Relationship of E-cadherin, Beta-catenin, N-cadherin, ZEB1 and αSMA as Epithelial Mesenchymal Transition markers with prognostic factors in early and advanced stage laryngeal squamous cell carcinomas

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Abstract

Objective: To investigate the relationship between E-cadherin, Beta-catenin, N-cadherin, ZEB1 and αSMA as eithelial mesenchymal transformation markers with tumor stage, lymph node metastasis (LNM) and overall survival (OS) in laryngeal squamous cell carcinomas (LSCC). Material and method: A total of 100 cases diagnosed with LSCC in our hospital between 2013-2020 were included in the study. Data about lymphovascular invasion (LVI), perineural invasion (PNI), necrosis and LNM were recorded by evaluating hematoxylin- eosin stained slides. Markers of E-cadherin, beta-catenin, N-cadherin, ZEB1 and αSMA were applied to the sections prepared from paraffin blocks of tumor samples. Results: Ninety-five male and five female patients were included in the study, and 38 of them exited. The average OS time of the cases was 35.8 months. A significant relationship was observed between OS with advanced tumor stage, presence of LNM and PNI. A significant relationship was found between increased tumor Zeb1 expression and advanced tumor stage. In univariate and multivariate analyses, a significant negative relationship with OS, and increased Zeb1 expression in tumor and tumor stroma was seen. Any relationship was not observed between E-cadherin, beta-catenin, N-cadherin and αSMA and OS. , Conclusion Among the EMT markers we evaluated in our study, it was seen that Zeb1, which is an EMT transcription factor, is associated with tumor stage, LNM, and OS. Remarkably, Zeb1 expression observed in tumor stroma was also significant for OS. Any similar data reported for LSCCs have not been encountered in the literature, and it was thought that it would be appropriate to support our findings with further studies to be performed on this subject.

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Results: Ninety-five male and five female patients were included in the study, and 38 of them exited. The average OS time of the cases was 35.8 months. A significant relationship was observed between OS with advanced tumor stage, presence of LNM and PNI. A significant relationship was found between increased tumor Zeb1 expression and advanced tumor stage. In univariate and multivariate analyses, a significant negative relationship with OS, and increased Zeb1 expression in tumor and tumor stroma was seen. Any relationship was not observed between E-cadherin, beta-catenin, N-cadherin and αSMA and OS. ,

Conclusion

Among the EMT markers we evaluated in our study, it was seen that Zeb1, which is an EMT transcription factor, is associated with tumor stage, LNM, and OS. Remarkably, Zeb1 expression observed in tumor stroma was also significant for OS. Any similar data reported for LSCCs have not been encountered in the literature, and it was thought that it would be appropriate to support our findings with further studies to be performed on this subject.

Key words. Laryngeal carcinoma, E-cadherin; Beta-catenin; N-cadherin; ZEB1, SMA

What is already known about this topic; Signal activation of epithelial mesenchymal transition (EMT) in the tumors is thought to be associated with invasion, metastasis, recurrence and the development of resistance to treatment.

What does this article add: There are controversial results regarding the relationship between the presence of EMT and the prognosis in laringeal squamous cell carcinomas (LSCC). In our study, it was noted that only as an EMT-TF, Zeb1 is associated with poor prognosis in LSCC. In addition Zeb1 expression observed in tumor stroma is also significant for overall survival.

Ρελατιονσηιπ οφ Ε-ςαδηεριν, Βετα-ςατενιν, Ν-ςαδηεριν, ΖΕΒ1 ανδ αΣΜΑ ας Επιτηελιαλ Μεσενςηψμαλ Τρανσιτιον μαρκερς ωιτη προγνοστις φαστορς ιν εαρλψ ανδ αδανςεδ σταγε λαρψνγεαλ σχυαμους ςελλ ςαρςινομας

1.Introduction Laryngeal cancer (LC) is the most frequently observed malignant tumor of the larynx, and laryngeal squamous cell carcinoma (LSCC) accounts for approximately 90% of the cases [19]. LSCC is a tumor with a high risk of metastasis and a dismal prognosis. Despite advances in diagnosis and treatment methods, 5-year survival rates for LSCC are still not at the desired level [1,2]. The reason for the inadequacy in treatment may be related to complex molecular changes that may cause treatment resistance and difficulties in detecting molecular markers that will enable predetermination of tumor aggressiveness [3]. Therefore, studies on genetic regulatory networks involved in progression of LSCC have gained momentum in order to develop individualized treatment methods and to determine more appropriate treatment methods for the patient [1,2]

Epithelial mesenchymal transformation (EMT); is defined as the loss of typical properties of epithelial cells such as adhesion and apical-basal polarity (ie. E-cadherin, alpha and beta-catenin), and gaining a mesenchymal- like phenotype (ie. N-cadherin, vimentin, αSMA) [2,4,5]. Signal activation of EMT in the tumor is thought to be associated with invasion, metastasis, recurrence and the development of resistance to treatment [6]

There are studies in the literature on EMT in squamous cell carcinomas of head and neck (HNSCC). However, it has been reported that it would be more appropriate to evaluate LSCCs separately from HNSCCs because of the different embryonic origin of the larynx and different clinical and biological behaviors they demonstrate [7]. In this study, we aimed to investigate the relationship between E-cadherin, Beta-catenin, N-cadherin, ZEB1 and markers of αSMA in LSCC with tumor stage, lymph node metastasis (LNM) and overall survival (OS).

2.Material and method :

A total of 100 patients with complete clinical follow-up data who underwent partial / total laryngectomy with a diagnosis of LSCC in our hospital between 2013-2020 were included in the study. Age, gender,

tumor location, tumor stage, and survival information of the patients were obtained from the departments of Otorhinolaryngology and Radiology. Hematoxylin - eosin stained slides of the cases in our archive were re-evaluated by two pathologists (ÜK, SE). Data about lymphovascular invasion (LVI), perineural invasion (PNI), necrosis and lymph node metastasis (LNM) associated with the tumor were recorded.

2.1.Immunohistochemistry:

All tumor specimens were fixed in 10% buffered formalin and embedded in parafin according to standard procedures. Serial 5µm- thick sections were placed on positively charged slides. Immunohistochemical (IHC) detection of E- cadherin (Mouse mAb clone NCH-38, IR05961-2, DAKO), β- catenin (Mouse mAb clone beta-cat-1, IR70261-2, DAKO), N- cadherin (Mouse mAB clone D-4, sc-8424, Santa Cruz, CA, USA), smooth muscle actin (Mouse mAb, Clone 1A4, DAKO), and Zeb1 (Rabbit pAb, ab87280, ing ABCAM) were performed. All IHC stainings were performed by an autostainer Link AS48 (DAKO, Denmark) which uses the envision flex system. The sections were observed and photographed using an Nicon Eclipse Ni microscope equipped with a Nicon digital sight DS-U3 camera.

2.2.Immunohistochemical evaluation:

Cytoplasmic and / or membranous staining was considered for the detection of E-cadherin and beta-catenin in tumor cells. Immunoreactive scoring (IRS) was performed based on staining percentage and intensity using a semi-quantitative approach. Staining density was evaluated as follows: 0; no staining, 1; weakly positive, 2; moderately positive, 3; strongly positive. Percentages of tumor cells stained were determined as follows: <5%, no staining, 1: <6-25%, 2; 26-50%, and 3; 51-100% staining. While calculating the IRS score, staining pattern, and intensity scores were multiplied and two groups were formed based on the product of the multiplication as downregulation ([?]3), and overexpression (>3) [7].

Percentages of nuclear and cytoplasmic staining in tumor cells for N-cadherin were scored separately based on the percentage of stained cells as follows: 1: 0-20%: 2: 21-40%, and 3: > 40% [8].

For Zeb1, percentage of nuclear staining in tumor cells was scored as follows: 1: 0-9%: 2: 10-50%, and 3:> 50%. Staining intensity was evaluated as follows: 0; no staining, 1; weakly positive, 2; moderately positive, 3; strongly positive. The final score was grouped as negative ([?]1) and positive (> 1) based on the product of multiplication between scores of staining percentage and intensity [9].

Zeb1 nuclear positivity in fibroblasts observed in the stroma surrounding the tumor was also scored. The percentage of stained cells was scored as follows: 1:<10%: 2:11-50% and 3:> 50%.

Cytoplasmic and / or membranous staining in fibroblasts located in the surrounding stroma with αSMA was considered and scored as follows: 1: <10%; 2: 11-50% and 3: > 50%.

2.3.Statistical analyses:

Statistical analyses were performed with SPSS v.24. The chi-square test was used the determine correlations among the variables. Statsitically analyses of prognostic effects of pathological tumour stage, LNM, LVI, PNI, necrosis and EMT markers on OS were performed. Univariate OS were estimated using the Kaplan-Meier method and log-rank statistics. Multivariate Cox regression models addresses OS. Statsitical significance was set at $p<0.05$.

3.Results:

Partial laryngectomy was performed in 37 and total laryngectomy in 63 cases. Ninety-five cases were male, 5 were female, and the median age of all cases was 63 (min: 43-max: 84, SD: 9.72) years. Sixty –one patients survived, and 38 cases exited, while survival information of one case was not available. The median OS time of the cases was 35.8 months (min: 2-max: 118, SD: 24.7).

Sixty–five glottic, 22 supraglottic, 1 subglottic, and 12 transglottic tumors were examined The average size of the tumors was 2.64 cm (min: 0.60 cm-max: 8cm, SD: 1.35). Distribution of pathological stages of the tumors was as follows: stage 1; n=22, stage 2; n=6; stage 3; n=22, stage 4A; n=35: and stage 4B; n=15.

LNM was detected in 34 cases. Histopathological examination revealed LVI in 25, PNI in 22 and necrosis in 30 of the cases. The data are summarized in Table 1.

Immunohistochemically (IHC) E-cadherin was overexpressed in 93 and downregulated in 7 tumors. Membranous staining was observed in 82, cytoplasmic and membranous staining in 16, and only cytoplasmic staining in 2 cases. Overexpression of beta-catenin was observed in 60, and its downregulation in 40 cases. Membranous staining was seen in 29, cytoplasmic and membranous staining in 66, and only cytoplasmic staining in 5 cases. Staining intensity with N-cadherin was scored as follows: Score 1 in 93, score 2 in 6, and score 3 in one tumor sample. Nuclear staining was noted in 18, cytoplasmic staining in 14, cytoplasmic and membranous staining in 1 case, while no staining was observed in 67 cases. Zeb1-negativity was detected in 91, and Zeb1-positivity in 9 tumor cells.

Percentage of Zeb1 positivity in the stroma surrounding the tumor was also evaluated. Accordingly, Zeb1 positivity was observed below 10% in 19, between 10-50% in 48, and over 50% in 33 cases. The density of fibroblasts in the surrounding stroma was scored using αSMA. Accordingly, the staining density was [?] 10% in 19, between 10-50%, in 48 and and above 50% in 41 cases.

3.1.Relationship between clinicopathological data and survival

When the pathological tumor stage and survival was compared, respective number of patients in stages 1 (n=5: 22.7%), 2 (n=1: 16.7%), 3 (n=8: 36.4%), 4A (n=12: 35.3%), and 4B (n=12: 80%) lost their lives. According to log -rank test, a significant negative correlation was observed between advanced pathological stage and OS (p=0.001) (Figure 1). Twenty-one (61.8%) patients with LNM and 17 (26.6%) cases without metastasis exited. A significant negative correlation was observed between the presence of LNM and OS (p<0.001) (Figure 2).

Tumor locations and survival rates were also compared. Indicated number of patients with glottic (n=20:31.3%), supraglottic (n= 12: 54.5%), transglottic (n= 5:41.7%) tumors, and one patient with subglottic tumor lost their lives. There was no statistically significant relationship between OS and tumor location (p=0.123, log-rank test).

Thirteen (59.1%) cases with, and 25 (32.5%) without PNI did not survive. There was a significant negative correlation between the presence of PNI and OS (p = 0.007). Twelve (48%) patients with, and 26 (35.1%) without LVI died. Fourteen (46.7%) cases with, and 24 (34.8%) without detectable necrosis exited. There was no significant relationship between OS, LVI and necrosis (p=0.337, p=0.107, respectively).

3.2.Relationship between histopathological data and tumor stage:

The relationship between LVI, PNI, necrosis and tumor stages was evaluated by chi-square analysis, and the data are summarized in Table 2. LVI was present in respective number of cases in stage 1 (n=1: 4.5%), stage 3 (n= 2 :9.1%) , stage 4A (n= 14 :40%), and stage 4B (n=8 :53.3%). LVI was not observed in stage 2 cases. A statistically significant correlation was found between advanced stage and the presence of LVI (p<0.001). PNI was observed in indicated number of cases in stage 2 (n=1: 16.7%), stage 3 (n=6: 27.3%), stage 4A (n=11: 31.4%), and stage 4B (n=4: 26.7%). PNI was not observed in stage 1 cases. A statistically significant relationship was found between advanced stage and the presence of PNI (p = 0.024). Tumor necrosis was detected in respective number of LSCC cases in stage 1 (n=3: 13.6%), stage 2 (n=1: 16.7%), stage 3 (n=4 :18.2%), stage 4A (n=16 :45.7%), and stage 4B (n=6: 40%). A statistically significant relationship was found between advanced stage and the presence of tumor necrosis (p=0.047).

3.3.Relationship between histopathological data and LNM

The relationship between LVI, PNI and necrosis and LNM was evaluated by chi-square test, and the data are summarized in Table 2. LVI was observed in 16 (47.1%) and PNI in 13 (38.2%) of the cases with LNM. A statistically significant correlation was observed between LNM, LVI and PNI (p <0.001, p=0.005, respectively). LNM was present in 14 of the cases (41.2%) with tumoral necrosis. There was no statistically significant relationship between the presence of necrosis and LNM (p=0.08).

3.4.Relationship between EMT markers and OS

Thirty-five (38%) cases with E-cadherin overexpression and 3 (42.9%) cases with E-cadherin downregulation died. There was no statistically significant relationship between E-cadherin expression and OS (p=0.741). Twenty (35%) cases with membranous, 9 (56.2%) cases with cytoplasmic and membranous, and one of the 2 cases with only cytoplasmic staining lost their lives. There was no significant relationship between staining patterns in tumor cells and OS (p=0.550).

Twenty-five patients (41.7%) with beta-catenin overexpression and 13 cases (33.3%) with its downregulation lost their lives. There was no statistically significant relationship between beta- catenin expression and OS (p=0.402). Fourteen (50%) cases with membranous, 21 (33.3%) with cytoplasmic and membranous, and 2 (40%) with only cytoplasmic staining did not survive. There was no statistically significant relationship between staining patterns and OS (p=0.09).

Thirty-five (38.5%) score 1 and 3 (50%) score 2 cases with N-cadherin expression lost their lives. One score 3 case survived. There was no significant relationship between N-cadherin expression and OS (p=0.843). Nine (52.9%) cases with nuclear, 5 (35.7%) cases with cytoplasmic, and 24 (36.4%) cases with negative staining did not survive. One patient with cytoplasmic and membranous staining survived. There was no statistically significant relationship between N-cadherin staining pattern and OS (p=0.467).

Six (66.7%) cases with, and 32 (35.6%) cases without Zeb1 expression in the tumor exited. A significant negative correlation was observed between Zeb1 positivity in the tumor and OS (p=0.006) (Figure 3).

The percentages of Zeb1 positive fibroblasts in the stroma around the tumor were <10% in 5 (26.3%), 11-50% in 17 (36.2%), and >50% in 16 (48.5%) deceased patients. A significant negative correlation was observed between OS and the percentage density increase in Zeb1 positive fibroblasts around the tumor (p=0.008) (Figure 4).

Alpha-SMA expression was not observed in tumor cells. The relationship between the density of αSMA-positive fibroblasts in the surrounding stroma and survival was evaluated. Indicated number of patients with staining densities of [?] 10% (n=14: 41.2%), 11-50% (n=8: 34.8%), and >50% (n=16: 38%) did not survive. There was no significant correlation between tumor circumference αSMA positive stromal fibroblast density and OS (p=0.66). Both tumoral and stromal Zeb1 expressions were significantly associated with OS in univariate analysis. This significant relationship was also demonstrated by Cox regression analysis (p=0.019, p=0.023) (Table 3).

3.5.Relationship between EMT markers and tumor stage

The status of EMT markers in the tumor, and distribution of tumor stages are summarized in Table 2. Nine Zeb1- positive tumors were in stage1 in 1 (11.1%), stage 3 in 1 (11.1%), stage 4A in 2 (22.2%), and stage 4B in 5 (55.6%) cases. A significant correlation was found between increasing tumor stage and tumor Zeb1 positivity (p=0.035). There was no statistically significant relationship between expression of E-cadherin and beta-catenin in the tumor, Zeb1 positivity in the surrounding stroma, and αSMA density and tumor stage (p=0.802, p=0.499, p=0.102, p=0.373, respectively).

3.6.Relationship between EMT markers and LNM

E-cadherin was found to be downregulated in one (2.9%) and beta-catenin in 12 (35.3%) cases with LNM. Tumoral N-cadherin expression was rated with scores 1 in 33 (97.1%), and 2 in one (2.9%) case with LNM. There was no significant correlation between E-cadherin, beta-catenin and N-cadherin expressions in the tumor and LNM (p=0.254, p=0.491, p=0.776, respectively). There was also no significant relationship between staining patterns and LNM (p=0.255, p=0.328, p=0.176, respectively).

LNM was present in 8 (88.9%) of 9 cases with tumoral Zeb1 positivity. A statistically significant correlation was found between Zeb1 positivity in the tumor and LNM (p<0.001). Zeb1 positivity in the surrounding stroma of the tumor and LNM were compared. In 2 (5.9%) of 34 cases with LNM, the density of Zeb1 expression in the surrounding stroma was below 10% in 2 (5.9%), 11-50% in 18 (52.9%), and over 50% in 14

(41.2%) patients. There was no significant relationship between Zeb1 positivity and LNM in the surrounding stroma (p=0.051). There was no statistically significant relationship between density of αSMA positivity in the tumor periphery and LNM (p=0.379). The data are summarized in Table 2.

4.Discussion

The pathogenesis of laryngeal cancer (LC) which is an important cause of morbidity and mortality, has not been fully elucidated [10]. Despite the developments observed in current treatment methods, the desired satisfactory results have not been obtained in the treatment of LC (11). Understanding the molecular mechanisms involved in the development of LC will be conceivably an important step in developing effective treatment methods [11]. Metastasis is the most important life-threatening risk factor for cancer patients and accounts for more than 90% of cancer-related deaths [12]. Studies have shown that in carcinomas, mesenchymal features induced by EMT play a role in many steps of the invasion- metastasis cascade [13,14]. EMT, which is characterized by the loss of intercellular junctions is defined as the loss of epithelial phenotype in cells (such as E-cadherin, alpha-catenin, beta-catenin) and the acquisition of mesenchymal phenotype (such as N-cadherin, vimentin, αSMA) [2,5]. It is thought that with the activation of EMT programs in tumor cells, tumor cells acquire many features of stem cells and climbing the steps leading to metastasis of the tumor is facilitated [15].

E-cadherin is a glycoprotein located in the membrane of normal epithelial cells. Intercellular adhesion is provided with the protein complex formed by binding the cytoplasmic domains of E-cadherin with beta-catenin [2]. Beta-catenins are bridges that mediate the binding of E-cadherin to the actin cytoskeleton. Deletions or mutations in the cytoplasmic tail of the E-cadherin result in the breakdown of cell-cell adhesion complexes at the location of the beta-catenin binding site [16]. The released cytoplasmic beta-catenin then displaces the nucleus and interacts with target genes that play a role in cell proliferation [16]. As stated in many stuides, downregulation / loss of E-cadherin and beta-catenin in the cell membrane and expression of nuclear beta-catenin are frequently detected in most types of cancer which suggests that E-cadherin and beta-catenin may be key molecules in tumor development and progression [2, 10].

In the literature, different results have been reported on the ways of evaluating the expressions of E-cadherin and beta-catenin especially in LSCCs and the importance of staining patterns. In their LSCC series of 82 cases, Greco et al reported that in univariate analysis, cytoplasmic and membranous E-cadherin overexpression was associated with shorter OS in advanced stage patients (T3-T4), and cytoplasmic E-cadherin positivity was associated with poor disease-specific survival (DSS) [7]. The authors also stated that in the multivariate analysis, only cytoplasmic E-cadherin overexpression continued to be a negative prognostic factor for OS [7].

On the other hand, in a LC series of 289 cases, Psyri et al. reported that in univariate analysis, disease –free survival (DFS) was longer in cytoplasmic and membranous E-cadherin expressers compared to cytoplasmic E-cadherin expressers [16]. According to the univariate analysis, it is also said that OS is longer in those who express cytoplasmic E-cadherin and beta-catenin than those expressing only beta-catenin. However, it has been reported that these findings have lost their importance in multivariate analysis [16]. Greco et al. reported that both cytoplasmic and membranous staining with beta-catenin can be demonstrated in univariate analysis, and decreased cytoplasmic and membranous staining is associated with higher histological grade [7]. Cytoplasmic overexpression of beta-catenin has been determined as a positive prognostic factor for DSS. In multivariate analysis, cytoplasmic beta-catenin overexpression has been reported to be associated with prolonged DFS [7].

Zhu et al. evaluated E-cadherin staining in their LSCC series of 76 cases, and determined lack of membranous staining in less than 90% of tumor cells [2]. They reported that membranous staining in tumor tissue decreased and diffuse cytoplasmic staining was observed. For beta-catenin, positive cytoplasmic and nuclear staining, but negative membranous staining was reported. They indicated that increased nuclear and cytoplasmic beta-catenin positivity with decreased membranous E-cadherin staining was associated with the presence of LNM, T4 tumor or poorly differentiated tumors [2]. In univariate analysis, negative expression

of E-cadherin and positive expression of beta-catenin were associated with a decrease in OS, whereas in multivariate analysis, only beta-catenin continued to be an important factor in OS [2]. In a LSCC series of 37 cases realized by Rocco et al, only membranous staining of E-cadherin was considered positive, and they evaluated the cases with percentages [17]. In their study, they couldn't detect a significant relationship between E-cadherin and pT, pN, and tumor grade, however, they associated decreased expression of E-cadherin with disease recurrence and shorter DFS [17].

Studies performed have shown that there is no widely accepted evaluation criterion for E-cadherin and beta-catenin in LSCCs. Studies have demonstrated that the staining patterns of markers may be significant in terms of patient survival, but in multivariate analyzes conducted especially for E-cadherin, markers have lost their significance.

In our study, expressions of tumoral E-cadherin and beta-catenin were evaluated by IRS scoring. There was no significant relationship between downregulation of E-cadherin, beta-catenin and tumor stage, LNM, and OS. Membranous, cytoplasmic and membranous, and only cytoplasmic staining with E-cadherin was seen in 82%, 16%, and 2% of the cases, respectively. OS time was longer in cases with membranous staining, and the lowest OS times were observed in cases with only cytoplasmic staining. However, the findings were not statistically significant which was thought to be due to the limited number of cases with only cytoplasmic staining.

Membranous staining with beta- catenin was observed in 29, cytoplasmic, and membranous staining in 66 and only cytoplasmic staining 5 cases. Besides, the survival was longer in cases with cytoplasmic and membranous staining, and the shortest survival was observed in those with only cytoplasmic staining. However, the results were not statistically significant.

N-cadherin, another member of the cadherin family, is expressed mainly in mesenchymal cells and nerve tissue. N-cadherin stimulates cell motility and migration by interacting with epidermal growth factor receptor-1 and members of the fibroblast growth factor receptor family [18]. N-cadherin is also associated with the MAPK / ERK signaling pathway, which plays a role in tumorigenesis [18]. Unlike E-cadherin, upregulation of N-cadherin increases the migration and invasion capacity of tumor cells [18]. N-cadherin, which is an indicator of the mesenchymal phenotype, is not normally found in epithelial cells. Studies have reported that abnormal N-cadherin expression in epithelial cells is associated with malignancy and tumor progression [18]. It is also stated that increased expression of N-cadherin in the cell membrane or cytoplasm is associated with the progression and metastasis of solid tumors [9]. Zhu et al. evaluated the expression of N-cadherin in LSCCs, and reported that the cytoplasmic N-cadherin in tumor cells was associated with the tumor T stage [2]. However, any significant relationship was not observed with LNM. An association between N-cadherin expression and lower survival rates was reported in univariate analysis which lost its significance in multivariate analysis [2]. In the LSCC series of Greco et al., cytoplasmic and membranous N-cadherin expression in the tumor was taken into consideration, and N-cadherin expression was associated with the histological tumor grade but not with DSS and OS [7]. On the other hand, Rocco et al. considered only membranous staining in their LSCC series of 37 cases, and reported that in most of their cases either N-cadherin staining was not observed or only low levels of immunoreactivity was seen [17].They also reported lack of any relationship between N-cadherin expression and pT, pN, and tumor grade, however, they noted a significant relationship with N-cadherin expression and disease recurrence [17]. In an oral SCC series consisting of 94 cases, Domenico et al. demonstrated cytoplasmic staining with N-cadherin in dysplastic cells and revealed different staining patterns in carcinomatous cells according to the invasion pattern [8]. In their study, they had also observed cytoplasmic and nuclear staining in the droplet invasion pattern, and membranous and cytoplasmic staining in single cell invasions [8]. In our series, similar to the study of Domenico et al, N-cadherin expression was observed in 32 cases, and also nuclear expression in tumor cells was noted. Nine (52.9%) of 17 patients with nuclear N-cadherin expression and 5 (36.7%) of 14 patients with cytoplasmic staining lost their lives. One case with cytoplasmic and membranous staining survived. Considering all cases, there was no significant relationship neither between N-cadherin expression and OS, nor between disease stage and LNM.

EMT is tightly regulated directly or indirectly by transcription factors (TFs) such as Zeb1, Zeb2, Snail, KLF8 and Twist (5,6,19). In addition, there are complex signal networks that regulate TF, such as transforming growth factor- beta [5]. Differences between the potency of these factors have been shown. Besides, as indicated in some studies, the same TF may induce different cellular responses in different carcinoma types [20-23]. ZEB1 is one of the critical members of the zinc finger E-box binding transcription family. Abnormal expression of Zeb 1 has been demonstrated in various tumors including pancreas, lung, liver, and breast carcinomas [19]. Zeb1 is the key factor that regulates EMT in invasive tumor cells and enables tumor cells to acquire a proinvasive and stem cell-like phenotypic characteristics [6]. It is also reported that ZEB1 also facilitates epigenetic silencing of E-cadherin [6].

Wan et al. published a meta-analysis of the results of EMT- inducing transcription factors (EMT-TF) in 2257 cases with HNSCC compiled from 22 articles [24]. Cases with oral SCC, tonsil SCC, LSCC and nasopharyngeal SCC were included in the analysis. As a result of the meta-analysis, it was found that EMT-TF (Zeb1, SNAI1, SNAI2, twist1) overexpression was associated with poor OS. They reported that similar results were observed in LSCCs when tumor subgroups were evaluated individually. In addition, EMT-TF was seen to be associated with DFS, T stage, LNM, distant metastasis, tumor differentiation, and disease recurrence [23]. In the LSCC series of Zhu et al. Zeb2 expression was reported to be associated with LNM, tumor T stage and differentiation [2]. Zeb2 expression was found to be an independent risk factor for OS in univariate and multivariate analysis [2]. Contrarily, in the LSCC series of Rocco et al, any significant relationship between Zeb1 expression in the tumor and pT, pN, tumor grade, disease recurrence and DSS could not be detected [17]. Data on the prognostic significance of Zeb1 expression in the tumor microenvironment, especially in breast carcinomas have been indicated in the literature. It has been shown that Zeb1 in basal-like breast cancers regulates the levels of various inflammatory cytokines such as IL6 / 8 and contributes to the formation of the tumor microenvironment. Increased stromal Zeb1 expression has been associated with extracellular matrix remodeling, immune cell infiltration and angiogenesis [19]. In our series, a statistically significant relationship was observed between tumoral Zeb1 expression and LNM, advanced stage disease and poor OS. In addition, as a remarkable finding, Zeb1 expression observed in the surrounding stroma had a significant relationship with OS. Alpha- SMA is the major component of contractile microflaments used to detect mesenchymal cells, especially myofibroblasts. During the process of EMT, TGF- β stimulates αSMA expression in transitioning epithelial cells, which has been reported to be associated with increased tumor invasion and decreased survival [24]. Benzour et al. evaluated alpha- and gamma- SMA expressions in hepatocellular carcinomas, and reported gamma- SMA was expressed only in tumor cells. They also observed αSMA positivity in only stromal component [25]. Based on this observation, they stated that only gamma-SMA may be expressed in hepatic progenitor cells [25].

In our LSCC series, αSMA expression was not detected in tumor cells. However, a positive reaction with αSMA was detected in the stroma surrounding the tumor. As a remarkable finding, these results were parallel to the data reported by Benzour et al. about αSMA which made us think that, similar to the theories of the researchers, LSCC progenitor cells may also express different SMA subtypes instead of αSMA.

It is known that factors secreted from the tumor, such as TGF- β, activate fibroblasts and these factors have different genetic characteristics from normal fibroblasts [26]. It has been stated that these cancer-related fibroblasts can be found in the "network pattern" in the whole tumor stroma or in the "spindle pattern" around the tumor islands [27]. In our series, in the group with lower density (<10%) of αSMA -positive fibroblasts consistent with the spindle pattern around the tumor islands, OS times were prolonged than the other two groups, without any statistically significant intergroup difference.

5.Conclusion

In the studies investigating the relationship between EMT and prognosis in LSCCs in the literature, any accepted common evaluation criterion to determine the expression of EMT markers has not been encountered. Perhaps for this reason, quite different results were obtained in the studies. In our study, it was noted that as an EMT-TF, Zeb1 is associated with tumor stage, LNM, and OS. In addition Zeb1 expression observed in tumor stroma is also significant for OS. There is a limited number of studies on Zeb1 expression in LSCCs in

the literature. Zeb1 expression in tumor stroma has not been studied so far. For this reason, we thought that Zeb1 expression in both the tumor stroma, and tumor should be taken into consideration in the evaluation of EMT in LSCCs and it would be appropriate to support this suggestion with novel studies.

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Table Legends:

Table 1: Baseline characteristics of the 100 patients with LSCC

Table 2: Relationship between LNM and pathological tumour stage with EMT markers and histopathological findings (Chi-square analysis)

Table 3: Multivariate analysis of Zeb1 in tumor and Zeb1 in stromal expression predicting OS

Figure Legends:

Figure 1: (A) Loss of membranous expression of E-cadherin (x200), (B Cytoplasmic and membranous staining of beta-catenin (x200), (C) Cytoplasmic expression of N-cadherin (x400), (D) Smooth muscle actin expression in stroma (x200), (E) Zeb-1 staining in tumor and stroma (x200), (F) Zeb-1staining in stroma (x100)

Figure 2: Kaplan Meier curves of OS stratified according to pathological stage, Lymph node status, Zeb1 expression in tumour and Zeb1 expression in stroma

Table I

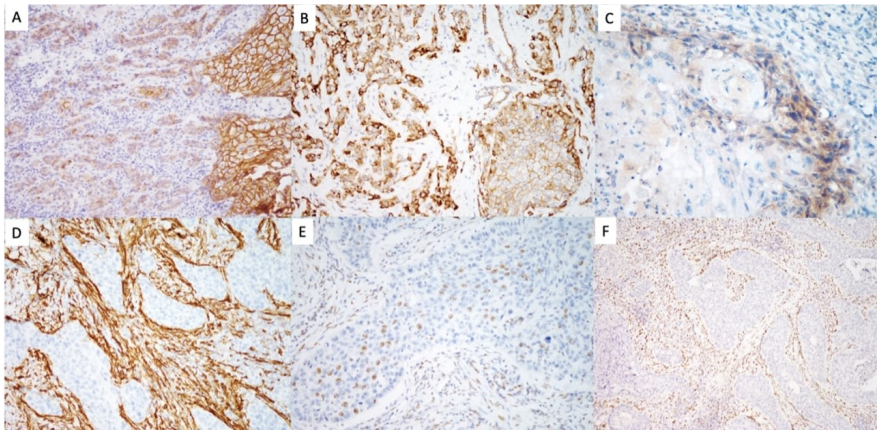
Caracteristics	**Value**
Age yr,median (range)	63 (43-84)
Gender (F/M)	5/95
Survey (alive/dead)	61/38
OS mean (range)	35.8 month (2-118)
Tumor localization Supraglottic Glottic Subglottic Transglottic	22 65 1 12
Patological Stage I/II/III/IVA/IVB	22/6/22/35/15
LNM (-/+)	66/34
LVI (-/+)	75/25
PNI (-/+)	78/22
Necrosis (-/+r)	70/30

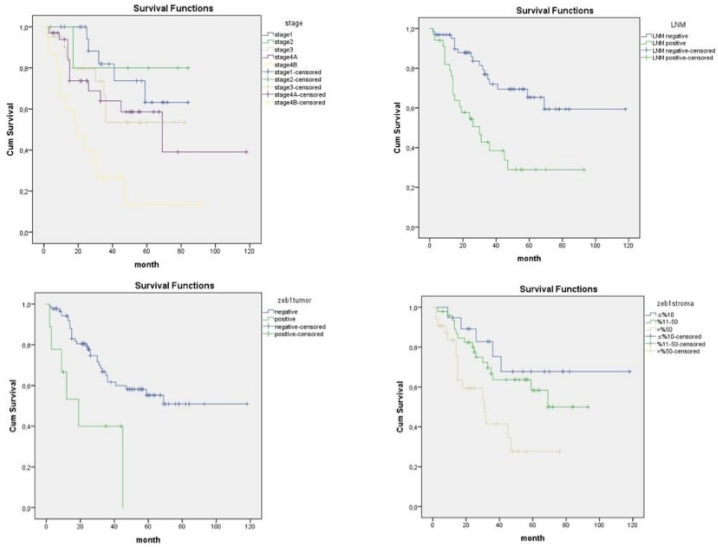
Table II

	LNM (n) (-/+)	**pStage (n)** (I/II/II/IVA/IVB)
LVI negative positive p value	57/18 9/16 **<0.001**	21/6/20/21/7 1/0/2/14/8 **<0.001**
PNI negative poisitive p value	57/21 9/13 **0.005**	22/5/16724/11 0/1/6/11/4 **0.024**
Necrosis negative positive p value	50/20 16/14 0.080	19/5/18/19/9 3/1/4/16/6 **0.047**
E-cadherin downregülasyon overekspresyon p value	6/1 60/33 0.417	3/0/1/2/1 19/6/21/33/14 0.802
Beta-catenin downregülasyon overekspresyon p value	28/12 38/22 0.491	9/2/10/16/3 13/4/12/19/12 0.499
N-cadherin Score 1 Score 2 Score 3 P value	60 33 5/1 1/0 0.776	20/6/21/32/14 2/0/1/2/1 0/0/0/1/0 0.98
Zeb1 tumour Negative Positive p value	65/26 1/8 **<0.001**	21/6/21/33/10 1/0/1/2/5 **0.035**
Zeb1 stroma <%10 %11-50 >%50 p value	17/2 30/18 19/14 0.051	7/2/3/7/0 12/3/12/13/8 3/1/7/15/7 0.10
αSMA stroma <%10 %11-50 >%50 p value	25/9 17/8 24/17 0.383	12/3/6/10/3 4/1/8/9/3 6/2/8/16/9 0.373

Table III

Variables	Total n	n of die	HR (95%Cl)	Multivariate p value
Zeb1 tumour negative positive	89 9	32 6	2.908 (1.195-7.073)	**0.019**
Zeb1 stroma <%10 %11-50 >%50	19 47 32	5 17 16	References 1.395 (0.509-3.820) 3.292 (1.183-9.162)	0.518 **0.023**





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