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Original article

Do Immunohistochemical Studies Have a Role in Predicting Prognosis of Laryngeal Squamous Cell Carcinomas? CD44 and Fascin Experience

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Abstract. Background and objectives: The diagnosis of laryngeal squamous cell carcinoma (LSCC) can be made easily based on histopathological findings, but the relationship between morphological findings and prognosis is not clear. In addition to morphological findings, the use of novel markers may contribute to the development of new treatment strategies and improved patient prognosis. CD44, which is a cancer stem cell marker, and Fascin-1, an actin-binding protein has been associated with poor prognosis in many tumors. The aim of this study was to investigate the relationship between CD44 and Fascin-1 expression and clinicopathologic parameters in LSCC and their roles in the determination of clinical behavior and prognosis. The aim of this study is to investigate whether CD44 and Fascin have a relationship with clinicopathological parameters and have a role in determining clinical behavior and prognosis in LSCC. Methods: 130 patients who were operated in our hospital for LSCC between 2012 and 2018 were included in this study. Fascin-1 and CD44 stains were applied immunohistochemically to the paraffin blocks of the tumors. Immunostained specimens were scored according to the intensity of staining and the percentage of staining for each marker. Overall scores were summed and was designated as immunoreactivity score (IRS). Finally, IRS was categorized into two groups; Low and High CD44/Fascin IRS. Results: There were no statistically significant differences between low and high CD44 and Fascin IRS groups in terms of clinicopathologic parameters, overall and disease-free survival ($p > 0.05$). Conclusion: Immunhistochemical studies are not yet sufficient to predict patient prognosis. Morphological findings still remain of priority and importance for pathologists. (www.actabiomedica.it)

Key words: Larynx, squamous cell carcinoma, CD44, Fascin

Introduction

Laryngeal squamous cell carcinoma (SCC) is the most common carcinoma of the respiratory system after lung carcinomas (1). While the diagnosis of laryngeal squamous cell carcinoma can be made easily based on histopathological findings, the relationship between morphological findings and prognosis is not clear (2).

Although significant improvements have been made in the surgical and radiotherapy techniques of laryngeal cancers, no significant change has been detected in the prognosis of the patients and the five-year life expectancy is approximately 60% (3).

In addition to morphological findings, various cytogenetic and molecular abnormalities have been shown to have prognostic significance (4). Using novel markers may contribute to the development of

new treatment strategies and improved patient prognosis (5). One of these, CD44, is a cancer stem cell marker (6). It is a surface adhesion molecule released from stem cells that program the properties of cancer cells such as regeneration, progression, invasion, local recurrence, resistance to chemotherapy, and radiotherapy (7). CD44 expression has been associated with tumor progression in many tumors (8).

Fascin-1 is an actin-binding protein that plays a role in many physiological functions of the cell and assists the cell motility in determining the risk of metastasis in many tumors (9). In laryngeal cancers, a positive correlation has been found between Fascin-1 expression and tumor grade, and lymph node metastasis in some studies (10).

The study aimed to investigate the relationship between CD44 and Fascin-1 expression and clinicopathological parameters in laryngeal squamous cell carcinoma and its role in the determination of clinical behavior and prognosis.

Materıals and Methods

The surgical specimens of 130 patients who had undergone partial and total laryngectomy for laryngeal cancer in our hospital between 2012 and 2018 were included in the study.

Demographic characteristics, tumor localization, tumor diameter, cervical lymph node metastasis, total follow-up time, presence of recurrence, distant metastasis were obtained from the original pathology reports, and patient files. Moreover, missing information pertaining to the patients was filled through telephone interviews with patients themselves or their relatives.

We chose a representative block from each tumour for immunohistochemical analysis. A 2-mm section from each formalin-fixed, paraffin-embedded tumour tissue block which containing all morphological features of tumours was automatically immunostained using CD44 (Mouse monoclonal antibody, ready to use, Genetex) and Fascin (Mouse monoclonal antibody, FCN01-SSK2, ready to use, Thermo Scientific), using the avidin-biotin-peroxidase technique. The sections were incubated with the primary antibody at room temperature for 60 minutes.

Hodgkin lymphoma tissue for Fascin and tonsil tissue for CD44 were used as external control and also vascular endothelium for Fascin; inflammatory cells for CD44 were used as an internal control. The staining reaction was evaluated using a scoring system that was modified by Boxberg et al (21). Immunostained specimens were independently examined by two investigators and, in the instance of a discrepancy, by the third individual; the investigators were blinded to the origin of the specimen. Specimens were scored according to the intensity of staining (1, none to weak; 2, moderate; 3, strong) and the percentage of tumor cells stained (1,0–5% positive; 2, 5–25% positive; 3, 25–50% positive; 4, 50–75% positive; 5, 75–100% positive) for each marker. Overall immunohistochemical scores for Fascin and CD44 expression were calculated for each case by summing the scores for staining intensity and percentage. This score was designated as an immunoreactivity score (IRS). Possible IRS ranged from 2 to 8. Finally, we categorized the IRS into two groups; 4 and below: low CD44/Fascin IRS and over 4: high CD44/Fascin IRS.

CD44/ Fascin IRS was compared according to following parameters: Age, sex, localization, tumor diameter, histological grade, lymph node metastasis, clinical stage and overall survival and disease-free survival of the cases.

In this study, statistical analysis was performed by NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA). Descriptive statistical methods (frequency and percentage distributions) were used to evaluate the data, and the chi-square test was used to compare qualitative data. Survival analysis of CD44, Fascin IRS groups were evaluated by Kaplan Meier and Log-Rank test. The results were evaluated with a significance level of $p < 0.05$ and a 95% confidence interval.

Results

Of the 130 patients, 128 were male (98.46%) and 2 were female (6.2%). The ages of the patients ranged from 42 to 89 years. 42 cases (32.31%) were supraglottic, 42 cases (32.31%) were glottic, 1 case subglottic (0.77%), 45 cases (34,46%) were in transglottic location.

Cervical lymph node metastasis was present in 48 (36.92%) of the cases. Extracapsular dissemination was seen in 27 (54.00%) of the patients with neck metastasis. The patients had a follow-up period of 12–86 months and 90 (69.23%) of the patients were alive without recurrence or metastasis at the time of statistical analysis, 13 (10.00%) survived with relapse or metastasis, 20 (15%) 38) patient died because of cancer; 7 patients (5.38%) died because of other reasons.

90 (69.23%) of the cases were keratinized type SCC, 26 (20.00%) were nonkeratinized type SCC and 14 (10.77%) were basaloid type SCC. The majority of the cases were evaluated as Grade 2 (91, 70.00%), 22 cases (16.92%) as Grade1, and 17 cases (13.08%) as Grade 3 according to WHO criteria.

There was no significant difference in age and gender between the low and high CD44/Fascin IRS groups. ($p > 0.05$). According to the WHO grading system, no statistically significant difference was observed between the groups ($p > 0.05$). There was no statistically significant relationship between tumor localization, histological subtypes, lymph node metastasis, T and N stage, and CD44/Fascin IRS groups. ($p > 0.05$).

There was also no statistically significant difference between CD44 / Fascin IRS low and high groups in terms of lymphovascular and perineural invasion, presence of necrosis, amount of stroma, and lymphocytic response accompanying tumor ($p > 0.05$) (Table 1,2).

Table 1. Clinicopathological variables by overall CD44 expression score

		≤4 CD44 n:59		>4 CD44 n:71		p
Age	<65	42	71.19%	49	69.01%	0.788
	>65	17	28.81%	22	30.99%	
Sex	Female	1	1.69%	1	1.41%	0.895
	Male	58	98.31%	70	98.59%	
Localization	Supraglottic	17	28.81%	25	35.21%	0.778
	Glottic	20	33.90%	22	30.99%	
	Subglottic	1	1.69%	0	0.00%	
	Transglottic	17	28.81%	20	28.17%	
	Glottic+Subglottic	4	6.78%	4	5.63%	
WHO Grade	Grade 1	11	18.64%	11	15.49%	0.635
	Grade 2	42	71.19%	49	69.01%	
	Grade 3	6	10.17%	11	15.49%	
T Stage	T1	6	10.17%	4	5.63%	0.279
	T2	31	52.54%	39	54.93%	
	T3	20	33.90%	20	28.17%	
	T4	2	3.39%	8	11.27%	
N Stage	N0	35	59.32%	47	66.20%	0.538
	N1	5	8.47%	4	5.63%	
	N2	6	10.17%	10	14.08%	
	N3	13	22.03%	10	14.08%	
AJCC Stage	Stage 1	5	8.47%	4	5.63%	0.397
	Stage 2	17	28.81%	27	38.03%	
	Stage 3	17	28.81%	16	22.54%	
	Stage 4a	7	11.86%	14	19.72%	
	Stage 4b	13	22.03%	10	14.08%	

(*continued*)

		≤4 CD44 n:59		>4 CD44 n:71		p
Lymphovascular invasion	No	33	55.93%	47	66.20%	0.231
	Yes	26	44.07%	24	33.80%	
Perineural invasion	No	50	84.75%	61	85.92%	0.851
	Yes	9	15.25%	10	14.08%	
Necrosis	No	18	30.51%	31	43.66%	0.123
	Yes	41	69.49%	40	56.34%	
Lymphocyte host response	Mild	10	16.95%	16	22.54%	0.707
	Modarete	30	50.85%	35	49.30%	
	Intense	19	32.20%	20	28.17%	

Chi-square test

Table 2. Clinicopathological variables by overall Fascin expression score

		≤4 Fascin n:39		>4 Fascin n:91		p
Age	<65	2955.546 pt	74.36%	62	68.13%	0.478
	>65	10	25.64%	29	31.87%	
Sex	Female	1	2.56%	1	1.10%	0.534
	Male	38	97.44%	90	98.90%	
Localisation	Supraglottic	10	25.64%	32	35.16%	0.127
	Glottic	12	30.77%	30	32.97%	
	Subglottic	1	2.56%	0	0.00%	
	Transglottic	11	28.21%	26	28.57%	
	Glottic+Subglottic	5	12.82%	3	3.30%	
WHO Grade	Grade 1	8	20.51%	14	15.38%	0.681
	Grade 2	27	69.23%	64	70.33%	
	Grade 3	4	10.26%	13	14.29%	
T Stage	T1	3	7.69%	7	7.69%	0.980
	T2	20	51.28%	50	54.95%	
	T3	13	33.33%	27	29.67%	
	T4	3	7.69%	7	7.69%	
N Stage	N0	25	64.10%	57	62.64%	0.736
	N1	3	7.69%	6	6.59%	
	N2	3	7.69%	13	14.29%	
	N3	8	20.51%	15	16.48%	
Lymphovascular invasion	No	23	58.97%	57	62.64%	0.694
	Yes	16	41.03%	34	37.36%	
Perineural invasion	No	35	89.74%	76	83.52%	0.357
	Yes	4	10.26%	15	16.48%	

		≤4 Fascin n:39		>4 Fascin n:91		p
Necrosis	No	15	38.46%	34	37.36%	0.906
	Yes	24	61.54%	57	62.64%	
Tumor-Stroma ratio	Stroma-poor	34	57.63%	48	67.61%	0.812
	Stroma-rich	25	42.37%	23	32.39%	
Lymphocyte host response	Mild	6	15.38%	20	21.98%	0.664
	Modarete	20	51.28%	45	49.45%	
	Intense	13	33.33%	26	28.57%	

Chi-Square Test

In some of the cases, more intense staining with CD44 was observed on the invasive margin (Figure 1, 2). The pictures showing staining with CD44 and Fascin of the cases are shown in figures 3-8.

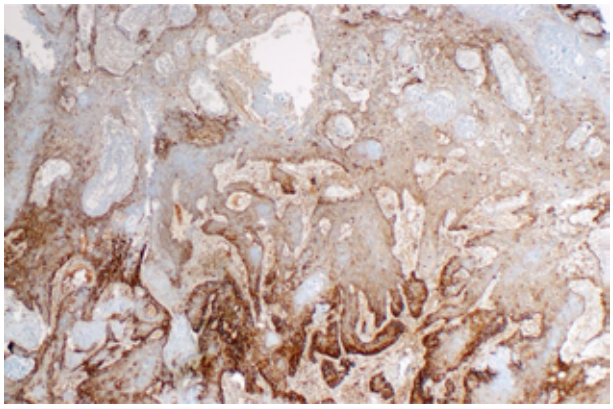

Figure 1. More intense CD44 expression at the invasive margin compared to the main tumor mass (CD44x40).

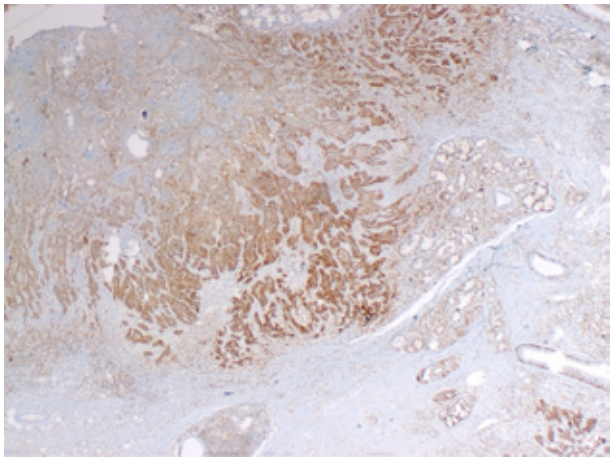

Figure 2. More intense CD44 expression at the invasive margin compared to the main tumor mass (CD44 x100).

Figure 3. High intensity and prevalence of CD44 immunoreactivity in the tumor (CD44 x40).

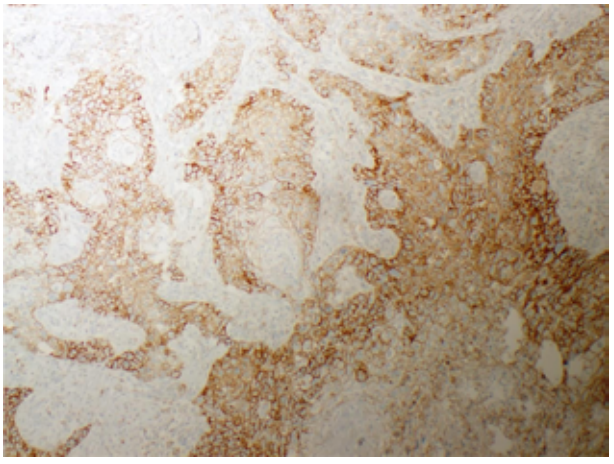

Figure 4. CD44 immunoreactivity with moderate intensity and prevalence in the tumor (CD44 x100).

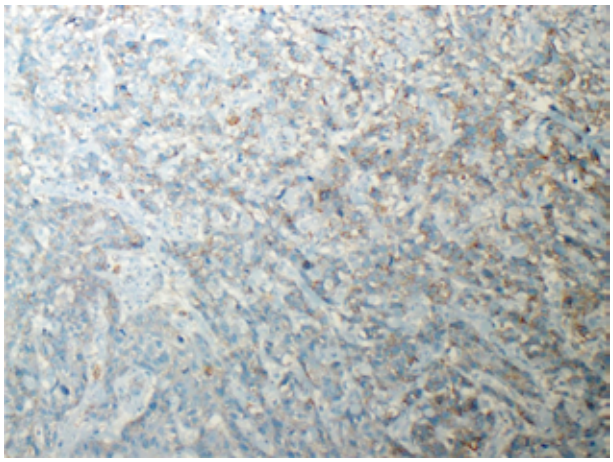

Figure 5. Low-intensity CD44 immunoreactivity in the tumor (CD44 x200).

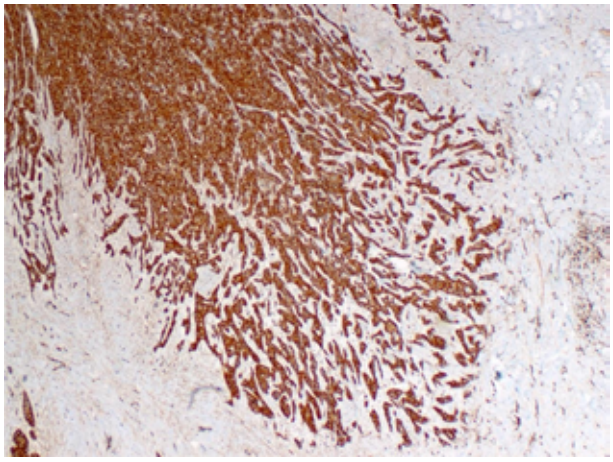

Figure 6. Intense immunoreactivity with Fascin-1 in the entire tumor (Fascin-1 x40).

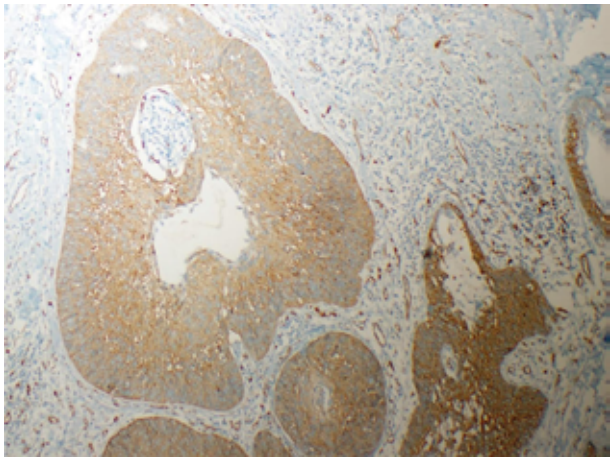

Figure 7. Moderate immunoreactivity with Fascin-1 in the tumor (Fascin-1 x100).

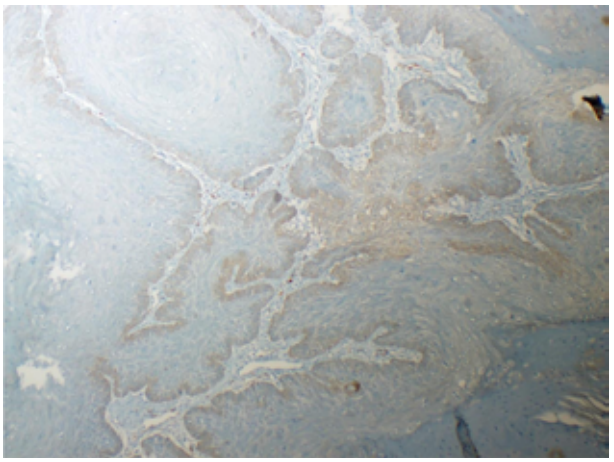

Figure 8. Low intensity and percentage of immunoreactivity with Fascin-1 in the tumor (Fascin-1x40).

Relationship between CD44 expression and survival of patients

The 5-year disease-free survival of the CD44-IRS-low group was found to be 65.11–80.29%, and the 5-year disease-free survival of the CD44-IRS-high group was 63.57–77.27%. Additionally, the 5-year overall survival of the low CD44 IRS group was found to be 64.41–80.12%, the 5-year overall survival of the high CD44 IRS group was 62.65–76.92% and no statistically significant difference was observed between the survival time of the groups ($p>0.05$). The survival curve of the cases is shown in Figures 9 and 10.

Relationship between CD44 expression and survival

The 5-year disease-free survival of the low Fascin IRS group was 63.29–83.03%, and the 5-year disease-free survival of the high Fascin IRS group was 63.39–75.79%. In addition, the 5-year average survival rate of the low Fascin IRS group was 64.48-83.23%, and the 5-year average survival rate of the high Fascin IRS group was 64.33–76.21%.>0.05). Disease-free and general survival curves of the cases are shown in Figures 11 and 12.

Discussion

Laryngeal squamous cell carcinoma (SCC) is the most common carcinoma of the respiratory system

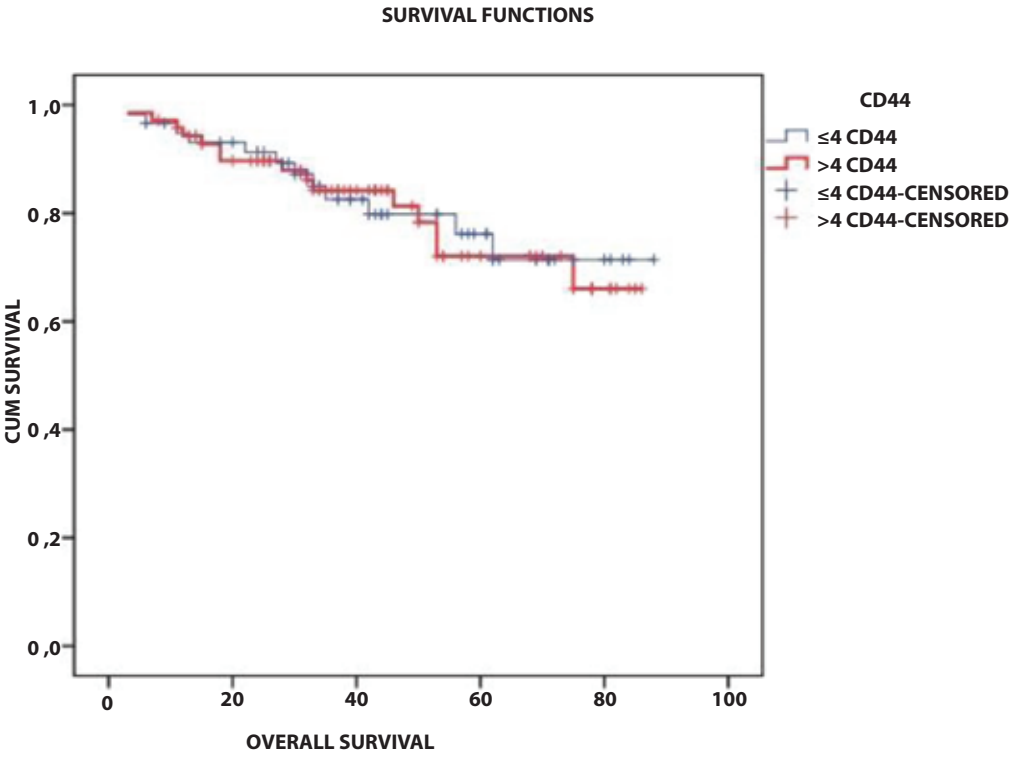

Figure 9. Overall survival curve of CD44 IRS groups (Kaplan Meier, p>0.05).

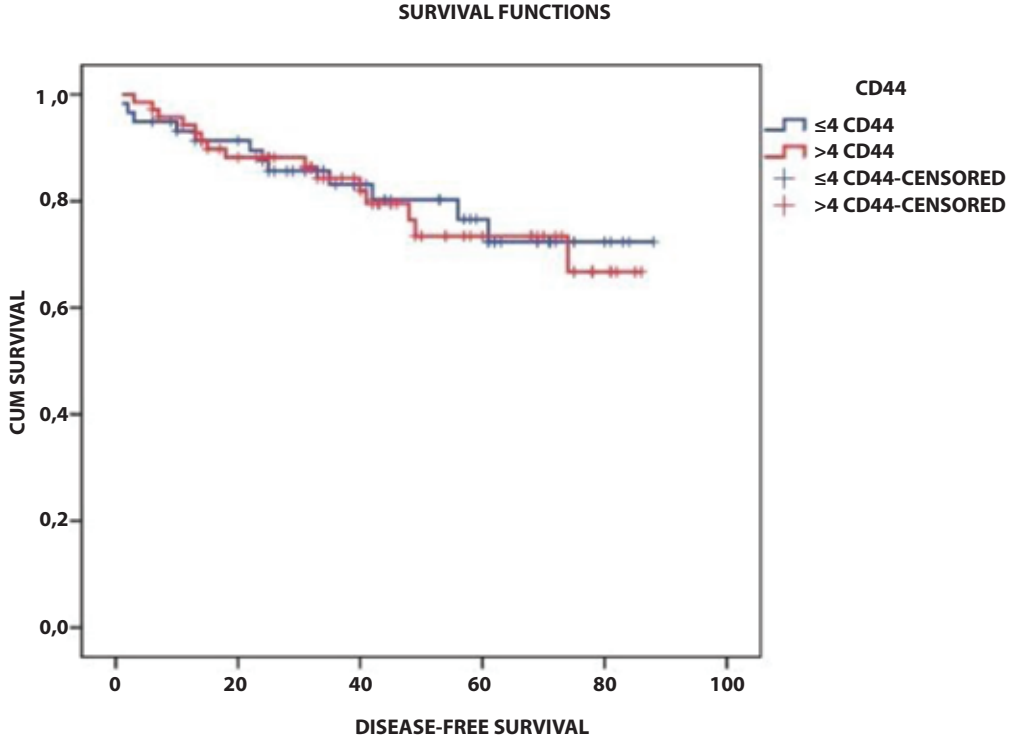

Figure 10. Disease-free survival curve of CD44 IRS groups (Kaplan Meier, p>0.05).

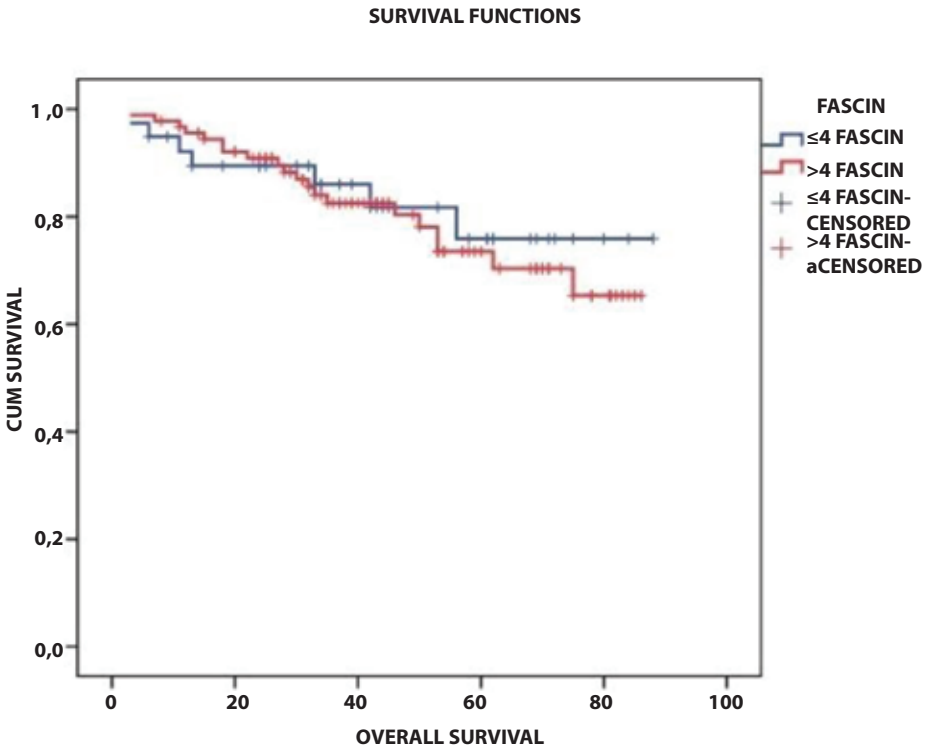

Figure 11. Overall survival curve of Fascin IRS groups (Kaplan Meier, p>0.05).

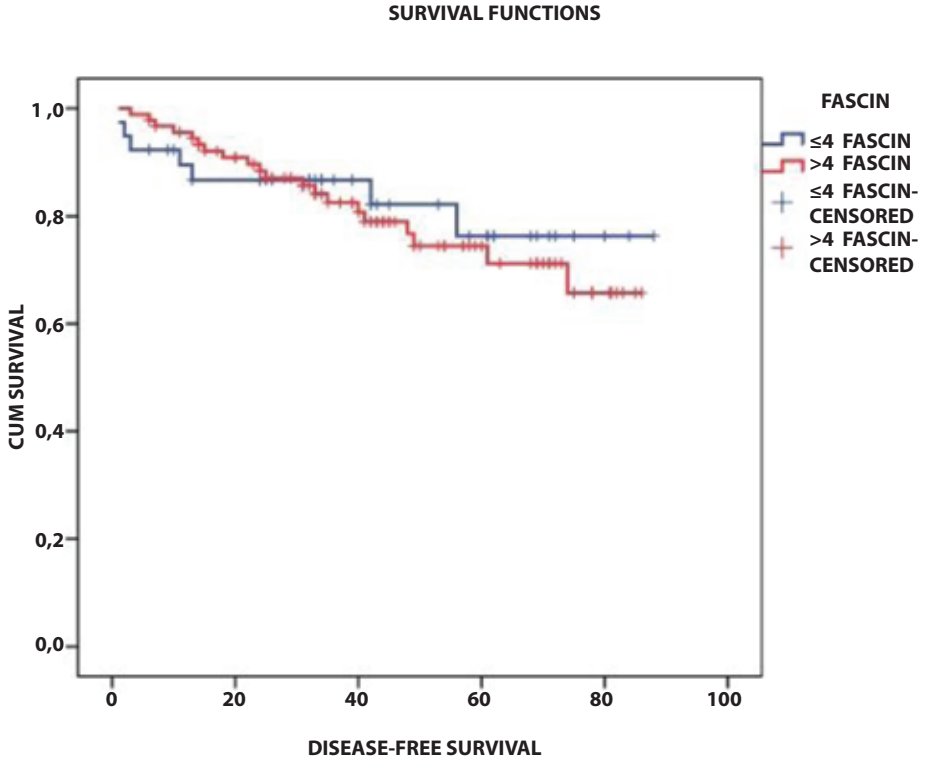

Figure 12. Disease-free survival curve of Fascin IRS groups (Kaplan Meier, p>0.05).

after lung carcinomas (1). Laryngeal SCC is graded by WHO into 3 groups as well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (11). This grading system which was proposed by Broders in 1920 is based on cellular pleomorphism, similarity to normal squamous epithelium; (12).

Cervical lymph node metastasis and/or local recurrence occurred in 10% of the patients who were included in our study, and 15.38% of the patients died because of laryngeal cancer.

Predicting poor prognosis in these patients could contribute to their survey. However, morphological parameters used in reporting to predict prognosis may prove to be insufficient. Therefore, in addition to morphological findings, immunohistochemical and molecular markers have also been investigated to predict prognosis (16). If the behaviour of the tumor can be predicted with by new biomarkers, different methods can be applied in the management of laryngeal carcinoma.

In our study, we investigated the expression levels of CD44 which is a cancer stem cell marker and Fascin proteins that play a role in cell mobility, by immunohistochemical method.

Epithelial cells detach from the tumor and lose their epithelial properties, gain mesenchymal features, and cancer stem cell characteristics. These cells, characteristically same with cancer stem cells, are capable of regeneration and metastasis unlike other cells of the same tumor. Therefore, cancer stem cells are responsible for tumor growth, invasion and recurrence. The presence of cancer stem cells has been identified in various cancers such as melanoma, lung, breast, colon, and stomach cancer (17). CD44 is a cancer stem cell marker and its expression is associated with tumor progression, recurrence, and chemotherapy resistance, similar to the characteristics of cancer stem cells (8).

It is possible to detect CD44 by a variety of methods such as Elisa, RT-PCR, mRNA analysis, and immunohistochemistry. Since the immunohistochemical method is simple, reproducible, and cost-effective, our study is based on the immunohistochemical method. Although CD44 expression is associated with poor prognosis in many tumors, its prognostic value in head and neck tumors remains unclear (18–20).

Boxberg et al. investigated the association of CD44 expression with histomorphologic parameters and clinicopathologic factors in oral SCCs. They found a significant association between CD44 expression and tumor budding, small tumor cell nest, and poor differentiation (21).

In this study, CD44 expression was evaluated separately in the main tumor mass and invasive margins of the tumor; CD44 expression in the main tumor mass was associated with poor prognosis, whereas CD44 expression at the invasive margin was not associated with prognosis.

In our study, CD44 expression was evaluated by grouping it as low and high according to the immunoreactivity score (IRS) based on the percentage and intensity of staining in the main mass of the tumor.

In addition, CD44 expression was found to be stronger in some cases at the invasive margin of the tumor. Therefore, the intensity of CD44 expression at the invasive margin was also evaluated in all cases. Higher expression of CD44 in the invasive tumor cell may be the cause of stronger staining at the invasive margin. As the pT stage of the cases increased, more patients were found in the high CD44 IRS group, but this was not statistically significant. Of the 10 patients in the pT4 stage, 8 were in the high CD44 IRS group, but the majority of patients (pT1: 10 patients, pT2: 70 patients, pT3: 40 patients, pT4: 10 patients) were clustered in the pT2 stage in our study. CD44 expression can be evaluated as more reliable in studies that employ a broader sample of patients data in all stages.

In contrast to studies in the literature that high CD44 expression is associated with poor prognosis, there are also studies suggesting that low CD44 expression is associated with poor prognosis in different localizations of the head and neck (6, 7, 22–24). And also, in some studies, no correlation was found between CD44 expression and prognosis (20). In our study, no correlation was found between CD44 expression and overall and disease-free survival of the patients.

Due to conflicting results in the literature, the relationship between CD44 expression and prognosis should be reviewed with new comprehensive studies consisting of a higher number of patients.

Fascin is an actin-binding protein, involved in cell motility which was first detected in 1970 (25). There are many studies in the literature on whether to use it as a biomarker to predict the behavior of the tumor and guide its treatment.

In a review study by Hashimoto et al. investigating the role of Fascin in human cancer cells, it was reported that Fascin is not only released from malignant cells but also from healthy epithelial cells. Thus it has been suggested that Fascin expression has a limited place in targeted therapies, yet it may detect early stages of the aggressive clinical course of tumors (26).

In the literature, Jawhari et al. found that cells that express the Fascin molecule at a higher rate had higher adhesion ability and showed more invasive properties in colonic cancer cells, in vitro (27). Cao et al. investigated the relationship between expression of 15 cell skeletal proteins, including Fascin, and patients' survival in esophageal SCCs. They found that Fascin molecule alone was not significant in patient survival. In the same study, the use of all proteins that made up the cell skeleton as a cocktail had been found to yield significant results in the determination of prognosis (24). There are other studies in the literature in which Fascin expression alone does not produce significant results (28). Therefore, the use of Fascin protein in combination with other proteins that play a role in the cell skeleton, may give more beneficial results.

Zhang et al. examined the distribution of Fascin expression between normal epithelium, hyperplastic epithelium, atypical hyperplasia, carcinoma in situ and invasive carcinoma; They found that Fascin expression increased because of proliferative activity of the cell. They suggested that increased Fascin expression in progression from normal epithelium to invasive carcinoma may be valuable in recognizing early stage esophageal SCC. However, no significant association was found between Fascin expression and prognostic parameters in the same study (29). Conversely to the study of Zhang et al., studies are suggest that increased Fascin expression levels correlate with tumor grade, stage and metastasis and poor prognosis in esophageal, non-small cell lung carcinomas, breast, colon, ovarian, pancreatic and prostate cancers (27, 30, 31).

Durmaz et al. evaluated Fascin expression in a group of 30 patients with laryngeal SCC and found that patients with high Fascin expression had a worse prognosis and they concluded that Fascin expression increased as the T, N, clinical-stage increased (32). In our study, no statistically significant difference was found between pT1, pT2, pT3, pT4 stages in terms of Fascin expression. This result contradicts with the findings of Durmaz et al. Such a discrepancy may arise from the fact that their study conducted using a smaller sample of patients.

In a study of 216 patients, Gao et al. found that Fascin, Ezrin and Paxillin immunohistochemical expression correlates with advanced stage (pT3, pT4), poor differentiation, lymph node involvement, and advanced clinical stages (III, IV) (10). In our study, no statistically significant difference was found between Fascin expression and pT, pN stage, and differentiation.

The reason that our findings do not correspond to the study of Gao et al. may be due to the tendency of our cases to cluster into the early stages. More reliable results can be achieved with larger case series representing all stages. Gao et al. also observed more Fascin expression in patients with supraglottic localized laryngeal SCC than glottic involvement. In our study, Fascin IRS was found to be high in 32 of 42 supraglottic laryngeal SCC cases. Similarly, Fascin IRS was found to be high in 30 of 42 glottic cases. Laryngeal SCC is seen in different localizations in different geographical regions. Therefore, whether Fascin expression varies by localization should be supported by new multicenter data. Furthermore, since lymphatic drainage of the larynx differs according to localization, it may be more appropriate to compare tumors in the same location for reliable results.

Papaspryrou et al. found no significant relationship between Fascin expression and pT stage, pM stage, sex, localization, disease-free survival, and overall survival in oropharynx SCCs however, they found that Fascin expression was associated with the pN stage of patients (33).

In our study, no significant correlation was found between Fascin expression and age, sex, localization, pT, pN, pM stages of the patients.

Zou et al. find that Fascin and E-cadherin expression was associated with the pT stage, lymph node metastasis, and histologic grade in patients with laryngeal SCC (34).

In our study, Fascin IRS was found to be high in 34 of 48 patients with cervical metastasis, but this finding was not statistically significant. In addition, there was no significant association between WHO grade groups and Fascin IRS low and high groups. This may

be due to the clustering of our cases in grade 2 and the low reproducibility of the WHO grading system among pathologists.

Few studies investigate the relationship between Fascin expression and other parameters that may have a role in prognoses such as lymphovascular invasion, perineural invasion, necrosis, tumor stroma ratio and tumor accompanying lymphocyte infiltration. In our study, the relationship between these parameters and fascin expression was investigated, but no significant result was obtained.

Conclusion

The WHO grading system currently used in laryngeal carcinomas is based on subjective criteria and has low reproducibility among pathologists. Therefore, it is necessary to look for a new marker or morphological finding in prognosis estimation. In our study, CD44 and Fascin expression were not associated with prognosis. In conclusion, immunohistochemical studies may not be useful in predicting prognosis; morphological findings remain of priority and importance for pathologists.

New and comprehensive immunohistochemical and molecular studies are needed to find new markers that may be effective in predicting prognosis and treatment management.

Conflicts of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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