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Adhesion Molecule CD44 as a Prognostic Factor in Laryngeal Cancer

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Abstract. *Background: Loss of expression of CD44 has been shown to be a factor of poor prognosis in some types of tumors. The purpose of this study was to analyze this event in relation to the survival of patients with laryngeal cancer. Patients and Methods: The expression of adhesion molecule CD44 was studied in 137 patients with laryngeal cancer. Data were gathered on clinical (primary tumor location, pyriform sinus involvement and tongue base damage) and pathologic (T, N, differentiation, inflammatory response, tumor thickness, surgical margin involvement, and CD44 expression) parameters. Immunohistochemical studies were carried out using DF1485 anti-CD44 monoclonal antibody. Results: In 29 tumors (21.1%) <25%, in 18 (13.1%) 25%-49%, in 42 (30.6%) 50%-74%, and in 48 (35.0%) ≥75% of the neoplastic cells expressed CD44. A Cox proportional risks multivariate analysis identified CD44 expression and surgical margin involvement as the parameters most associated with survival (p<0.001). Conclusion: The reduced expression of CD44 behaves as a marker of a poor laryngeal cancer prognosis.*

The study of prognostic factors that influence the survival of patients with laryngeal cancer is of great interest because the prognostic value of the TNM system has been reported to be inadequate in patients with head and neck carcinoma (1-3). Efforts have been made to identify clinicopathological and molecular parameters (4-6) that enable the prognosis of these patients to be objectively predicted.

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Little is known about the molecular mechanisms underlying the infiltration and destruction of adjacent tissues and the metastatic expansion of tumor cells (7). One precondition may be a change in expression of intercellular adhesion molecules on the tumor cell surface (8-10). The CD44 molecule is known to be a major factor in cell-cell interactions and cell adhesion. The standard form of this molecule is CD44s, while there are variant isoforms (CD44v) derived from post-transcriptional splicing of CD44 gene mRNA (11, 12). It seems reasonable to hypothesize that loss of CD44 expression releases neoplastic cells from their adhesion to neighboring cells, favoring their invasiveness. Therefore, loss of expression of this molecule should behave as a factor of poor tumor prognosis. Some studies of laryngeal and head and neck cancer support this hypothesis (13-16). The present study analyzed the influence of tumor expression of CD44s on the survival of patients with laryngeal cancer.

Patients and Methods

A survival study was conducted on 137 patients with laryngeal cancer treated at the University Hospital of Valme, Sevilla (Spain) from 1988 to 1997 and followed up until 2003. The mean age was 60.0 years (range 37-85 yrs), and all were male.

The clinical data of the patients were obtained from hospital medical records and included primary tumor location (supraglottic, glottic, subglottic, transglottic, and pyriform), pyriform sinus involvement and tongue base damage.

Measurement of the pathologic T parameter was obtained from the pathology report in the medical records. Histopathological data and tumor thickness measurements were obtained by hematoxylin-eosin staining of formalin-fixed and paraffin-embedded operative tissue sections. Tumor involvement of cervical lymph nodes (pathologic N) was assessed according to IUAC and AJCC criteria (17). The degree of differentiation, the inflammatory response and the status of the surgical margin were also evaluated. Tumor thickness was measured using a method reported elsewhere (4). The histopathological studies were all carried out by a single pathologist (IRA).

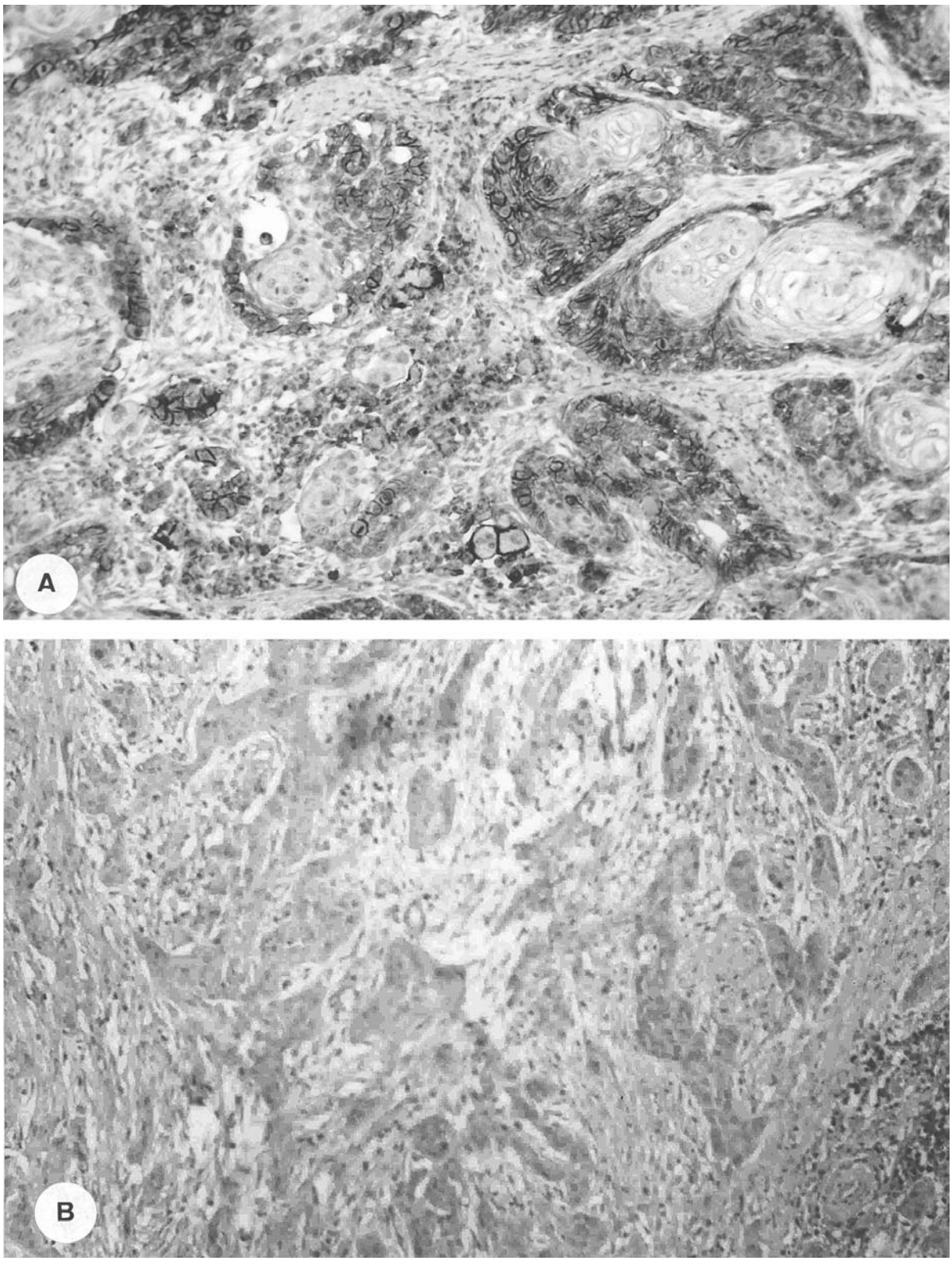

Figure 1. *Positive (A) and negative (B) CD44 expression in laryngeal cancer. Hematoxylin-eosin staining of formalin-fixed and paraffin-embedded surgical tissue sections (10x).*

Table I. *Univariate influence of age and clinical variables on survival of patients with laryngeal cancer (n=137).*

Variable	n	Cumulated survival proportion ±se at 36 months	G: Global test[a] T: Tendency test[b] P: Paired comparisons[c]
All	137	0.775±0.037	
Age (yrs)			
37-49	19	0.722±0.122	G: χ^2=3.88 (3 gl), p=0.275
50-59	45	0.739±0.068	T: $\chi^2_{tend.}$=0.07, p=0.799
60-69	56	0.852±0.048	
70-85	17	0.631±0.121	
Primary tumor location			
Supraglottic	48	0.742±0.064	G: χ^2=4.91 (3 gl), p=0.179
Glottic and subglottic	46	0.818±0.058	
Transglottic	27	0.877±0.067	
Pyriform	16	0.570±0.138	
Sinus pyriform involvement			
No	112	0.804±0.038	G: χ^2=3.17 (1 gl), p=0.075
Yes	24	0.613±0.110	
Unknown	1		
Tongue base involvement			
No	129	0.794±0.037	G: χ^2=7.54 (1 gl), p=0.006
Yes	7	0.333±0.193	
Unknown	1		

[a]Log/rank test to compare curves by categories in each variable.
[b]Tendency log/rank test (for variables with at least three ordered categories).
[c]Only statistically significant comparisons (p<0.05) are marked with the symbol "≠".

Table II. *Univariate influence of pathologic variables on survival of patients with laryngeal cancer (n=137).*

Variable	n	Cumulated survival proportion ±se at 36 months	G: Global test[a] T: Tendency test[b] P: Paired comparisons[c]
Pathologic T			
T1	5	0.800±0.179	G: χ^2=18.10 (3 gl), p<0.001
T2	17	0.816±0.096	T: $\chi^2_{tend.}$=7.10, p=0.008
T3	85	0.851±0.040	P: T2, T3≠T4
T4	29	0.481±0.103	
Unknown	1		
Pathologic N			
N0	109	0.807±0.039	G: χ^2=12.08 (2 gl), p=0.002
N1	17	0.791±0.111	T: $\chi^2_{tend.}$=8.76, p=0.003
N2-N3	10	0.400±0.155	P: N0≠N2-N3
Unknown	1		
Degree of differentiation			
Good differentiation	97	0.779±0.044	G: χ^2=2.21 (2 gl), p=0.331
Moderate differentiation	17	0.647±0.116	T: $\chi^2_{tend.}$=0.00, p=0.998
Poor differentiation	23	0.850±0.081	
Inflammatory response			
Low	26	0.960±0.039	G: χ^2=7.48 (2 gl), p=0.024
Medium	63	0.757±0.057	T: $\chi^2_{tend.}$=7.10, p=0.008
High	48	0.694±0.068	
Low≠Medium, High Depth of invasion			
<3 mm.	44	0.857±0.054	G: χ^2=6.44 (2 gl), p=0.040
3-7 mm.	15	0.929±0.069	T: $\chi^2_{tend.}$=4.78, p=0.029
>7 mm.	72	0.689±0.057	P: <3 mm.≠>7 mm.
Unknown	6		
Surgical margin involvement			
Free	122	0.835±0.035	G: χ^2=33.94 (1 gl), p<0.001
Positive	11	0.121±0.113	
Unknown	4		
CD44 tumor expression (cells percent)			
<25%	29	0.526±0.096	G: χ^2=30.50 (3 gl), p<0.001
25-49%	18	0.429±0.128	T: $\chi^2_{tend.}$=21.04, p<0.001
50-74%	42	0.923±0.043	P: <25%, 25-49%≠50-74%,
≥75%	48	0.913±0.042	≥75%

[a]Log/rank test to compare curves by categories in each variable.
[b]Tendency log/rank test (for variables with at least three ordered categories).
[c]Only statistically significant comparisons (p<0.05) are marked with the symbol "≠".

For immunohistochemical staining, 4 μm sections were cut from the paraffin blocks. After blockage of endogenous peroxidase with H_2O_2 in methanol for 30 min, the sections were immersed in citrate buffer (pH 6.0) in a microwave-resistant container. The anti-CD44 antibody used was clone DF1485 from Dako (Dako Corporation, Carpinteria, CA, USA). Sections were incubated overnight. Immunoperoxidase detection was employed using the ABC method (Dako) and diaminobenzidine substrate. Counter-staining was performed with hematoxylin. Antigen retrieval methods were used in this study. Staining of infiltrating lymphocytes was considered as a positive internal control. A carcinoma section that had not received the primary antibody was used as a negative internal control. For immunohistochemical evaluation of the CD44 molecule we considered the membrane expression (Figure 1), calculating the percentage of positive epithelial cells with respect to the total number of cells encountered in 20 representative high-power fields. We took no account of the intensity of the staining. We formed groups of tumors according to the percentage of stained cells (<25%, 25%-49%, 50%-74% and ≥75%) (7).

Table III. *Factors associated with survival of patients with laryngeal tumors by Cox's regression (n=133)*[a].

Variable	β (se)	e^{β} (95%-CI)	Wald F p-value
Surgical margin involvement			16.487 <0.001
Free (reference category)	0.00	1.00	
Positive	1.79 (0.44)	6.02 (2.53-14.30)	
Tumor expression of CD44 (% cells)			16.376 <0.001
<25%, 25-49% (reference category)	0.00	1.00	
50-74%, ≥75%	1.73 (0.43)	5.62 (2.43-12.96)	

[a]After excluding 4 cases with missing values. See Methods for an explanation of the model.

Table IV. *Association of pyriform sinus involvement and pathologic T grade with CD44 expression in 137 tumors.*

Variable		Tumor expression (% cells) (n=137)			
	n	<25%	25-49%	50-74%	≥75%
Sinus pyriform involvement					
No	112	18.80%	11.60%	30.40%	39.30%
Yes	24	29.20%	20.80%	33.30%	16.70%
Unknown	1				
Association[a]			p=0.029		
Pathologic T					
T1	5	20.00%	20.00%	40.00%	20.00%
T2	17	23.50%	5.90%	23.50%	47.10%
T3	85	16.50%	12.90%	30.60%	40.00%
T4	29	31.00%	17.20%	34.50%	17.20%
Unknown	1				
Association[b]			r_s=-0.16, p=0.069		

[a]Chi-square test or Fisher's exact test (dysplasia and hyperplasia) and Mann-Whitney test (tumor expression).
[b]Mann-Whitney test (dysplasia and hyperplasia) and Spearman correlation (r_s) (tumor expression).

The statistical analysis was performed using SPSS for Windows, version 12.0 (SPSS Inc. Chicago, Illinois, USA). Survival times of the patients was gathered until 2003, distinguishing between complete (death from laryngeal cancer) and incomplete (alive at last available date) times. No deaths for other reasons were recorded during the follow up. Survival analysis was performed by means of the Kaplan-Meier method (18). A study was conducted of the association between survival curves and different variables, grouping some categories to permit the analysis (Tables I and II) and using global log-rank tests or, for variables with three or more ordered categories, tendency log-rank tests. When there was statistical significance, pair-comparisons were carried out (18). Possible confounding factors were investigated using the Cox proportional hazards model (19). Potential variables were classified as described above, considering one of the categories as indicator, and were included in the model according to their statistical significance (forward stepwise method, with $p<0.05$ to enter and $p>0.10$ to exit the model, according to the likelihood ratio). During the construction of the model, variables that showed a correlation greater than 0.75 with previously included variables were excluded to avoid collinearity effects. The categories of the variables in the final model were further collapsed to obtain narrower confidence intervals.

Results

The distribution of age and clinical variables are shown in Table I. The majority of patients in the series were 50-69-years-old (101 cases). The localization of the laryngeal carcinoma was predominantly supraglottic (48 cases) or glottic and subglottic (46 cases). The pyriform sinus and tongue base were involved in 24 and 7 cases, respectively. Pathological variables and CD44 expression are listed in Table II. Most patients had large tumors at the diagnosis (T3=85 cases, T4=29 cases), and there was involvement of cervical lymphatic nodes in 27 cases. In general, the tumors were well-differentiated (97 cases) with considerable invasion depth (invasion depth of >7 mm in 72 cases). In most cases, the surgical margins were tumor-free (122 cases). Forty-eight patients, 35% of the total, presented CD44 expression in ≥75% of cells (Table II). No patient presented distant metastases (data not shown). The mean 36-month survival rate for the whole series was 77.5% (standard error = 3.7%).

An analysis of the influence of age and clinical parameters on survival is shown in the last two columns of Table I. Only involvement of the tongue base by the tumor clearly influenced the survival of the patients ($p=0.006$). CD44 expression and all histopathological parameters studied, except for degree of tumor differentiation, were significantly associated with survival (Table II). In the present series, a greater tumor expression of CD44 was associated with a better tumor prognosis. Multivariate

analysis showed that the parameters with the greatest influence on survival were CD44 expression and surgical margin involvement (Table III). Once these variables were included in the model, no other variable entered.

CD44 expression loss was significantly ($p=0.029$) associated with pyriform sinus involvement and showed a relationship close to significance ($p=0.069$) with the pathologic T stage of the tumor (Table IV).

Discussion

Among a series of 137 patients with laryngeal cancer, 90 (65.6%) presented CD44 expression in at least 50% of the neoplastic cells, within the range reported in studies of cancer oral, laryngeal cancer, and head and neck cancer (7, 14, 15, 20-22). One of the most important aspects of CD44 expression in laryngeal cancer is its influence on patient survival. In the present study, a reduction in CD44 expression had a negative effect on survival according to the univariate analysis ($p<0.001$) and also entered as a variable in the Cox proportional hazards model. It seems reasonable to conclude that the loss of CD44 expression releases cells from their adhesion to neighboring cells and favors their invasiveness, thereby behaving as a factor of poor prognosis. Our results support this hypothesis. However, the findings of other research groups have been contradictory. Some studies of oral cancer showed that a reduction in the expression of CD44v7 and CD44v9 had a negative influence on both survival and disease-free period (7, 20). Our group previously demonstrated that loss of CD44s expression is an early event in lingual carcinogenesis (23). According to one study (16), although loss of expression affects only one tumor group, it increases the risk of death. This observation is also valid for the loss of expression of CD44s and CD44v6 in patients with laryngeal carcinoma, also significantly associated with a reduction in survival (16). In contrast, the relationship between CD44 and survival is different in some other tumors. Stoll *et al.* (7) described three types or groups of tumors according to the relationship of CD44 expression to survival. CD44 expression was correlated with poor prognosis in one group, which included cancer of stomach, colon, breast, cervix, vulva, and malignant lymphomas. In a second group, including ovarian and bronchial carcinomas, CD44 expression had no effect on survival or the effect was not clearly established, such as in renal carcinoma and malignant melanoma. In the third group, loss of CD44 expression was correlated with a poor prognosis, such as in neuroblastoma, epidermal skin tumor and, as commented on above, oral and laryngeal carcinomas. On the other hand, a review of the literature (7) showed that many tumors have been classified into one or other group on the basis of only one or two studies. This clearly seems inadequate, especially given the variability in the method of assessing CD44 expression and in the CD44 isoforms studied. Aberrant, non-functional forms of CD44 may also have been produced by certain neoplastic cell types. It is important to bear in mind that the immunohistochemical expression of a protein does not necessarily imply function (24) and *in vitro* studies (25) showed that CD44 expression in squamous cells was independent of function. Finally, it was also proposed (15) that differences in the prognostic significance of CD44 may reflect a specifically regulated expression in each organ that differs according to the organ in question or even according to the pathologic type of the tumor.

A previous study by our group (4) demonstrated the importance of tumor thickness as an independent prognostic variable in tongue cancer. Tumors with a depth greater than 3 mm had a significantly worse prognosis than those invading less than 3mm. Mackay *et al.* (11) reported an increase in invasion and cell migration of a malignant melanoma cell line after treatment with anti-CD44s monoclonal antibodies. Sato *et al.* (26) showed that the invasiveness of OSCC cell lines expressing high levels of CD44v9 (HSC-2 and HSC-3) significantly increased after treatment with anti-CD44v9 monoclonal antibodies, whereas the invasiveness of lines weakly expressing CD44v9 were not affected by the antibody treatment. Similar results were reported by Kanke *et al.* (15).The present investigation showed that laryngeal tumors with greater losses of CD44 expression did not present significantly greater invasion depths (Spearman correlation coefficient $r_s=-0.06$, $p=0.516$). Although our interpretation in tongue cancer was that loss of CD44 expression, by favoring cellular detachment, increases the invasiveness of the tumor and behaves as the original reason for the increased mortality in patients with deeper tumors (27), some anatomical features of the larynx that are clearly different from those of the tongue (purely muscular organ with no invasion barriers) probably explain this difference. However, no published study has investigated the influence of CD44 expression loss on the invasive capacity of laryngeal cancer.

In our view, the present findings of a significant association between loss of CD44 and involvement of the pyriform sinus ($p=0.02$), and of a relationship close to significance between loss and pathological T stage ($p=0.06$) indicate that tumors of the larynx that lose CD44 expression have a greater growth and expansion potential. Nevertheless, the published research on laryngeal carcinomas is contradictory. Thus, some studies reported a direct and statistically significant relationship between CD44 expression and clinical (28) or pathological (29) tumor size, one group (30) found no relationship between CD44 expression and tumor size, and others demonstrated that reduced CD44 expression appeared with significantly higher frequency in

tumors of larger size (21), more advanced stage (31), and higher mitotic index (21). In this context, Ostwald *et al.* (32) observed major differences in the intensity of CD44 labeling depending on the area of the tumor nidus studied, with a high intensity of expression by cells of the external peripheral area and a low or absent expression in internal areas. The authors (32) concluded that CD44 can be a valid proliferation marker and attributed its higher expression at the periphery of the nidus to the proliferative activity of malignant cells in these areas, which could indicate a tendency to expansive growth of the tumor nidus. On the other hand, the results observed by Ostwald *et al.* (32) may have an alternative interpretation. For Sato *et al.* (20), CD44 expression in well-differentiated nidi was similar to that in normal squamous epithelium, with positive expression at the periphery being equivalent to the basal and suprabasal expression of normal epithelium and absent expression at the center equivalent to the lack of expression in the upper layers of normal epithelium. Therefore, CD44 expression at the periphery of tumor niduses with absent expression at their center may indicate a good tumor differentiation and a consequent reduction in cell proliferation activity. Our findings indicate that tumors with greater loss of CD44 expression more frequently involve adjacent structures (specifically the pyriform sinus) and can probably reach a larger size. In our view, since tumors with little or no CD44 expression deviate from the normal differentiation and maturation pattern of healthy laryngeal epithelium, they may acquire characteristics that allow them to proliferate more intensely (21). However, further research is required on the relationship between proliferation markers and the expression pattern of these adhesion molecules.

In conclusion, the reduced expression of CD44 behaved as a marker of a poor tumor prognosis and demonstrated an independent influence on the survival of patients with laryngeal cancer.

Acknowledgements

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