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Irregular expression of hyaluronan and its CD44 receptor is associated with metastatic phenotype in laryngeal squamous cell carcinoma

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Abstract The distributions of hyaluronan (HA) and its CD44 receptor were studied in 24 normal, 27 dysplastic samples of laryngeal epithelium and in 172 squamous cell carcinomas (LSCC), using a specific probe prepared from cartilage proteoglycan (bHABC, biotinylated hyaluronan binding complex) and a monoclonal antibody (Hermes 3). HA and CD44 were expressed similarly in all normal and about 90% of dysplastic and neoplastic laryngeal epithelia. In the normal epithelium HA and CD44 were homogeneously distributed throughout the epithelium, whereas the most superficial layers were negative. This was in contrast to the picture in dysplastic epithelium and well-differentiated invasive carcinomas, which were entirely HA and CD44 positive. Local areas with a low signal for HA and CD44 were present in 11% and 22% of the samples with dysplasia, and in 27% and 28% of those with carcinoma, respectively. The presence of this staining irregularity was associated with poor differentiation of the carcinoma, a significantly elevated mitotic index and a high frequency of nodal spreading and metastases. Furthermore, the irregular staining showed a trend towards poor disease-free survival, suggesting that an altered metabolism of HA is a common feature in LSCC and is associated with an aggressive growth pattern.

Key words Hyaluronic acid · CD44 receptor · Adhesion · Laryngeal neoplasms · Prognosis

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Introduction

Hyaluronan (HA) is an unbranched polysaccharide consisting of repeating disaccharide units of *N*-acetyl-glucosamine and glucuronic acid [37]. The molecular mass of newly synthesized HA usually exceeds 10^6 Da [37]. Unlike other glycosaminoglycans, which are produced in the Golgi apparatus, HA is extruded through the plasma membrane as it is synthesized [21]. HA is almost ubiquitously distributed in various human tissues. Although many simple epithelia express little HA, such stratified squamous epithelia as the epidermis display a strong signal for HA in all the cell layers [35, 36, 41].

Many functions have been attributed to HA, including cell migration, proliferation and differentiation during embryonic development, wound healing and inflammation [37]. An extracellular environment rich in HA is a characteristic feature of many tumours, and the stromal content of HA is correlated with invasiveness [16]. It has been suggested that host tissue HA provides an expanded, loose matrix, which is easy to penetrate and serves as a substratum for HA-receptor-bearing tumour cells [4]. In many adenocarcinomas, the stroma is rich in HA while the epithelial cells show little or no HA [3, 41]. In contrast, well-differentiated squamous cell carcinomas of the oesophagus [41] and skin [36] are strongly HA positive. In squamous cell carcinomas, poor differentiation is frequently associated with a reduced HA signal in the carcinoma cells [41].

The tumour-growth-facilitating activity of HA may be mediated via CD44, one of the HA-binding cell surface

receptors [24]. CD44 is expressed as different splice variants of the gene, giving rise to a number of core protein [26] and glycosylation [39] isoforms. The standard isoform (CD44s) lacking all of the exons from the alternatively spliced region [26] is present in almost all normal human tissues and also in a variety of neoplasms [24]. The variant isoforms of CD44 are found preferentially in epithelial tissues [11], and their expression seems to be upregulated in many tumours [24]. It was originally suggested that the isoforms containing variant exons have a reduced capacity to bind HA [32], but the affinity for HA is also influenced by the post-translational modifications of the molecule and its cell surface organization [17]. In various squamous epithelia HA and CD44 closely colocalize [35, 42], suggesting that CD44 is the major HA receptor in these tissues.

The present study was undertaken to reveal the distribution of both HA and its CD44 receptor in parallel in normal laryngeal tissues and primary squamous cell carcinomas of the larynx (LSCC). In addition, the prognostic significance of changes in HA and CD44 expressions was evaluated in 172 LSCC patients with a long-term follow-up.

Materials and methods

Representative samples of primary tumours were obtained from 172 patients with LSCC diagnosed between 1975 and 1995. They were fixed in 10% buffered formalin immediately after removal, maintained in formalin overnight and embedded in paraffin according to a standard procedure. In addition, samples of normal (n=24) and dysplastic (n=27) epithelia were obtained from laryngectomy specimens. The material consisted of 44 well, 85 moderately and 43 poorly differentiated tumours using the WHO classification [29]. The clinical stage and exact location of the primary tumours were evaluated by the same otorhinolaryngologist (JVi) according to the TNM classification [30]. Only patients with histologically verified lymph node metastases were considered to have a node-positive disease (pN1–3).

The median and mean age of the patients was 64 years (range 31–85). There were 7 (4%) women in the study cohort. Curative radiotherapy with a median dose of 66 Gy was given to 82 patients (48%), while twenty-two patients (13%) underwent surgical resection as the only treatment. Combined radiotherapy and surgery were given to 66 patients (38%), including 7 who received neoadjuvant chemotherapy; 2 patients did not receive any treatment.

The biotinylated hyaluronan-binding region and link protein complex (bHABC), used as an HA-specific probe, was prepared from bovine articular cartilage as previously described [36, 42]. Proteoglycans were extracted from bovine knee articular cartilage with 4 mol/l guanidium chloride. After dialysis against water they were allowed to reassociate with HA and were digested with trypsin. The trypsin-resistant ternary complex between the HA-binding region of aggrecan, link protein, and HA was purified using sequential hydroxyl apatite and size exclusion chromatography. The proteins in the complex were then biotinylated and separated from HA using dissociative gel filtration in 4 mol/l guanidium chloride, and dialyzed. Polyacrylamide gel electrophoresis of the probe showed only bands corresponding to the HA-binding region of aggrecan and link protein [36].

The sections were deparaffinized and rehydrated according to a standard procedure. After being washed with PBS (pH=7.2), the sections were incubated with 0.3% H_2O_2 in 30% methanol for 10 min to block tissue peroxidases, washed with 0.1 M Na-phosphate buffer, (pH=7.4) and incubated in 1% (w/v) bovine serum albumin in the phosphate buffer for 30 min to block nonspecific binding. The sections were incubated with bHABC (5 µg/ml, diluted in 1% bovine serum albumin) overnight at 4°C, washed thoroughly with the buffer, and treated with avidin–biotin–peroxidase (Vector Laboratories, Irvine, Calif; 1:200 dilution) for 1 h at room temperature. The sections were washed with the buffer and incubated in 0.05% DAB (3,3'-diaminobenzidine, Sigma Chemical Co., St Louis, Mo.) and 0.03% hydrogen peroxide in the phosphate buffer for 5 min at room temperature. The sections were dehydrated and mounted in DPX.

The specificity of the staining was controlled by predigesting sections with *Streptomyces* hyaluronidase (100 turbidity reducing unit/ml 0.1 M sodium acetate buffer, pH 5, for 3 h at 37°C) in the presence of protease inhibitors [36]. The digestion experiment also included controls incubated under otherwise similar conditions but lacking the enzyme. Other controls were stained using a probe preincubated with HA oligosaccharides (11–12 disaccharide units) at an oligosaccharide/bHABC ratio of 1:3 (W/W), to block the HA binding site and reveal the possible nonspecific attachment of the probe to the sections (Fig. 1C).

To stain CD44, sections were deparaffinized, rehydrated and washed with PBS. Endogenous peroxidase was blocked by 5% hydrogen peroxide for 5 min, followed by washing for 5 min with PBS. The sections were preincubated in 1% bovine serum albumin for 30 min and then incubated overnight at +4°C with the primary antibody Hermes 3 [13] (kindly donated by Prof. S. Jalkanen, University of Turku, Finland) diluted 1:100 in PBS with 1% bovine serum albumin, washed twice with PBS, and incubated for 30 min with a biotinylated secondary antibody in PBS. The sections were washed three times in PBS for 5 min, incubated for 40 min in a preformed avidin–biotin peroxidase complex, washed twice for 5 min with PBS, and the colour developed with DAB and H_2O_2 as described above. The sections were dehydrated, cleared and mounted in DPX. Samples of normal human skin were used as positive controls. In negative controls the antibody was omitted.

Volume-corrected mitotic index (M/V) was measured from haematoxylin-eosin-stained sections by the method and formula originally introduced by Haapasalo et al. [8]. The M/V index expresses the number of mitotic figures per square millimetre of neoplastic epithelium in the microscope. Mitotic figures were counted in 10 consecutive fields of the invasion front areas with highest mitotic frequency. Counting was done using an objective magnification of 40× (field diameter 400 µm). Areas of inflammation, necrosis and dysplastic epithelia without distinct invasion were avoided. The M/V indexes were already available from the database used in our previous work [10].

The localization of HA and CD44 was evaluated in the normal epithelium in addition to dysplasias and invasive carcinomas. The distribution of the staining was scored as homogeneous if all areas showed the same staining intensity, and irregular if the staining intensity varied inside the sample. The intensity of the homogeneous stainings was scored in four categories ranging from negative to strongly positive (–/+/++/+++). The strong staining of either normal or dysplastic epithelium was used as an internal control in the section.

The association between the HA and CD44 staining and the clinicopathological variables were tested using Chi-square and exact tests as appropriate. The differences in M/V indexes between the groups with different staining patterns was assessed using the Mann-Whitney U test. The patterns of overall survival (OS) and disease-free survival (DFS) were estimated by means of the product-limit method (Kaplan-Meier). Corrected survival rates were used; that is to say only deaths caused by LSCC were taken as outcome events and all other deaths were censored events. Multivariate analyses were performed using a Cox proportional hazards model and likelihood ratio tests. All variables in the models were considered as categorical variables by means of the Wald backward method.

The research plan was approved by the Research Ethics Committee of Kuopio University and Kuopio University Hospital. For access to data held by the Finnish Cancer Registry, the study was approved by the Finnish Ministry Social and Health (permission NR 117/07/95).

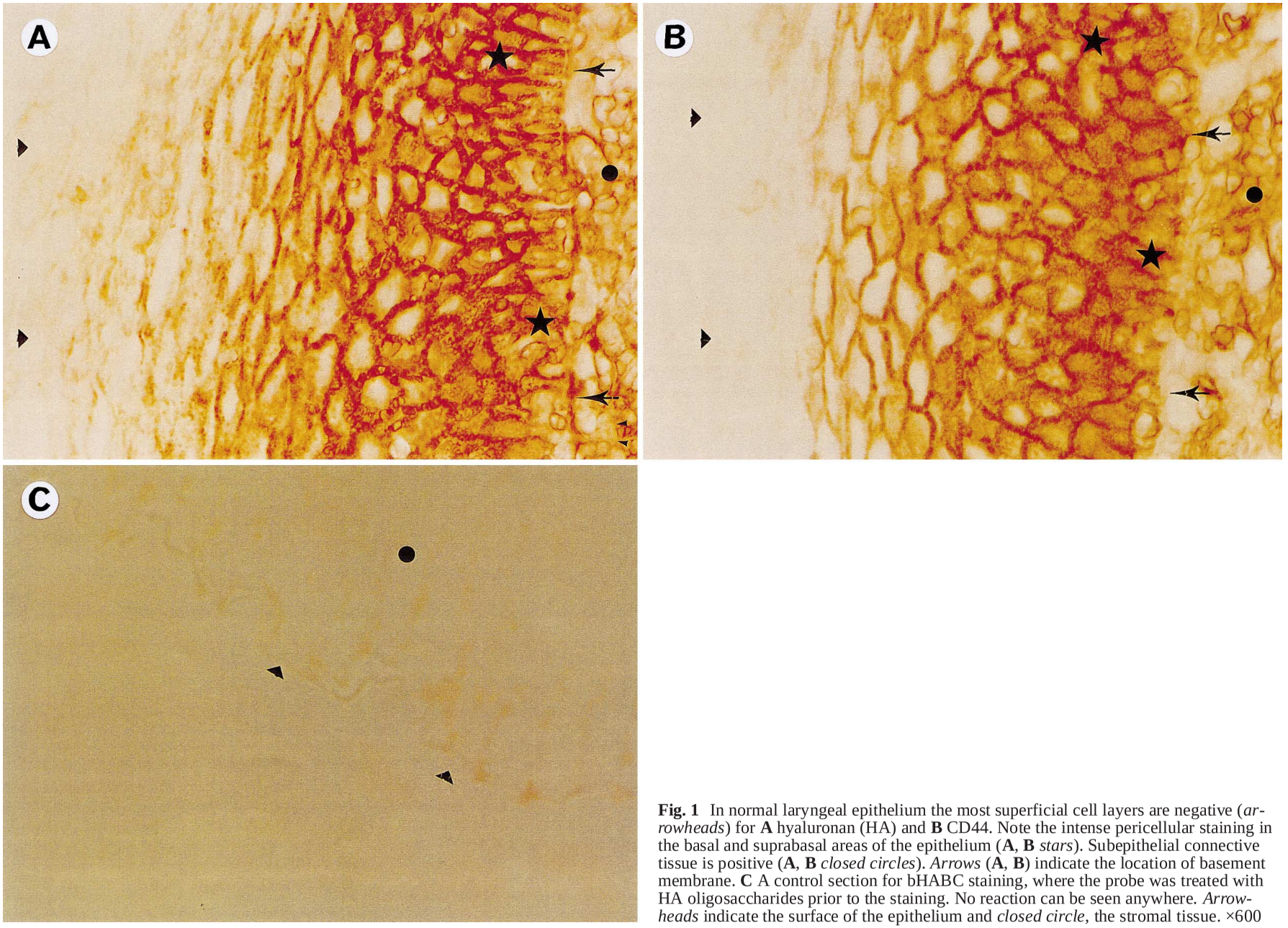


Fig. 1 In normal laryngeal epithelium the most superficial cell layers are negative (*arrowheads*) for **A** hyaluronan (HA) and **B** CD44. Note the intense pericellular staining in the basal and suprabasal areas of the epithelium (**A**, **B** *stars*). Subepithelial connective tissue is positive (**A**, **B** *closed circles*). *Arrows* (**A**, **B**) indicate the location of basement membrane. **C** A control section for bHABC staining, where the probe was treated with HA oligosaccharides prior to the staining. No reaction can be seen anywhere. *Arrowheads* indicate the surface of the epithelium and *closed circle*, the stromal tissue. ×600

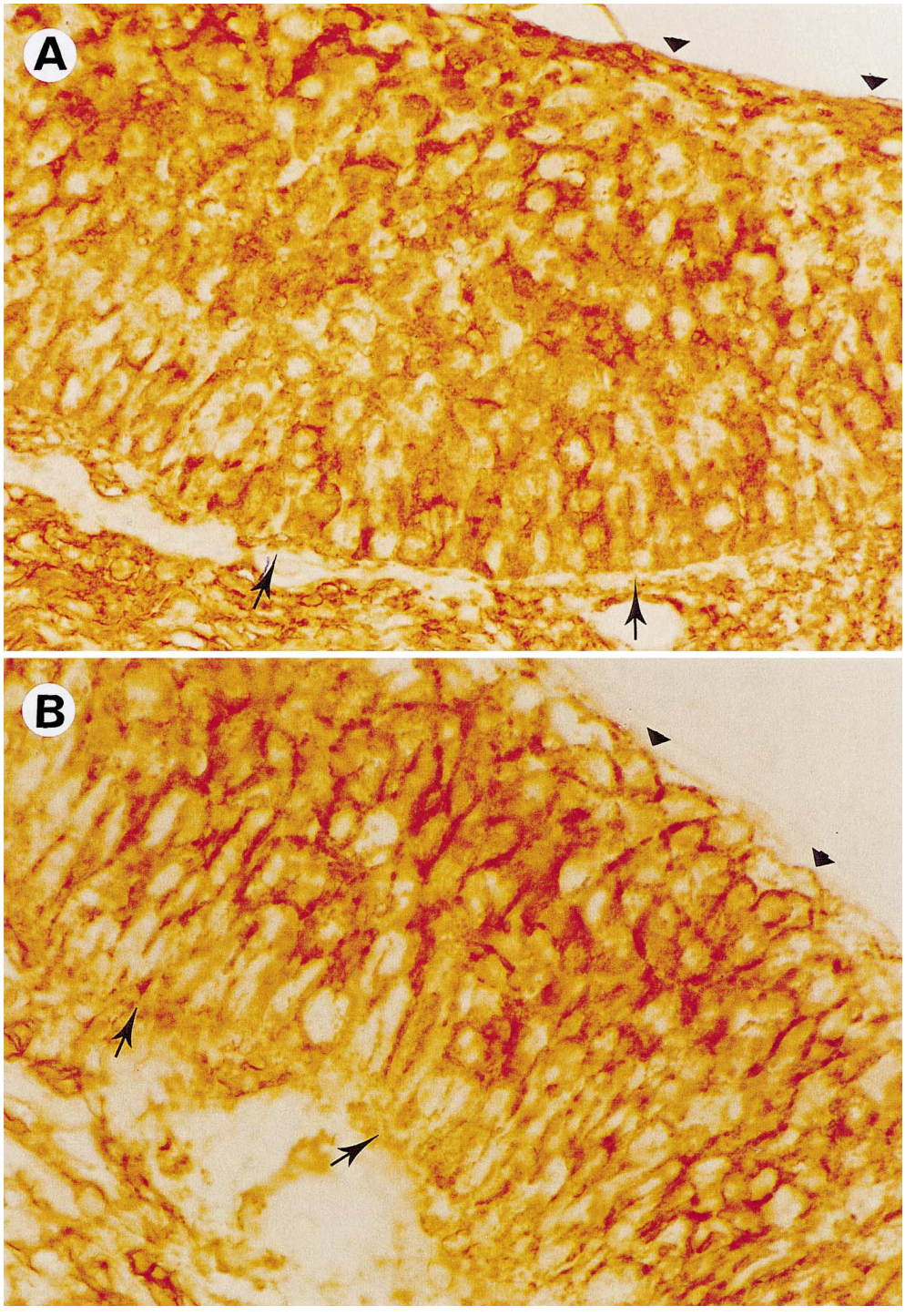


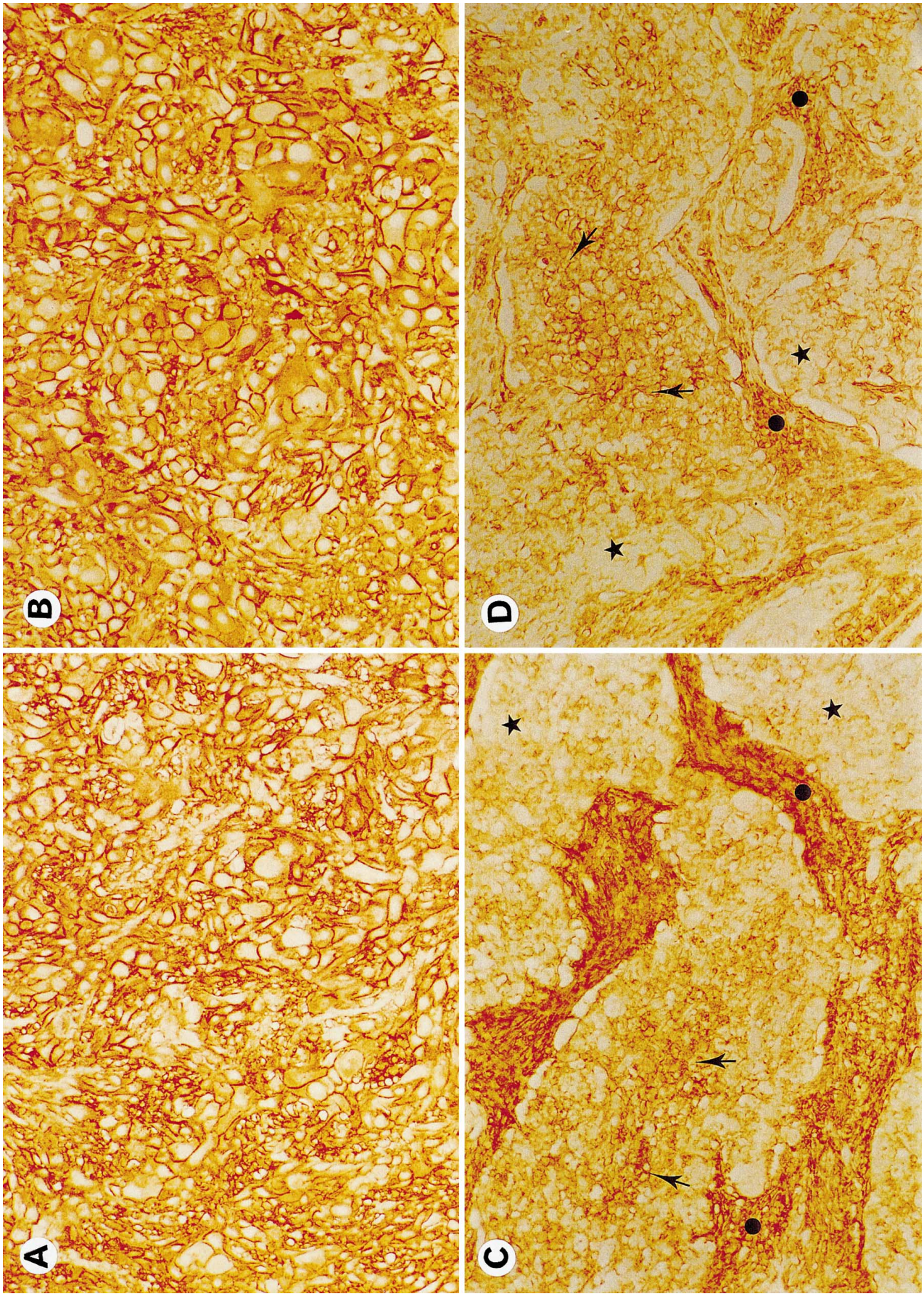
Fig. 2 All cell layers are positively stained for **A** HA and **B** CD44 in dysplastic laryngeal epithelium. *Arrows* indicate the location of basement membrane and *arrowheads*, the surface of the epithelium. ×400

Results

Normal epithelium showed a strong staining for HA (Fig. 1A) in all 24 samples studied. A homogeneous staining pattern was present in all layers of the epithelium except the most superficial layers, which were negative. The staining pattern of CD44 was identical to that of HA (Fig. 1B).

In dysplastic epithelium a distinct difference from the normal staining pattern was evident: the strong HA and CD44 positivity extended up to the most superficial layer (Fig. 2A, B). Localized areas with reduced signal for HA and CD44 were observed in some of the dysplasias. This irregular staining pattern of HA was seen in 3 of 27 samples (11%), and in 5 of 23 samples (22%) stained for

Fig. 3 A squamous cell carcinoma with intense homogeneous staining pattern for **A** HA and **B** CD44. The staining patterns are more variable, with areas showing both strong (*arrows*) and weak (*stars*) expression for **C** HA and **D** CD44 in carcinomas irregularly stained for HA and CD44. **C**, **D** same area of the tumour. Note also that stroma (*closed circles* in **C**, **D**) is positively stained for **C** HA and **D** CD44, whereas no stroma is seen in **A** and **B**. ×200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CD44. In four sample blocks there was no representative dysplastic epithelium left for immunodemonstration of CD44.

There was a representative area of invasive LSCC available from 161 patients for the demonstration of HA. The staining was distributed throughout all layers of the epithelium. An irregular staining pattern (Fig. 3C) of HA was seen in 43 tumours (27%) and a homogeneous staining pattern (Fig. 3A) in 118 tumours (73%). Most of the homogeneously stained tumours showed either strong or moderate staining intensity (66% and 32% of cases, respectively), while only 2% showed weak positivity and none was totally negative.

Sections with representative invasive LSCC were available from 160 patients for the immunodemonstration of CD44. Irregular staining of CD44 (Fig. 3D) was seen in 45 of the tumours (28%). In homogeneously stained tumours (Fig. 3B) 73% showed strong, 24% moderate and 3% weak staining intensity. No tumours were totally negative for CD44.

In 90% of cases the staining patterns for HA and CD44 were identical, either both homogeneous (68%) or both irregular (22%). In 10% of the tumours irregular staining for either HA or CD44 was detected while the other stained homogeneously.

The irregular staining pattern of HA and CD44 was associated with poor histological differentiation ($P<0.0005$ for both), presence of nodal ($P<0.0005$ and 0.03, respectively) and distant metastases ($P=0.002$ and 0.02). However, no association between tumour stage (T) and HA or CD44 staining was seen (Table 1). In addition, irregular staining pattern for HA was seen more frequently in patients with supraglottic tumours (35%) than in patients with glottic tumours (19%); $P=0.02$. A similar trend was noticed in CD44 staining: 35% vs 21%, respectively ($P=0.05$).

The median M/V index for tumours with irregular staining of HA was 20.7 (range 0–67.5), and that for tumours with homogeneous staining was 14.2 (0–103.9) ($P=0.003$, Mann-Whitney U-test). Similarly, the median M/V index was 20.2 (range 0–103.9) for tumours with irregular CD44 staining and 13.4 (0–60.0) for those with homogeneous staining ($P=0.004$).

The median length of follow-up was 66.7 months (range 1–241). The follow-up was complete in all cases. At the end of follow-up 42 (24%) patients were alive and 58 (34%) had died of LSCC. No difference in OS was noticed in patients with different staining patterns for HA ($P=0.09$) or CD44 ($P=0.31$).

Cox's multivariate analysis included tumour stage (T1–T4), nodal status (N0–pN+), distant metastases (M0–M+), histological differentiation grade (grades 1–3), site of primary tumour (glottic, supraglottic, subglottic or transglottic), HA (homogeneous vs irregular), and CD44 staining (homogeneous vs irregular). Significant predictors of OS were tumour stage ($P=0.001$) and presence of distant metastases ($P<0.00005$).

Relapses of LSCC were detected in 55 cases (33%). The 5-year DFS for patients with irregular staining pattern for CD44 was 62% (95% confidence interval 45–75%) and 71% (61–78%) for homogeneous staining ($P=0.04$). Similarly, 55% (38–69%) and 72% (62–80%) of patients with irregular and homogenous staining for HA had no relapse after 5 years ($P=0.02$; Fig. 4).

In Cox's multivariate analysis, including tumour stage, nodal status, histological differentiation grade, site of primary tumour, and HA and CD44 staining, the only significant independent predictor of survival was nodal status (pN; $P<0.00005$).

Table 1 Association between clinicopathological features and homogeneous and irregular staining patterns of HA and CD44 in laryngeal squamous cell carcinoma. Numbers of patients in different groups are shown (*Homog*. homogeneous, *Irreg* irregular staining pattern)

Feature	CD44 staining		HA staining	
	Homog.	Irreg.	Homog.	Irreg.
Tumour stage				
Tis	2	0	1	1
T1	32	10	34	9
T2	25	16	28	13
T3	34	11	34	11
T4	22	8	21	9
	$P=0.4$[a]		$P=0.7$[a]	
Nodal status				
N–	99	32	105	27
pN+	16	13	13	16
	$P=0.03$[b]		$P<0.0005$[b]	
Distant metastasis				
M–	113	40	117	37
M+	2	5	1	6
	$P=0.02$[a]		$P=0.002$[a]	
Histological grade				
Grade 1	38	2	39	1
Grade 2	58	21	63	17
Grade 3	19	22	16	25
	$P<0.0005$[b]	$P<0.0005$[b]		

[a] Exact test
[b] Chi-square test

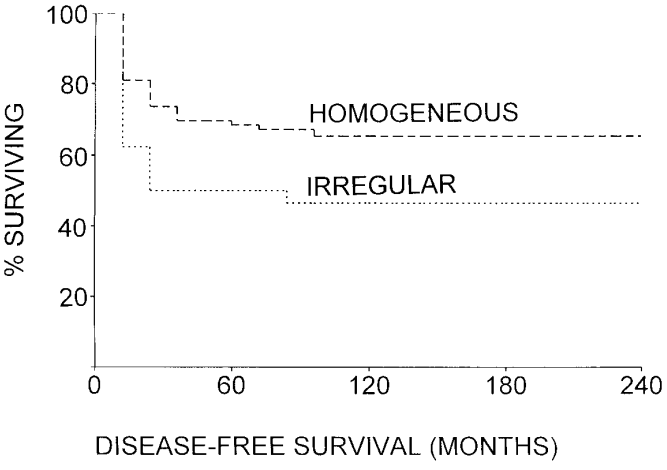

Fig. 4 Disease-free survival is poorer in patients with irregular ($n=55$) expression of HA than in patients with homogeneous ($n=72$) staining ($P=0.02$)

Discussion

The present study shows that HA and its cell surface receptor, CD44, are localized in a similar pattern in all normal and about 90% of dysplastic and neoplastic samples of laryngeal epithelia. In addition, while in the normal epithelium their distributions are homogeneous, malignant transformation is associated with focal reductions of both HA and its receptor. Furthermore, the loss of HA and CD44 in LSCC was associated with poor differentiation and increased mitotic index, and showed a four-fold enrichment in a subgroup with nodal or metastatic spread.

The HA staining pattern of the normal laryngeal epithelium with a strong pericellular signal in the basal and spinous cell layers and absence in the most superficial cell layers corresponds to that seen in other stratified epithelia, such as epidermis [34], gingiva [35], and oesophagus [41]. The pattern of CD44 expression was similar to that of HA, and it corresponds to the pattern of CD44 in earlier reports [9, 31]. During embryonic development a reduction in HA level often indicates the cessation of cell movements and the onset of normal cytodifferentiation [38]. The continued expression of HA and CD44 in the most superficial cell layers of all dysplastic epithelia indicates that failure to reduce HA and CD44 levels is an inherent feature of the differentiation defect in dysplasias.

The focal decrease in HA and CD44 staining detected in more than a quarter of the LSCC was particularly prevalent in poorly differentiated tumours, but also occurred in dysplasias. This suggests that the focal reduction of HA and CD44 level is a common and often early event in the set of properties that LSCC adopts during its development. Our results indicate that this property is associated with nodal and metastatic spreading of the cancer, and thus, with an unfavourable prognosis.

During dedifferentiation and the metastatic cascade some malignant clones may transform into a state with low HA production [15, 40]. The locally reduced content of HA might also result from enhanced uptake of HA and lysosomal degradation, or from activation of enzymatic [18] or nonenzymatic [1] extracellular degradation of HA. However, our findings of the parallel loss of CD44 is difficult to explain by a primary loss of HA alone, since there are no data available to suggest that the absence of HA would reduce the cell surface expression of CD44. More plausible explanations include separate but concomitant decrease in HA and CD44, acceleration of CD44 degradation, or inhibition of CD44 synthesis; the last two would lead to a secondary loss of HA from the cell surface.

One explanation for the simultaneous reduction of HA and CD44 is proteolytic cleavage of CD44 and subsequent inability of HA to stay on cell surface. The expression of CD44 is essential for the anchorage of HA onto the keratinocyte plasma membrane, as suggested by the failure of transgeneic mice lacking CD44 to retain HA in the epidermis [14]. Metalloproteinase activity, also expressed in head and neck tumours [19, 20], can cleave off the extracellular domain of CD44 [5] and cause shedding of CD44 and associated HA. Soluble, perhaps shed, forms of CD44 have been detected in the serum of cancer patients [22].

A general decrease of CD44 level has been reported in tumours of squamocellular origin [12, 23, 25, 27, 28, 31], a finding in line with the current results. Reduced expression of CD44 in endometrial carcinoma favours the release of cells from the tumour into the lymph vascular space [7]. Adhesive functions of CD44 in cell–cell and cell–matrix (HA) interactions may be important in maintaining the normal architecture of the stratified epithelia, and down-regulation of CD44 may allow the cell to detach from its surroundings and invade.

Interestingly, the irregular staining of HA and CD44 was significantly associated with mitotic activity. In accordance with the present data, a more proliferative state is associated with reduced expression of CD44 in epidermal [27] and oesophageal [6] neoplasms. However, similar unanimous correlation between proliferative activity and HA expression has not been found in epithelial cells [2, 33].

This is the first study to demonstrate the association between focally reduced expression of HA and CD44 in LSCC and general aggressiveness, as judged by histological grade and M/V index. The irregular staining pattern was also a significant, unfavourable prognostic factor for DFS in univariate analysis. From the clinical point of view, the presence of nodal and distant metastases remained superior to the molecular markers in predicting the outcome of head and neck neoplasms. However, markers in primary tumours, such as irregular HA and CD44 staining, predicting an increased likelihood of metastases, might have some clinical importance in the pretreatment staging of LSCC and in the follow-up of patients.

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