apparent in the stroma of cervical cancer but not in carcinomas in situ and in normal cervical mucosa [24]. Our findings are in agreement with most of the relevant studies and show that TN appears as a result of interactions between neoplastic epithelium and stroma during tumour development and may help to predict the risk of progression of benign or pre-cancerous lesions to cancer.

We also observed TN expression in epithelial tumour cells in some cases. In vitro studies have shown that exogenously added TN may influence the growth of breast cancer cell lines. In addition, a study of breast cancer tissues, using in situ hybridization, demonstrated that both cancer and stromal cells express TN mRNA, showing that TN is produced by cancer epithelial as well as by stromal mesenchymal cells [15]. It is thought, that TN in tumour tissue is synthesized by stromal fibroblasts which are induced by tumour cells [7] and, therefore, TN has been discussed as a consequence of a paracrine effect.

A significant association of TN with CD44 expression was observed in the group of invasive carcinomas. CD44 is a widely distributed receptor-type protein, overexpression of which has been found in various types of human tumours and is supposed to be implicated in tumour progression. In laryngeal squamous cell carcinomas, the role of CD44 expression 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's survival [33]. There are also studies in which CD44 expression does not appear to have a strong connection with the metastatic behaviour of the tumours [22]. In our previous study, concerning CD44 expression in laryngeal lesions, we found a gradually statistically significant increase of CD44 levels from hyperplastic to pre-malignant and malignant lesions [14]. The positive correlation of TN and CD44 expression probably means that both proteins may be involved in the complex mechanism of the development and progression of laryngeal cancer.

The concerted action of proteolytic enzymes, such as CD, that either promote or directly take part in ECM and basement membrane degradation and remodelling is considered of main importance in cancer invasion and metastatic spread. In our previous study, concerning CD expression in laryngeal lesions, a gradually statistically increased expression of the enzyme from keratosis, papillomas and dysplastic lesions to in situ or invasive carcinomas was observed [11]. It was interesting that in in-

vasive lesions, CD immunoreactivity was often more evident in the outer layer of tumour nests, suggesting that CD could have a role in tumour invasion by the digestion of extracellular matrix. In a very recent study of Jahkala et al. [17], concerning CD expression in early breast cancer, a TN-positive invasion border was associated statistically with CD expression in both carcinoma and stromal cells. In the present study, we found a trend for correlation between CD of neoplastic cells and stromal TN expression, but this relationship was not statistically significant.

Fibronectin is regarded as the major mesenchymal extracellular matrix glycoprotein involved in cell–matrix and cell–cell adhesion, cell migration and oncogene transformation [27]. Studies of fibronectin expression in breast carcinomas showed a strong expression and different distribution than with non-tumoral breast parenchyma [10, 13]. In the adult breast, fibronectin reactivity was considerable in the interlobular stroma, but it was weak or absent in the intralobular and immediate periductal matrix. In fibrocystic diseases and in intraductal carcinomas, fibronectin reactivity surrounded the abnormal epithelial structures and was also noted in the distal stromal regions. In invasive ductal and lobular carcinomas, a strong and extensive fibronectin staining was observed in the tumour stroma. An in situ hybridization study of human colon tissues showed a positive correlation between fibronectin mRNA expression and the depth of invasion as well as the frequency of lymph-node metastases, suggesting that fibronectin expression could be important for the remodelling process of neoplastic tissues during cancer development and progression [12]. In our study, fibronectin staining was more intense in the stroma of invasive tumours, when compared with the other studied lesions, and the positive cells were more widely spread throughout the interstitial matrix than TN-positive cells. Although the theory of a major fibronectin antagonising role of TN has been supported [6], in the present study, a trend for correlation between these proteins was found, but it was not statistically significant.

High PCNA and Ki-67 indices were correlated with aggressive behaviour in tumours of various sites. In laryngeal carcinomas, there are data that indicate that cell proliferation indices may be considered as reliable and reproducible indicators of biological aggressiveness. In addition, studies analysing 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. In vitro, TN participates in the control of cell proliferation and migration, and studies of TN expression in intraductal breast carcinomas have shown a strong correlation with Ki-67 expression [16]. Moreover, it is known that the ECM acts as a reservoir for a number of growth factors and their binding proteins, which are selectively accumulated and released [31]. It has also been suggested that growth factors in the ECM are particularly active, when complexed with other ECM molecules, this interaction often being essential to their activity [9]. In this study, the non-existing correlation between TN expression and proliferation-associated indices probably means that TN does not directly link with cell proliferation and that, indeed, a variety of growth factors may be involved in the regulation of TN expression.

In conclusion, TN 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 behaviour of these lesions. In addition, the determination of this stromal marker may prove useful for selecting the group of patients, especially with pre-malignant lesions, that is at greatest risk of progressing to cancer and that would therefore benefit from a close follow-up. This is clearly a subject for further studies.

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