ORIGINAL ARTICLE

Prognostic Value of CD44 Variant 6 in Laryngeal Epidermoid Carcinomas

Gülnur Güler, MD; Sarp Saraç, MD; Ayşegül Üner, MD, PhD; Erdem Karabulut, MD; Ayşe Ayhan, MD, PhD; Ogawa Hiroshi, MD

Background: CD44 variant exon 6 (v6) belongs to a family of transmembrane glycoproteins involved in cell adhesion.

Objectives: To determine the prognostic role of CD44v6 in laryngeal cancer and to examine its relation with other clinicopathologic prognostic factors.

Design: A retrospective cohort study was designed with 93 laryngeal cancer cases. They were selected randomly from patients treated with laryngectomy between January 1, 1983, and December 31, 1993.

Setting: Faculty of Medicine, Hacettepe University, Ankara, Turkey.

Patients: The ages of the patients ranged from 31 to 73 years. Eighty-eight patients were men and 5 were women. Three had stage I, 33 had stage II, 27 had stage III, and 30 had stage IV disease at the time of surgery.

Intervention: Histological sections of tumors and metastatic lymph nodes were reevaluated for several histopathological factors. Sections were stained using anti-CD44v6 monoclonal antibody by immunohistochemical methods.

Results: CD44v6 expression was seen only in the lower one third of the normal squamous epithelium but in all layers of dysplasia and in situ carcinoma. Besides a general evaluation of tumor staining, immunostaining was evaluated separately for cell groups located in the center of neoplastic islands (*nonbasal cells*), at the periphery of the neoplastic islands (*basal cells*), and at the infiltration zones (*marginal cells*). Decreased disease-free survival was noted when there was extensive staining in the general evaluation and in cases with extensive staining in marginal and nonbasal cells ($P=.03$). Using Cox regression analysis, the greatest dimension of the largest metastatic lymph node and extensive expression of CD44v6 in nonbasal tumor cells were independent prognostic factors.

Conclusion: Our results suggest that CD44v6 expression is an important prognostic factor in laryngeal cancer.

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From the Departments of Pathology, Faculty of Medicine, Hacettepe University, Ankara, Turkey (Drs Güler, Saraç, Üner, Karabulut, and Ayhan), and Seirei Mikatabara Hospital, Hamamatsu, Japan (Dr Hiroshi).

THE TNM classification system is widely used to determine the stage and appropriate therapy in laryngeal epidermoid carcinomas.[1] However, it is well known that tumors in the same stage may show different biological behavior.[2] For this reason, it is worthwhile to investigate new prognostic factors in laryngeal cancer.

The *CD44* gene, located on chromosome 11p13, encodes a large family of transmembrane glycoproteins. It is composed of at least 21 exons. Various isoforms of CD44 protein are produced via alternative splicing. Ten exons (1-5 and 16-20) are expressed in all tissue types, producing standard CD44 protein. The remaining 11 exons are added to form variant protein isoforms.[3,4] CD44 variant protein 6 (CD44v6) is produced by the insertion of exon 11 to the standard form. This variant form was first identified in rat pancreatic carcinoma cell lines and was found only in their metastatic clones.[5]

Many investigations have been performed to determine the prognostic value of CD44v6 in various human tumors. CD44v6 overexpression correlated with a worse prognosis in colorectal carcinoma, breast carcinoma, pancreas carcinoma, and non-Hodgkin's lymphoma.[6,7] Studies[3,8-10] that show no correlation or a positive correlation between CD44v6 overexpression and prognosis in different types of human malignancies have also been reported. The purpose of our study was to investigate the prognostic significance of CD44v6 expression in laryngeal epidermoid carcinomas.

MATERIALS AND METHODS

Ninety-three case records of laryngeal epidermoid carcinoma diagnosed between January 1, 1983, and December 31, 1993, were retrieved from the archives of the department of pathology. Various clinical features and therapy for these patients are summarized in **Table 1**.

All the sections were reexamined, and histopathological factors of the primary tumor and metastatic lymph nodes were reevaluated. Separate grades were assigned to each tumor based on nuclear, structural, and keratinization features. Dimensions of the tumor, tumor depth, histologic location of deepest invasion, infiltration pattern, presence of lymphovascular and perineural invasion, presence of dysplasia or in situ carcinoma in tumor margins, growth pattern of tumor, ulceration, necrosis, degree of cellular reaction against the tumor, mitotic activity in the tumor, presence of metastatic lymph nodes, number of metastatic lymph nodes, diameter of the biggest metastatic lymph node and metastatic focus, and presence of extracapsular invasion were considered.

A representative block was chosen for each case, and immunohistochemical staining was performed using a streptavidin-biotin-peroxidase complex procedure.[11] After deparaffinization and dehydration with xylene and alcohol, 4-mm-thick sections were incubated with 1% hydrogen peroxide in methanol for 15 minutes and immersed in phosphate-buffered saline for 5 minutes. For reducing unspecific background staining, blocking with normal serum diluted 1:40 in phosphate-buffered saline at pH 7.2 containing 1% bovine serum albumin for 20 minutes was performed. Sections were treated by microwave 3 times for 5 minutes each in citrate buffer (pH 6.0) at 620 W power. Subsequently, they were incubated for 1 hour with a 1:200 dilution of antihuman CD44v6 monoclonal antibody (Clone VFF-18; Bioproducts, Heidelberg, Germany). Subsequent to washing twice with phosphate-buffered saline at pH 7.2 for 5 minutes, biotinylated goat antimouse immunoglobulin (DAKO, Glostrup, Denmark) was applied at a dilution of 1:100 in phosphate-buffered saline with 1% bovine serum albumin at pH 7.2 for 30 minutes, followed by peroxidase-conjugated streptavidine in a 1:100 dilution medium for 30 minutes. Finally, peroxidase activity was visualized by diaminobenzidine, followed by counterstaining with hematoxylin. In negative controls, the primary antibody was replaced by dilution medium. Normal squamous epithelial staining in histologic sections was used as an internal positive control. Two pathologists, blinded to clinical results, evaluated immunostaining simultaneously. Staining properties of normal squamous epithelium, dysplastic epithelium, and in situ carcinoma were noted. The entire section was evaluated for immunohistochemical staining, which corresponds to at least 20 low-power fields. The proportions of positive neoplastic cells were evaluated in tumors and metastatic lymph nodes. Immunostaining was judged as negative (≤10%), focally positive (11%-89%), or extensively positive (≥90%). Immunostaining of neoplastic cells was appraised generally and separately as *basal cells*, *nonbasal cells*, and *marginal cells* (see the "CD44v6 Immunohistochemical Study" subsection of the "Results" section).

χ^2 Test, Fisher exact test, and independent sample *t* test were used to evaluate the data obtained from this study. The patients' disease-free survival data (DFS) were used to determine the possible correlation between the histopathological criteria, CD44v6 expression, and prognosis of the cases. Survival curves were constructed using the Kaplan-Meier method. The statistical significance of these data was analyzed by the log-rank test. Variables affecting survival were analyzed by the Cox proportional hazards regression model.

RESULTS

CLINICAL DATA

The ages of the patients ranged from 31 to 73 years (mean, 52 years). Eighty-eight patients (95%) were men and 5 (5%) were women. Clinical staging according to the criteria of the American Joint Committee on Cancer[12] revealed that 3 (3%) patients had stage I disease, 33 (36%) had stage II, 27 (29%) had stage III, and 30 (32%) had stage IV. Patients had been followed up for at least 2 years, with a median follow-up of 33.4 months.

From the evaluation of clinical data, lymph node status and stage of the disease had a statistically significant relation with DFS ($P=.01$ and $P<.01$, respectively). In 14 of 43 patients in whom lymph nodes were enlarged, histological examination did not prove metastasis, giving a false-positive rate of 33%. In contrast, metastasis was seen histologically in 16 of 50 patients in whom lymph nodes were not palpable, giving a false-negative rate of 32%. Age, sex, and clinical T value did not have a statistically significant relation with DFS.

There was a statistically significant difference in DFS between patients who received no additional therapy and those who received additional therapy in the form of chemotherapy or radiotherapy ($P<.01$).

HISTOPATHOLOGICAL FACTORS

The tumors were divided into 2 groups based on the pattern of invasion. The tumors with pattern 2 had infiltration as 1-layered cords or single tumor cells. All the remaining tumors were considered under pattern 1. According to these criteria, invasion patterns of the tumor correlated significantly with DFS ($P=.04$), and the presence of perineural invasion had a borderline significant relation with DFS ($P<.06$). Existence of histopathologically proven node metastasis was highly significant ($P<.01$). For this reason, other factors related to lymph node involvement were examined only in the group of patients who had lymph node metastasis. Even after elimination of the importance of histopathologically proven lymph node metastasis, the number of metastatic lymph nodes ($P=.02$) and the greatest dimension of the largest metastatic lymph nodes ($P=.01$) had statistically significant prognostic value.

CD44v6 IMMUNOHISTOCHEMICAL STUDY

In the immunohistochemical study, normal squamous epithelium showed membranous CD44v6 expression only in the lower one third. In contrast, in dysplasia and in situ carcinoma, all cell layers showed positive staining (**Figure 1**). In some tumors, the cell groups in the cen-

ter of the neoplastic islands (*nonbasal cells*) stained less intensely compared with the *basal cells* that were located at the periphery of the neoplastic islands (**Figure 2**). There was also more intense staining of the neoplastic cells located at the infiltration zone of the tumor (*marginal cells*) (**Figure 3**). In addition to a general examination, immunostaining of so-called basal, nonbasal, and marginal cells was evaluated separately.

Statistical analysis in the general examination revealed that extensive staining with CD44v6 correlated with a significant decrease in DFS ($P=.03$). Furthermore, a similar decrease in DFS was noted when the CD44v6 staining was extensive in marginal and nonbasal cells ($P=.03$ and $P=.02$, respectively) (**Figure 4**).

In the immunohistochemical study, the mean proportion of staining of the neoplastic cells was 70% in primary tumors and 85% in the metastatic lymph nodes. The proportion of lymph node metastasis staining was higher in the primary tumor staining, but this difference was not statistically significant ($P=.10$).

When the factors having a significant relation with DFS in univariate analyses (**Table 2**) were evaluated in Cox regression analysis, the greatest dimension of the largest metastatic lymph nodes and extensive expression of CD44v6 in nonbasal tumor cells were independent prognostic factors (**Table 3**). However, in the patients who received chemotherapy or radiotherapy, the statistically significant effect of CD44v6 expression in nonbasal cells on DFS was no longer apparent ($P>.05$).

Table 1. Clinical Features of the 93 Patients*

Clinical Feature	No. (%) of Patients
Location	
Glottic	3 (3.2)
Supraglottic	38 (40.9)
Subglottic	1 (1.1)
Transglottic	49 (52.7)
Not available	2 (2.2)
Stage (AJCC)	
I	3 (3.2)
II	33 (35.5)
III	27 (29.0)
IV	30 (32.3)
Type of laryngectomy	
Total	69 (74.2)
Supraglottic	18 (19.4)
Vertical	3 (3.2)
Subtotal	3 (3.2)
Neck dissection	
Right	21 (22.6)
Left	25 (26.9)
Bilateral simultaneously	7 (7.5)
Bilateral asynchronously	40 (43.0)
Radiotherapy	
Postoperative	32 (34.4)
Preoperative	7 (7.5)
Not received	54 (58.1)
Chemotherapy	
Received	15 (16.1)
Not received	78 (83.9)

*Some percentages do not sum to 100 because of rounding. AJCC indicates American Joint Committee on Cancer.

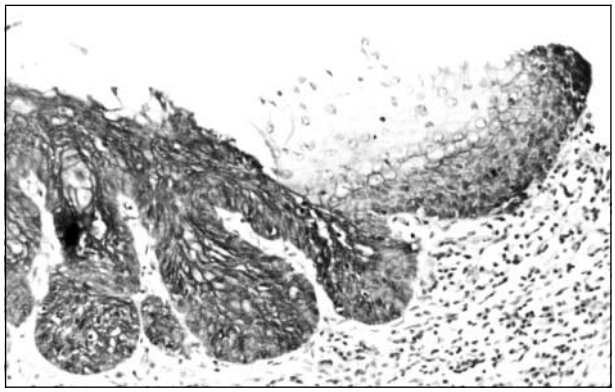

Figure 1. CD44 variant exon 6 expression in normal and neoplastic squamous epithelium (hematoxylin-eosin, original magnification ×400).

COMMENT

Overexpression of CD44 in adenomatous polyps and in the transitional zones near the tumor reported in colon cancers supports the view that expression of the CD44 variant forms begins early in colorectal carcinogenesis.[13,14]

Likewise, in our study, in normal squamous epithelium of the larynx, positive CD44v6 immunostaining could be seen only in the lower one third of the epithelium, with a change in the pattern in dysplasia and in situ carcinoma. All cell layers of dysplastic epithelium and in situ carcinoma stained positively in the cell membranes using CD44v6 antibody. Similar findings were reported in 2 other previous studies[9,10] of laryngeal cancer, suggesting its abnormal expression also begins early in the carcinogenesis of cancer of the larynx.

In the literature, it has been reported that the overexpression of CD44v6 correlated with a worse prognosis in various cancers, such as colon, breast, gastric cancer, and non-Hodgkin's lymphoma.[3,4,6,7] In contrast, other studies[3,8-10,15,16] have shown that overexpression of CD44v6 was associated with a better prognosis in adenocarcinomas of the lung, neuroblastomas, squamous cell carcinomas of the head and neck, cervical cancer, and transitional carcinomas of the bladder. CD44v6 immunostaining was investigated in laryngeal cancer in 2 previous reports. In one of them, increased CD44v6 expression was associated with a longer survival.[9] In the other one, Ostwald et al[10] reported that they could not find any relation between CD44v6 expression and metastatic behavior.

Down-regulation of CD44v6 expression during malignant transformation of squamous tissues was reported using a different anti-v6 antibody (Var3.1).[17,18] Similarly, Roye and colleagues[19] suggested that overexpression of CD44v6 may be involved in the development of esophageal dysplasia and carcinoma, but its expression was decreased in poorly differentiated esophageal epidermoid carcinoma. van Hal et al[20] investigated head and neck epidermoid carcinomas and cell lines using 3 different anti-v6 antibodies (U36, U39, and VFF-18). Immunohistochemistry was performed on tumors and cell lines, and v6-encoding splice variants were also characterized by screening a complementary DNA library of human head and neck squamous carcinoma cell lines, using reverse transcriptase polymerase chain reaction. In their investigation, there was

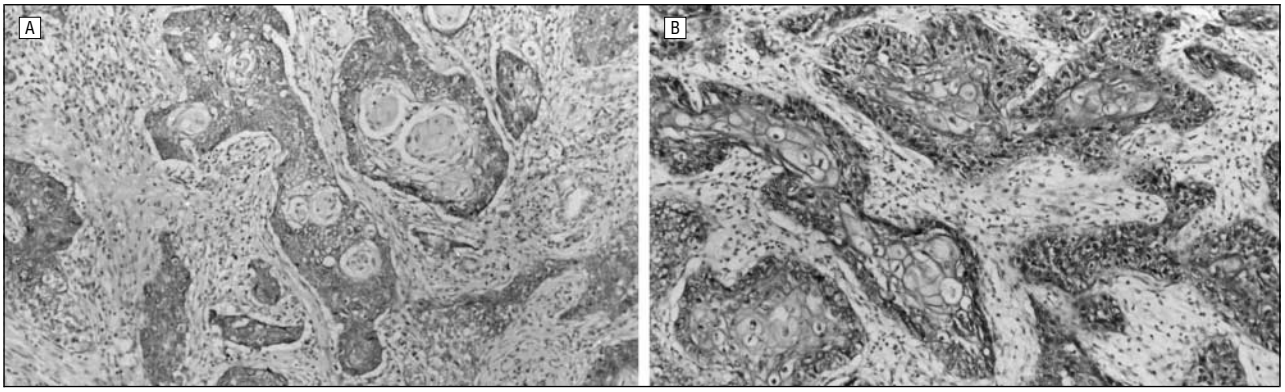

Figure 2. A, Nonbasal cell negativity in tumor with CD44 variant exon 6 (CD44v6, original magnification ×200). B, Extensive CD44v6 expression in nonbasal cells (CD44v6, original magnification ×100).

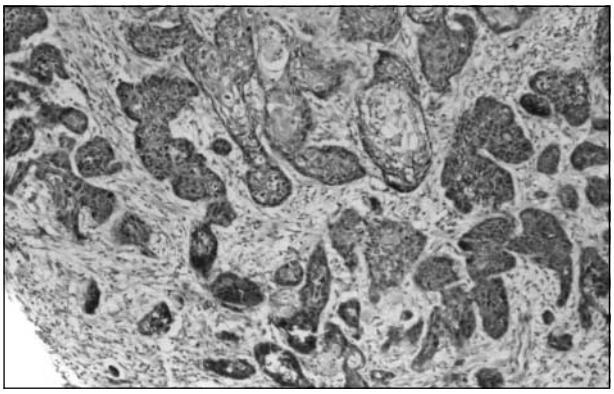

Figure 3. Extensive expression of CD44 variant exon 6 in marginal tumor cells (CD44v6, original magnification ×100).

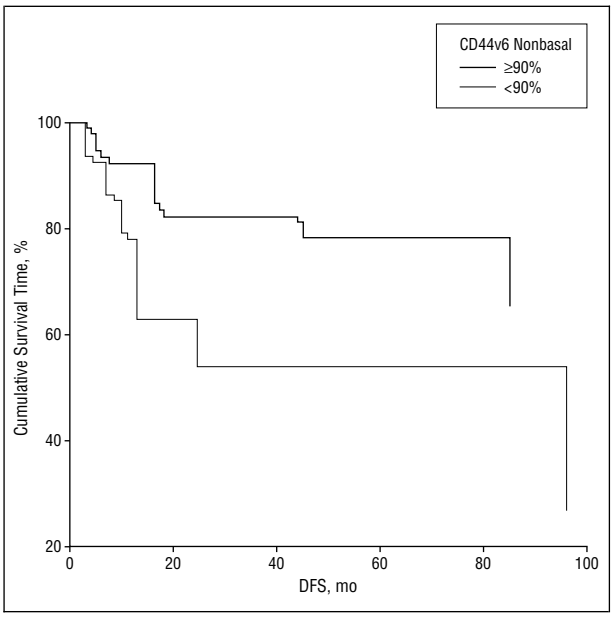

Figure 4. CD44 variant exon 6 (v6) positivity in nonbasal tumor cells and disease-free survival (DFS).

marginal or no down-regulation of CD44v6 in malignant tissue samples.

Although these discrepancies may be attributed, in part, to the differences in immunohistochemical methods and primary antibodies used, they may also be related to the methods of evaluation. For this reason, we tried to use a uniform evaluation system based on location and proportion of immunopositive cells. Furthermore, the different observations obtained in different studies may also stem from the heterogeneity of various clinical features, such as the location of the tumor and therapy of patients in these studies.

Table 2. Mean Values of Staining Proportion

Histological Section	Staining Proportion, %
Whole neoplastic cells	70
Basal neoplastic cells	80
Nonbasal neoplastic cells	60
Marginal neoplastic cells	80
Metastatic lymph nodes	85

Table 3. Multivariate Analyses of Prognostic Factors for Disease-Free Survival: Summary of Stepwise Results

Prognostic Factor	χ^2	Multivariate *P* Value	Relative Risk (95% Confidence Interval)
Greatest dimension of largest metastatic lymph nodes*	8.9	.003	4.6 (1.7-12.6)
≥90% Staining of CD44 variant exon 6 in nonbasal cells	20.5	.000	6.5 (2.9-14.6)

*Differences between the patients who have 0, ≤2-cm, and >2-cm metastatic lymph nodes.

Disease-free survival decreased significantly when the proportion of immunopositive nonbasal cells was 90% or greater, and this factor was an independent prognostic factor. This finding suggests that abnormal expression of CD44v6 may also have a role in the progression of laryngeal epidermoid carcinomas. In cases showing a staining pattern similar to that of normal squamous epithelium, the prognosis was better than in those with extensive nonbasal staining. Although, when all cases were included, the extensive nonbasal staining was an independent prognostic factor, such an effect was no longer apparent in the patients who received additional therapy. This finding suggests that patients with extensive nonbasal CD44v6 expression may benefit from additional radiotherapy or chemotherapy. If this series had included only the patients who

did not receive additional therapy, the significance of CD44v6 expression on DFS probably would have been more pronounced. In the previous 2 reports that dealt with CD44v6 expression in laryngeal cancer, different staining patterns in nonbasal and basal cells were also noticed, but they were not evaluated separately.[9,10]

Lipponen et al[15] used a similar method for evaluation of immunohistochemical staining of CD44v6 in transitional cell carcinomas of the bladder. Using a 15% cutoff value for positivity without performing extensive staining, they documented survival in patients with CD44v6-positive nonbasal cells.

In metastatic lymph nodes, although not statistically significant, the percentage of tumor cells expressing CD44v6 was higher compared with that in their primary tumors, indicating a further role in progression. Similarly, CD44v6 was expressed in 80% of the primary tumor samples, but in 100% of the metastatic tumors in colon cancer.[21]

Another finding of our study was a significantly shorter DFS that was associated with extensive CD44v6 staining in marginal tumor cells. Although we could not find any related finding in the literature, Sugino et al[22] reported a correlation between the gradual loss of standard CD44 protein expression and the invasive stage of the tumor in bladder carcinoma. Expression of the structurally complex *CD44* gene in cancer may be modulated by microenvironmental factors. Accordingly, expression patterns may change as the invading neoplastic cells encounter different mesenchymal components in the microenvironment.[23] In uterine cervical cancer, loss of expression of CD44v6 was also noticed in advanced invasive tumors.[24] However, we could not find any relation between extensive CD44v6 staining in marginal tumor cells and the depth of invasion.

In conclusion, an abnormal expression pattern of CD44v6 seems to begin early in the carcinogenesis of laryngeal epidermoid carcinomas. As the expression pattern deviates from that of normal squamous epithelium, the abnormality in expression increases, with faster progression of the tumor and a significantly shortened DFS. In the patients who received additional therapy, the effect of extensive CD44v6 expression in nonbasal cells was no longer obvious. This suggests that patients with this adverse prognostic factor may benefit from additional therapy.

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