Stage-related Decorin and Versican Expression in Human Laryngeal Cancer

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Abstract. *Background: The major proteoglycan of normal human larynx is aggrecan. In laryngeal carcinoma, aggrecan is depleted, with versican and decorin appearing in higher amounts. Materials and Methods: Proteoglycans in laryngeal carcinoma samples were characterized immunohistochemically and using Western blotting; their expression was examined by RT-PCR. Results: Aggrecan was totally removed in advanced cancer and its RT-PCR product was not identified. Both versican and decorin were overexpressed in cancer, versican much more than decorin. Decorin expression was higher than that of versican in the normal larynx; therefore, their disproportionate overexpression during cancer resulted in about equimolar expression. Both proteoglycans' expression correlated with their stage-related accumulation within the tissue. Conclusion: These data add to our previous findings and support the view that the levels of expression and the extent of accumulation and localization in the tumor stroma of structurally modified versican and decorin could be associated with the degree of aggressiveness of laryngeal carcinoma.*

Proteoglycans (PGs) are a class of glycosylated proteins with an important role in cell proliferation, differentiation and matrix synthesis. Cell membrane PGs and PGs produced from the surrounding cells are responsible for growth factor binding to their receptors and *via* the intracellular signaling pathway, the signal is transmitted to the nucleus and affects the cellular response (1). We have previously shown that the main component of extracellular matrix of normal human laryngeal cartilage (NHLC) is the large hyaluronan-binding proteoglycan aggrecan (2). Aggrecan, the largest cartilage extracellular PG, interacts non-covalently with hyaluronan and is stabilized by Link Protein to form large molecular weight aggregates that are responsible for the cartilage properties of compressibility and elasticity because they enable high concentrations of hydrophilic sulphated glycosaminoglycans to be entrapped within the tissue, thus enabling water molecules to penetrate the matrix. Other proteoglycans, like versican, decorin and biglycan are also found in NHLC, but in smaller amounts.

At the glycosaminoglycan level, a recent study demonstrated that in laryngeal squamous cell carcinoma (LSCC) the structural and compositional changes were closely associated with the tissue type (3). At the proteoglycan level, a dramatic loss of aggrecan observed at the late stage of laryngeal cancer, ~18-fold, was evidenced as a crucial event from the aspect of the macromolecular changes that occur during LSCC (4). In addition, an important cartilage remodeling at the aggrecan level in LSCC was observed *via* the extractability of the cancerous tissue (5). Moreover, a stage related loss of aggregable aggrecan in apparently normal cartilage (AANC) adjacent to cancer compared to the NHLC was noted, which was excessive in advanced stages of the disease (6). We have also indicated that increased accumulation of structurally modified versican and decorin is related to the progression of laryngeal cancer (7). Overexpression of versican was observed at early staged pharyngeal (8) and epithelial ovarian cancer (9), but in most of the cases versican expression was closely related with cancer progression (10-15). This type of proteoglycan is one of the main components of the extracellular matrix (ECM), where it provides hygroscopic properties to create a loose and hydrated matrix that is necessary to support key events in development and disease. Through direct or indirect interactions with cells and molecules, versican is able to regulate cell adhesion and survival, cell proliferation, cell migration and the ECM assembly (16). Versican interacts with its binding partners through its *N*- and *C*-terminal globular regions as well as its central GAG-binding region and it binds to both ECM components and cell surface proteins (17). These

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Key Words: Aggrecan, versican, decorin, Western blotting, immunohistochemistry, RT-PCR, proteoglycan.

Table I. *Nucleotide sequence of the primers used in RT-PCR experiments.*

Type of primer	Nucleotide sequence
Sense	
AGGRECAN	ATGCCCAAGACTACCAGTGG
VERSICAN	GATGTGTATTGTTATGTGGATCA
DECORIN	CGAGTGGTCCAGTGTTCTGA
GADPH	ACATCATCCCTGCCTCTACTGG
Antisense	
AGGRECAN	TCCTGGAAGCTCTTCTCAGT
VERSICAN	CATCAAATCTGCTATCAGGG
DECORIN	AAAGCCCCATTTTCAATTCC
GADPH	AGTGGGTGTCGCTGTTGAAGTC

multiple binding interactions play important roles in cell and tissue behaviour. Through its interaction with hyaluronan and other ECM partners, versican creates pericellular matrices that are required for cell proliferation and migration.

Overexpression of the V1 versican isoform in cultured fibroblasts increases both proliferation and apoptotic resistance (18). It has been shown that the versican G1 domain can enhance cell proliferation and reduce cell adhesion in different cell types (19, 20) and the versican G3 domain enhances tumour growth and angiogenesis (21). Hence the possibility that extended degradation of this proteoglycan is required to express the cancer phenotype cannot be excluded. In addition, it has been observed in patients with primary oral squamous cell carcinoma that increased stromal versican expression correlated with both increased risk for disease recurrence and shortened survival, however versican expression did not correlate with clinicopathological factors or tumour cell proliferation (13).

Not only versican, but also decorin plays an important role in cancer. This proteoglycan is up-regulated in various cancer types and is considered to be a tumour suppressor proteoglycan (1, 22). The tumour suppressor activity of decorin is achieved by its ability to cause inhibition of cancer cell growth due to up-regulation of $p21^{Cip/WAF-1}$ protein (23, 24) and by acting as a ligand for the epidermal growth factor (EGF) receptor (25, 26) and triggering apoptosis *via* caspase-3 activation (26). It is also prevents metastatic spread in at least breast cancer (27).

Our previous studies in laryngeal carcinoma demonstrated that both versican and decorin undergo specific modifications on both the protein core and GAG levels during the progression of laryngeal cancer, which may be related to the mild malignant phenotype of this cancer type (7). Therefore, the purpose of the present study was to confirm aggrecan, versican and decorin expression both in normal human larynx and LSCC at the mRNA level to examine whether the alterations in these cartilage components were at the metabolic or expression level.

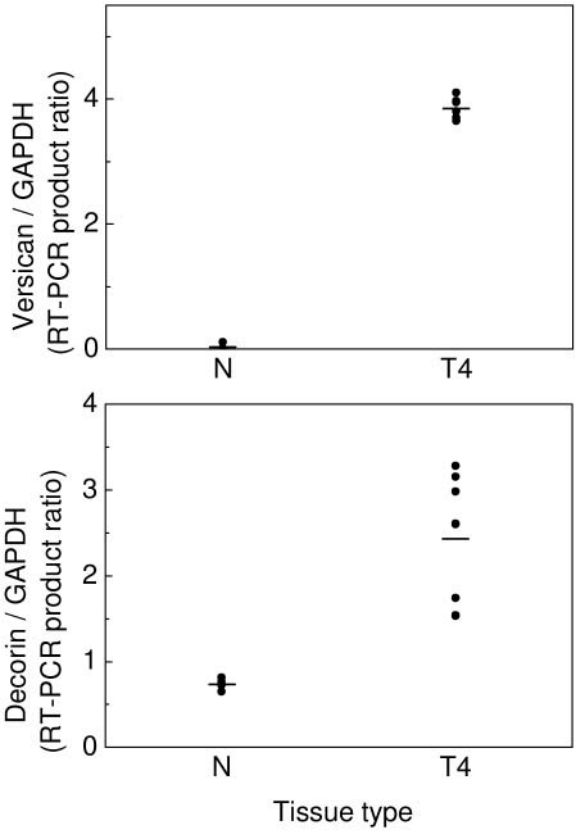

Figure 1. *Overexpression of versican and decorin in laryngeal cancer (T4 samples) as compared to normal larynx (N) by semiquantitative RT-PCR using GAPDH as house-keeping gene.*

Materials and Methods

Materials. Monoclonal antibodies against versican (2-B-1) and decorin (6-B-6) were obtained from Seikagaku America (East Falmouth, MA, USA). A polyclonal antiserum against aggrecan was prepared in rabbits, as described elsewhere (28). Total RNA extraction kits were obtained from Macherey-Nagel (Düren, Germany) and RT-PCR was performed using One Step RT-PCR kits from Qiagen (Valencia, CA, USA). All other chemicals used throughout the study were of the best available analytical grade.

Human samples. Human laryngeal cartilage was obtained from the larynx after total laryngectomy for laryngeal carcinoma. The patient population consisted exclusively of men with an age range of 44 to 74 years. The primary tumors were squamous cell carcinomas (SCC) of the larynx and the stages were distributed as 4 samples stage II, 6 samples stage III and 8 samples stage IV. Four healthy larynx samples were obtained after autopsy from men with an age range of 45 to 83 years. The samples were used directly or stored at –80˚C. The study design had the agreement of the Ethical Committee of the University Hospital of Patras, Greece, and informed consent was obtained from all patients entering the study.

Immunohistochemical staining of tissue sections. Specimens processed for light microscopy were pretreated as described elsewhere (6). Thereafter, the slides were incubated with primary antibodies

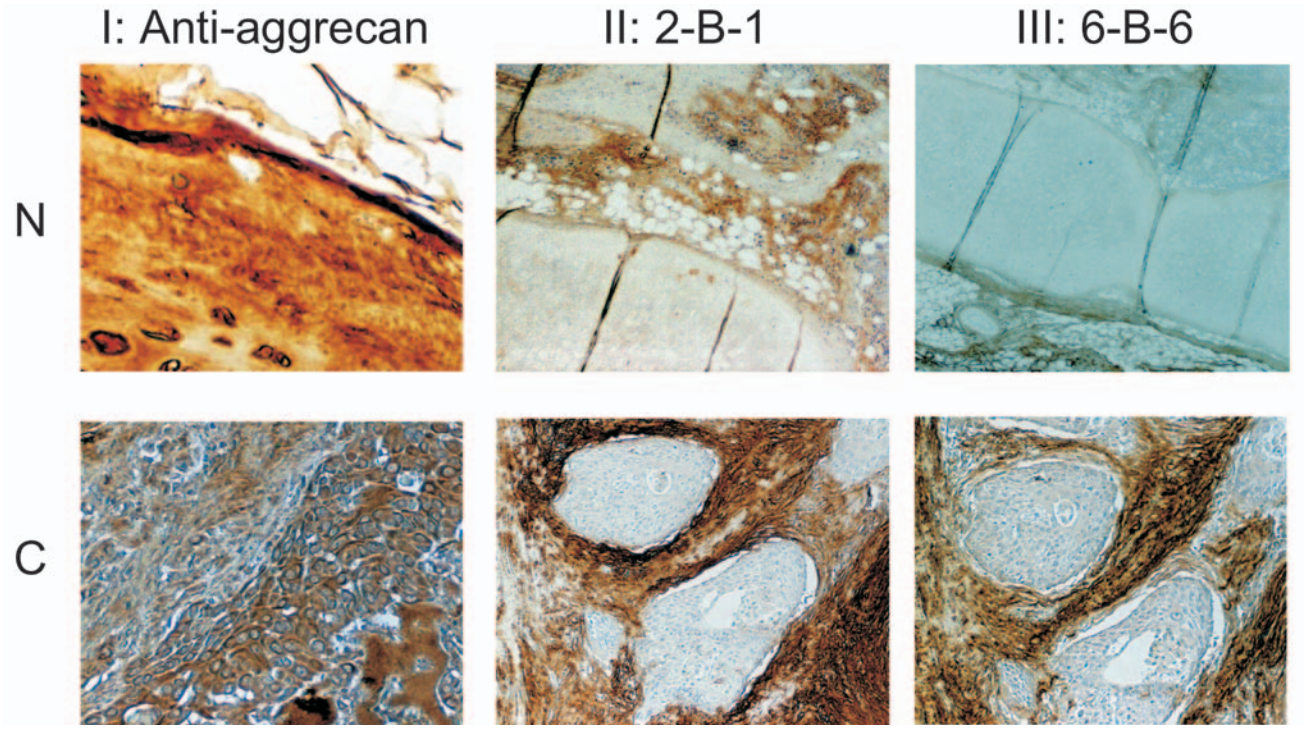

Figure 2. *Immunohistochemical analysis of aggrecan (panel I), versican (panel II) and decorin (panel III) in normal (N) and cancerous (C) T4 samples. Original magnification: N: x200; C: x400.*

(polyclonal anti-aggrecan, 1:500; 2-B-1, 1:1000; 6-B-6, 1:2000) diluted in phosphate-buffered saline (PBS) containing 1% (v/v) normal rabbit or swine serum overnight at 4˚C. The obtained antigen-antibody complexes were visualized by 30-min incubation at room temperature using biotinylated rabbit anti-mouse antibody or biotinylated goat anti-rabbit antibody diluted 1:200 and the avidin-biotin peroxidase technique (Dakopatts, Hamburg, Germany) according to manufacturer's instructions. The staining was developed with 3,3-diaminobenzidine (DAB)/hydrogen peroxide for 5 min at room temperature and slides were counterstained with hematoxylin.

Extraction of proteoglycans and Western blotting. Total proteoglycans were extracted from small pieces of tissue with 10 volumes/g wet weight of tissue by 4 M guanidine hydrochloride (GdnHCl) - 0.05 M sodium acetate (pH 5.8) solution containing proteinases inhibitors for 2x24 h periods, as described elswhere (4, 6, 7). The extracts were transferred to 0.1 M Tris-acetate pH 7.3 (1 mg of uronic acid/ml) and digested with keratanase and chondroitinase ABC in the presence of 0.1 unit of each enzyme for 4 h at 37˚C. The obtained core proteins were subjected to SDS-PAGE in 4-20% polyacrylamide gels and the separated bands were thereafter electrotransferred to PVDF membranes in 0.05 M Tris-HCl, pH 8.3, at a constant current of 80 mA at 4˚C for 20 h, followed by immunochemical detection as described elswhere (4, 6, 7).

RNA extraction and RT-PCR. Laryngeal specimens were pulverized in liquid nitrogen and subjected to total RNA extraction (29). Strand cDNA was synthesized from 100 ng of total RNA in 50 μl reaction components for the QIAGEN one-step RT-PCR kit, according to the manufacturer's instructions. This reaction mixture in addition contained 1 μM of the sense and antisense primers shown in Table I. The amplification was performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer Co., Waltham, MA, USA) and the reaction profile used for all primer sets was: 94˚C for 15 min for the activation of DNA polymerase and then 35 cycles at 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min to finalize the extension. The number of cycles was chosen so that reactions could be terminated during the linear phase of amplification. The reaction products were separated by electrophoresis in 2% (w/v) agarose gels contained SYBR gold stain to visualize the amplified cDNA fragments under UV. The gels were then scanned and the bands were analyzed densitometrically. Quantitative differences between cDNA samples were normalized by including GAPDH in all experiments.

Statistical analysis. Normality of distribution of values was tested with the Kolmogorov-Smirnov test. Results were statistically analyzed using the unpaired *t*-test to detect differences between groups. $P<0.05$ was regarded as statistically significant. For the statistical analysis, the Origin Pro 7.5 SRO statistical software was used (Origin Lab Corporation, USA).

Results

Aggrecan, decorin and versican expression and localization in advanced stage of laryngeal cancer. RT-PCR was used to analyze the expression of aggrecan, decorin and versican

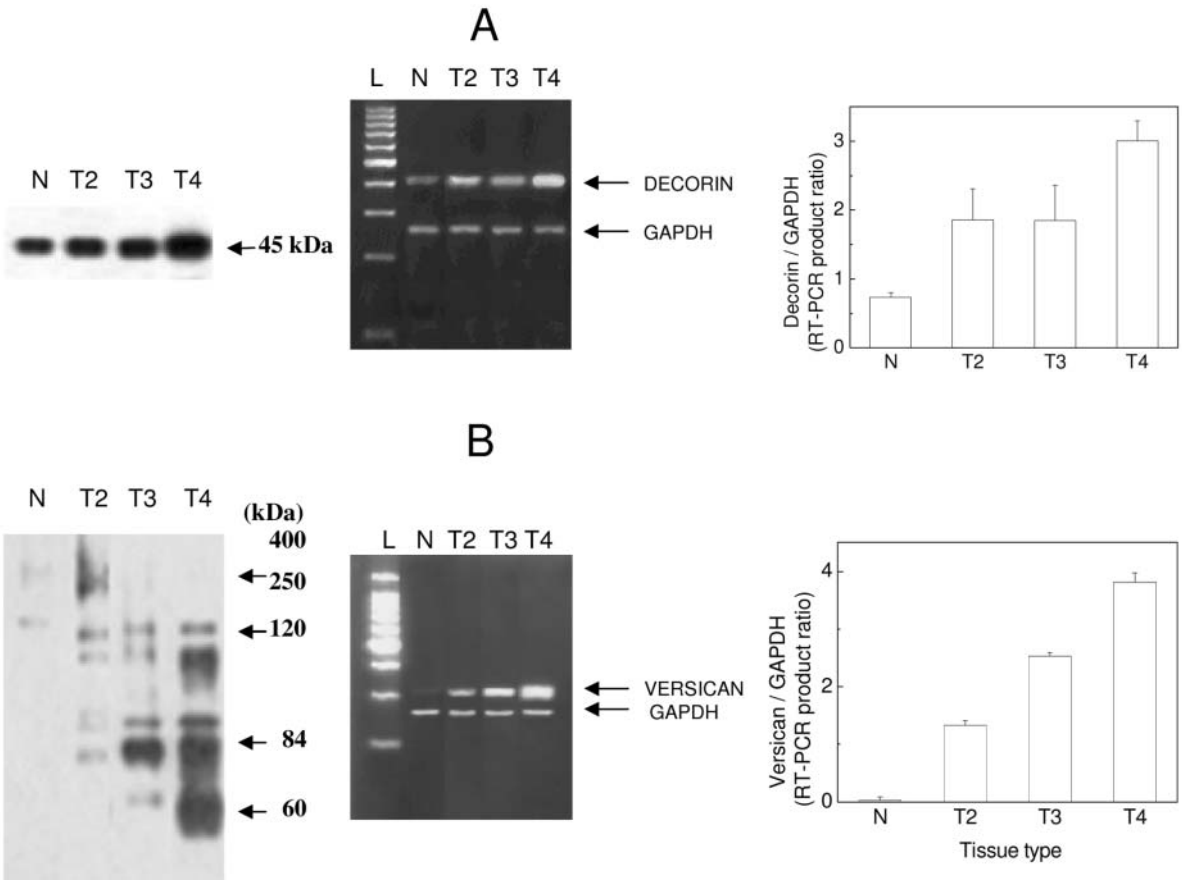

Figure 3. *Stage-related expression of the proteoglycans decorin (A) and versican (B) in laryngeal carcinoma. Samples were analyzed by Western blotting after chondroitinase ABC treatment (left) and RT-PCR (middle). The results of the RT-PCR were plotted against the stage of the samples (right).*

mRNA levels in normal human larynx and advanced laryngeal cancer (T4-staged) samples. The results indicated a very high increase of the expression of versican of approximately 140-fold, as well as a significant increase of decorin by about 2.5-folds in laryngeal cancer tissue compared to normal controls (Figure 1). In contrast, it was observed that aggrecan expression totally ceased in laryngeal cancer (not shown).

Localization of aggrecan, decorin and versican in normal larynx and laryngeal cancer (T4-staged) samples was examined by immunohistochemical analysis (Figure 2). Staining of tissue sections with the antiserum to aggrecan indicated its presence only in normal specimens (Figure 2, panel I N) and its almost total absence from the cancerous (Figure 2, panel I C). As expected, aggrecan staining was observed exclusively in the cartilaginous parts of normal laryngeal specimens, especially in the ECM. Epithelial tissue did not stain at all. On the other hand, T4-staged cancerous specimens stained topically with this antiserum, indicative of the gradual removal of aggrecan from these sites, with no replacement with newly synthesized macromolecules.

Staining of normal tissue sections with the MAb 2-B-1 (anti-versican) indicated its main presence in epithelial tissue and its concomitant absence from cartilage (Figure 2, panel II N). In T4-staged cancer sections, very strong versican staining was observed (Figure 2, panel II C), being located mainly pericellularly and peritumorally. Similar results were obtained when the tissues sections were stained with MAb 6-B-6 (anti-decorin). Staining of normal sections (Figure 2, panel III N) was observed especially in the perichondrium, whereas in T4-staged cancer specimens strong decorin staining was observed (Figure 2, panel III C), though not as strongly as compared with the respective tissue staining for versican, suggesting the pericellular and peritumoral localization of this proteoglycan.

Stage-related expression and accumulation of decorin and versican. In order to examine the stage-related expression and accumulation of decorin and versican, two different sets of laryngeal extracts were prepared, one for RT-PCR analysis and a second for Western blotting. The results of

the RT-PCR analysis of decorin in different stages of laryngeal cancer showed a two-fold increase of expression for both stages II and III and appriximately three-fold for stage IV (Figure 3 A, right). Western blotting analysis of decorin core protein indicated a significant increase in both stages II and III, of a similar extent, and an additional increase in stage IV as compared with normal tissue (Figure 3 A, left). The expression of versican was found to be more characteristic. In normal specimens, extremely small amounts of RT-PCR product were obtained, thereafter gradually increasing according to the stage of cancer, *i.e.*, 50-, 90- and 140-fold at stages II, III and IV, respectively (Figure 3 B, right). Western blotting analysis of versican core protein indicated an obviously stage-related increase in cancer specimens as compared with normal tissue (Figure 3 B, left), which was accompanied by marked structural changes of the proteoglycan. In particular, normal laryngeal extracts contained immunoreacting bands of very high molecular mass, migrated in the 250 to 400 kDa range. In addition, lower molecular size bands in the 120 to 180 kDa range were detected. The immunoreactivity profile of laryngeal cancer samples significantly differed from that of normal larynx, since a remarkable shift to lower molecular sizes with increasing disease stage was observed. The cancerous samples of stages III and IV showed abundant versican fragments/isoforms (2B1-reactive material) in the 60 to 120 kDa range, whereas the major bands (>250 kDa) detected in normal larynx and early stage II were either significantly lower (stage III) or totally absent (stage IV).

Furthermore, the comparison of the expression rate of decorin to that of versican (Figure 4 A), *via* the ratios of the RT-PCR products of decorin/GAPDH to versican/GAPDH, revealed that the levels of decorin in normal tissues were higher than those of versican, about 27-fold. The corresponding ratios in the cancerous tissues of stages II, III and IV were found to be 1.4, 0.7 and 0.7, respectively. These findings imply that versican appeared to predominate in stages III and IV of laryngeal cancer. The predominance of versican in these two latter stages was due to the different increase of its expression rate versus decorin, as revealed by the relative ratios of expression of versican to decorin compared with that of normal samples, being 2.0 in stage II and reaching 36 and 34 in stages III and IV, respectively (Figure 4 B).

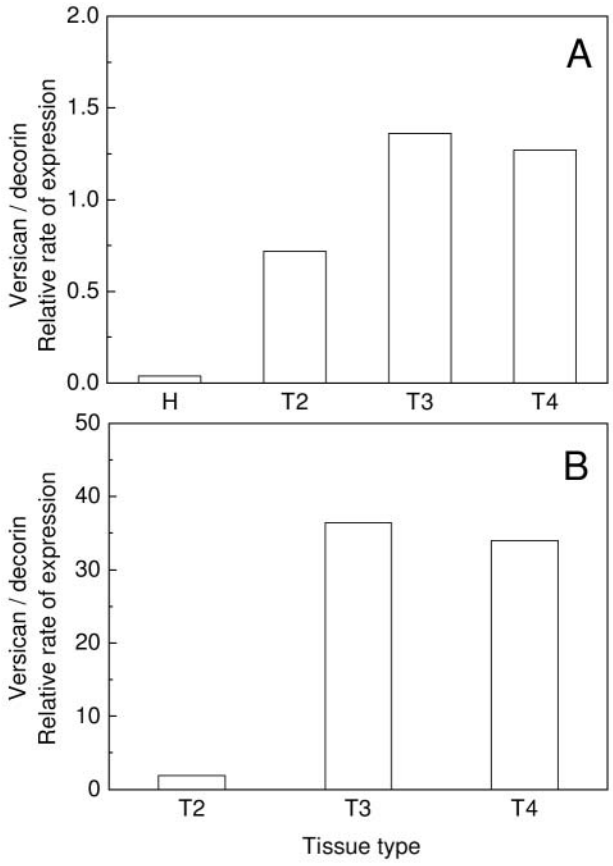

Figure 4. *Relative expression of versican to decorin. A) The ratio of versican to decorin expression in the samples was plotted. B) The ratio of versican to decorin in cancerous samples relative to healthy ones was plotted.*

Discussion

Squamous cell carcinoma (SCC) comprises more than 95% of laryngeal carcinomas and is obviously the most important laryngeal cancer (30). In our previous studies regarding the macromolecular changes occurring in laryngeal cancer, we had demonstrated that the extractability of the ECM macromolecules of the diseased tissue was perceptibly altered, thus implying an important cartilage remodeling in LSCC (5, 31). Moreover, we observed a significant destruction of cartilage in advanced (stage IV) LSCC, which was followed by a marked decrease of the large aggregating PG aggrecan compared to those from human normal larynx (HNL) (4, 6). In contrast to the loss of aggrecan in LSCC, a significant increase in accumulation of structurally modified versican and decorin was observed in the tumor-associated stroma with the progression of laryngeal cancer (4, 7).

In an initial set of experiments in the present study, we examined the expression and immunohistochemcal localization of aggrecan, versican and decorin in normal and cancerous (stage IV) specimens. The results on the expression level indicated a significant but disproportionate increase of expression of both versican and decorin in cancerous specimens compared to normal ones, whereas aggrecan expression totally ceased in cancerous specimens, indicating either the total absence of chondrocytes from the tissue or their inability to express aggrecan under such conditions. Immunohistochemical examination showed, as

expected, a strong immunoreactivity for aggrecan in sections of normal specimens but an inordinate reduction in reactivity in cancerous specimens. The removal of aggrecan from cartilage should be attributed to increased enzymatic activities (29, 32, 33). In contrast, versican and decorin presented increased reactivity in tumor-associated stroma in cancerous specimens compared to normal ones.

We then focused on the expression of versican and decorin in laryngeal cancer of different stages in order to examine simultaneously any correlation with the extent of accumulation of the proteoglycan in the cancerous samples, as evaluated by Western blot analysis of its core protein. The results indicated that in normal tissues the expression levels of decorin and its accumulation were very much higher than those of versican, approximately 27-fold. In comparison with the normal tissue, the expression levels of decorin in the cancerous samples of stages II and III presented a stable increase in both stages of approximately two-fold with an additional increase in the stage IV of approximately three-fold. This increase of decorin expression was associated with a simultaneous increase of its accumulation, as evaluated by Western blot analysis. Correspondingly, the expression levels of versican in the cancerous samples presented a characteristic stage-related increase, *i.e.*, 50-, 90- and 140-fold for stage II, III and IV, respectively. The overexpression of versican in comparison to decorin led the former to predominate in cancerous specimens of stages III and IV. Moreover, a simultaneous stage-related increase of accumulation of versican was also observed. In addition, Western blot analysis revealed an increased heterogeneity of versican with increasing LSCC stage as well as the clear predominance of versican protein cores/fragments of significantly smaller molecular sizes in late disease stages, in comparison to those found in normal tissue. On a quantitative level, the findings of this study indicated that the expression and accumulation of both versican and decorin in advanced laryngeal cancer (stage IV) were to about the same extent with light predominance of versican.

LSCC accounts for approximately one-fourth of all head and neck SCCs. More than one-half of LSCCs are present as local disease without metastasis, one-fourth are present as local disease with regional metastasis, and approximately 15% are first seen at an advanced stage with or without distant metastasis (30). The mild aggressive potential of LSCC, apart from the extensive presence of laryngeal cartilages, which act as physiological barriers to the spread of carcinoma cells (34), could be explained, at least in part, by both positive and negative roles that have been proposed for versican and decorin.

Our results, derived from the Western blotting analysis of the core protein of versican, demonstrated a stage-related increase of especially degraded proteoglycan, since the majority of the immunoreacting bands at the late stages migrated at approximately 84 kDa (stage III) and in the 60 to 120 kDa region (stage IV). The intense presence of versican fragments/isoforms reduced its positive effect by the restriction of the favourable environment for this cancer type. This was mainly due to the fact that the modified versican core protein created limited hydration, necessary for the formation of a highly malleable extracellular environment that could support a cell-shape change required for cell proliferation and migration because of the limited presence of their GAG side chains. The reduced aggressive potential of LSCC could also be explained by the increase of the tumor-inhibitory ability of decorin.

Collectively, in the present study, we provide direct evidence that versican and decorin presented a significant and stage-related expression in laryngeal cancer that was in good agreement with the extent of accumulation and localization of these molecules in the tumor stroma of the laryngeal cancer. These data together with our previous studies on LSCC support the view that the levels of expression and the extent of accumulation of structurally modified versican and decorin might be associated with the degree of aggressiveness of LSCC. In addition, some other such as the size of hyaluronan chains, the type and the degree of sulphation and the degree of glucuronate epimerization should be considered.

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Received August 16, 2007
Revised November 9, 2007
Accepted December 11, 2007