Original Article / Çalışma - Araştırma

Clinical significance of Ki-67, c-erbB-2 and E-cadherin expressions in open partial laryngectomy patients

Açık parsiyel larenjektomi hastalarında Ki-67 c-erbB-2 ve E-kaderin ekspresyonlarının klinik önemi

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ABSTRACT

Objectives: This study aims to analyze the correlation between biomarkers and risks of cervical lymph node invasion, recurrence, distant metastasis and survival regarding the clinicopathological variables in laryngeal cancers.

Patients and Methods: Forty-one patients with laryngeal cancers were examined retrospectively. The correlations evaluated between E-cadherin, Ki-67, c-erbB-2 expressions, tumor site, differentiation, the risk of cervical lymph node metastasis, perineural, perivascular, pericapsular invasion and recurrence as well as survival time.

Results: There was significant correlation between T-grade and the survival rates, and between pericapsular invasion and the lowest survival rates. There was no significant correlation between E-cadherin, Ki-67, c-erbB-2 expressions and clinicopathological variables of tumor. Positive correlation between strong stain of E-cadherin and Ki-67 proliferation index was determined.

Conclusion: These markers are not reliable prognostic and predictive factors for laryngeal cancers. E-cadherin expression was protected in well-differentiated and less invasive cancers, which maintain their cell-cell adhesions whereas it was reduced in undifferentiated cancers. Positive correlation between E-cadherin and Ki-67 proliferation shows that histopathological differentiation of laryngeal cancers is maintained in spite of the high proliferation index.

Keywords: C-erbB-2; clinicopathological variables; E-cadherin; Ki-67; Larynx cancer.

ÖZ

Amaç: Bu çalışmada gırtlak kanserlerinde klinikopatolojik değişkenler ile ilgili biyo-göstergeler ve servikal lenf nodu invazyonu, nüks, uzak metastaz ve sağkalım riskleri arasındaki ilişkinin analiz edilmesi amaçlandı.

Hastalar ve Yöntemler: Larenks kanseri olan 41 hasta retrospektif olarak incelendi. E-kaderin, Ki-67, c-erbB-2 ekspresyonları, tümörün yeri, farklılaşması, servikal lenf nodu metastazı, perinöral, perivasküler, perikapsüler invazyon riski ile nüks ve sağkalım süresi arasındaki ilişki istatistiksel olarak değerlendirildi.

Bulgular: T-evresi ile sağkalım oranları, perikapsüler invazyonu ve en düşük sağkalım oranları arasında anlamlı ilişki bulundu. E-kaderin, Ki-67, c-erbB-2 ekspresyonları ile tümörün klinikopatolojik değişkenleri arasında anlamlı ilişki bulunmadı. Güçlü E-kaderin boyaması ile Ki-67 proliferasyon indeksi arasında pozitif ilişki saptandı.

Sonuç: Bu göstergeler larenks kanseri için güvenilir prognostik ve öngördürücü faktörler değildir. E-kaderin ekspresyonu, hücre-hücre adezyonlarını sağlayan farklılaşmış ve daha az invaziv kanserlerde iyi korunup farklılaşmamış kanserlerde azaltılmıştır. E-kaderin ve Ki-67 proliferasyonu arasındaki pozitif ilişki gösteriyor ki larenks kanserlerinin histopatolojik farklılaşması, yüksek proliferasyon indeksine rağmen sürdürülmektedir.

Anahtar Sözcükler: C-erbB-2; klinikopatolojik değişkenler; E-kaderin; Ki-67; larenks kanseri.

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Tumor node metastasis (TNM) classification, tumor location and neck lymph node involvement are the parameters significantly affecting prognosis in laryngeal squamous cell carcinoma (SCC). Although there are some other prognostic and predictive factors such as histopathological differentiation, perineural and perivascular invasion, more accurate and objective variables are needed to identify the survival in these patients. High risk for recurrence after open partial laryngectomies is still a reality and more useful parameters should be studied for a rational choice between different types of surgery or other multimodality therapeutic strategies.[1] Amongst these parameters are biological factors, mainly molecules such as tumor suppressor genes and oncogenes. E-cadherin acts as a tumor suppressor and it suppresses invasion and metastasis.[2] It is an adhesion molecule that provides connections of intercellular areas and impairment of its expression creates a basis for development of tumors.[3] C-erbB-2 is a tyrosine kinase growth factor and oncogene whose overexpression has been observed in the development of many malignancies; it may therefore play a role in cell transformation and tumor pathogenesis.[4] Ki-67 protein is a nuclear protein and is a marker of proliferation.[5] The purpose of this study is to analyze the correlations between these biomarkers and the clinical parameters such as the risk of cervical lymph node invasion, recurrence, distant metastasis and survival time.

PATIENTS AND METHODS

Forty-one patients (40 males, 1 female; mean age 63 years; range 44 to 81 years) with laryngeal SCC diagnosed and treated by open partial laryngectomy between 2009 and 2012 were studied retrospectively. The study was approved by the Medical Faculty of Çanakkale Onsekiz Mart University Ethic Committee and informed consent of all participants were obtained. The clinical history, smoking and alcohol habits, age, gender, physical examination, involved anatomical region, and tumor staging was recorded according to TNM classification. The study was conducted in accordance with the principles of the Declaration of Helsinki. All patients underwent curative partial laryngectomy, vertical or supraglottic according to the location of the tumor. Neck dissection was performed for all supraglottic cases. Pathological specimens of all 41 patients included in the study were analyzed by the Pathology Department of our institution. Forty-one biopsies of laryngeal tumors were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. Four-micrometer sections from paraffin embedded tissues were processed. The histological grading of malignancy was analyzed under light microscopy (200x magnification) at the front of invasion with hematoxylin-eosin staining. Histopathologic characteristics of the group such as differentiation, perineural and perivascular tumor invasion, lymph node metastasis and pericapsular invasion were evaluated. The relationship between all biomarkers was studied.

Immunohistochemical study

Additional sections were prepared on polylisin coated slides for immunohistochemical staining and Ki-67 (Dako, Monoclonal Mouse Anti-Human), c-erbB-2 (Dako, Polyclonal Rabbit Anti-Human) ve E-cadherin (Dako, Monoclonal Mouse Anti-Human) antibodies were used. All immunohistochemical staining processes were done using an automatic immune staining device (Leica-Bondmax). All specimens were analyzed and photographed under a light microscope (Carl Zeiss Axioscope photomicroscope, Carl Zeiss Axiocam ICc3 3.3 Mp digital camera and Axiovision Software). The correlations were statistically evaluated between E-cadherin, Ki-67, c-erbB-2 biomarker expressions and tumor site and differentiation, and the risk of cervical lymph node metastasis, perineural, perivascular and pericapsular invasion, as well as recurrence and survival.

Immunohistochemical assessment for E-cadherin

The percentages of positive cells were determined according to the most stained area. Staining between 0% and 50% was considered as weak staining while staining between 50% and 100% was accepted as strong staining.

Immunohistochemical assessment for Ki-67

The percentages of positive cells were determined according to the most stained area. Classification of the nuclear staining were as follows; 0 for non-stained samples, 1+ or weak for less than 10% stained samples, 2+ or moderate for samples that were stained between 11% and 50%, 3+ or strong for samples that were stained more than 50%.

Immunohistochemical assessment for c-erbB-2

Initially stain distributions were assessed under high magnification. Then, positive cell count in all fields was carried out. Membranous and cytoplasmic staining was accepted as positive. Samples were classified as follows; 0 for non-stained samples, 1+ or weak for less than 20% stained samples, 2+ or moderate for samples that were stained between 21% and 50%, 3+ or strong for samples that were stained more than 50%.

Statistical analysis

Statistical analysis was performed by IBM SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). Distribution of E-cadherin, c-erbB-2 and Ki-67 (for more than 10%) levels according to clinicopathologic parameters like tumor location and stage, cervical lymph node, recurrence, survival time, and histopathologic parameters like perineural, perivascular and capsule invasion, were analyzed by chi-square tests. Associations between Ki-67 (for median index) and the clinicopathologic variables were evaluated by ANOVA and Student-t test. Kendall tau-b test was used for the correlations between these biomarkers, E-cadherin, c-erbB-2 and Ki-67 (for all criteria) and the clinicopathologic variables.

RESULTS

Of all patients, 40 (97.5%) had positive history of smoking and 32 (80%) used both alcohol and smoke. The tumors were classified as supraglottic in 16 cases (39%) and glottic in 25 (61%) whereas there were no subglottic cases. According to TNM criteria, there were 11 (26.8%) T_{1a}, three (7.4%) T_{1b}, 22 (53.6%) T_2, and five (12.1%) T_3 tumors. Based on pathological examination seven patients (17.5%) had positive cervical lymph nodes with stage N_1 and all these cases with regional metastasis were supraglottic cancers. Clinical follow-up ranged from 7 to 46 months with a mean time of 24 months. Locoregional recurrence developed in five patients (12.2%) and four of them died. The survival rate was 90.2%. Squamous cell carcinoma was detected in all the cases and 17 of them were well differentiated, 15 were moderately differentiated, and nine poorly differentiated. Strong E-cadherin staining was observed in 30 patients (73.2%) with weak expression in 11 (26.8%) (Figure 1). There was no significant expression difference for tumor location, T stage, regional metastasis and tumor differentiation ($p>0.05$) (Table 1). Also no significant association was found for perineural, perivascular and pericapsular invasion, recurrence and survival ($p>0.05$) (Table 1). Twenty-six (64.4%) specimens showed positive staining for c-erbB-2 while 15 (36.6%) demonstrated no staining (Figure 2). There was no significant expression difference for tumor location, T stage, regional metastasis and tumor differentiation ($p>0.05$) (Table 2). Also, no significant association was seen for perineural, perivascular and pericapsular invasion, recurrence and survival ($p>0.05$) (Table 2). In all cases, positive reaction was detected for Ki-67 (Figure 3). There was no statistically significant

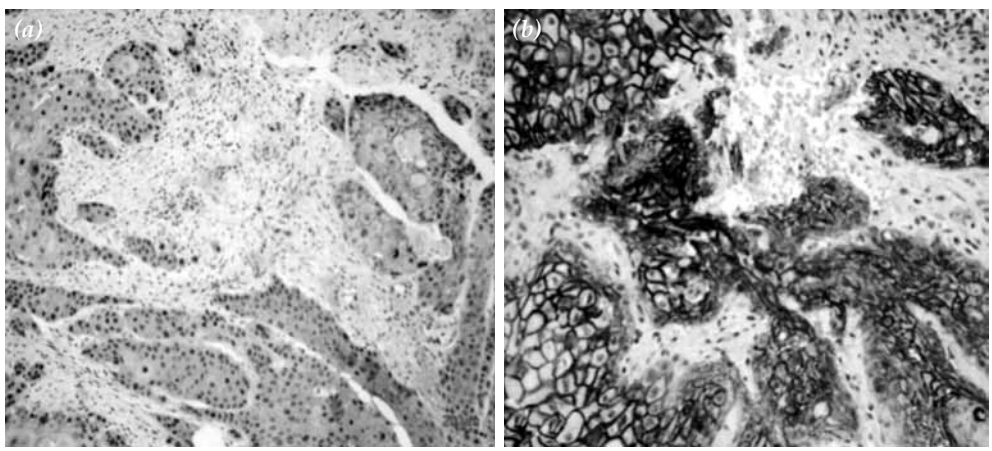

Figure 1. *Immunohistochemical staining of E-cadherin in islands of tumor.* ***(a)*** *(+) positive staining x100,* ***(b)*** *(+ +) positive staining x200.*

Table 1. Statistical data between E-cadherin and the clinicopathological values

E-cadherin	Weak staining (+)		Strong staining (++)		
	n	%	n	%	*p*
Tumor location					
Supraglottic tumors	2	12.5	14	87.5	0.152
Glottic tumors	9	36	16	64	
Tumor, node, metastasis					
T_1	5	33.3	10	66.7	
T_2	5	23.8	16	76.2	0.473
T_3	1	20	4	80	
T_4	-	-	-	-	
Tumor differentiation					
Poor	3	33.3	6	66.7	0.614
Moderate	2	13.3	13	86.7	
Well	6	35.2	11	64.8	
Perivascular invasion					
Presence	1	33.3	2	66.7	1.000
Absence	10	26.3	28	73.7	
Perineural invasion					
Presence	1	20	4	80	1.000
Absence	10	27.7	26	72.3	
Pericapsular invasion					
Presence	-	-	2	100	1.000
Absence	11	28.2	28	71.8	
Lymph node					
Positive	-	-	7	100	0.160
Negative	11	32.3	23	67.7	
Recurrence					
Presence	1	20	4	80	1.000
Absence	10	27.7	26	62.3	
Ex					
Presence	-	-	4	100	0.559
Absence	11	29.7	26	71.3	

correlation between strong Ki-67 expression (>50%) and tumor location, T stage, regional metastasis and tumor differentiation (p>0.05) (Table 3). Also no significant association was showed between strong staining of Ki-67 and perineural, perivascular and pericapsular invasion, recurrence and survival (p>0.05) (Table 3). Ki-67 proliferation index was 41.8 in specimens that showed weak staining for E-cadherin, whereas it was 60.3 in strong E-cadherin expression cases. A statistically significant correlation between strong stain of the E-cadherin and Ki-67 proliferation index was determined (p<0.05).

DISCUSSION

Larynx carcinomas form 2-3% of all malignant tumors in organisms. It is the most common malignant tumor in the head-neck region with a rate of about 25%.[6] More than 95% of larynx carcinomas are SCC. General prognostic factors to be considered include tumor stage, anatomic location, histological differentiation and presence of metastasis in the neck, with

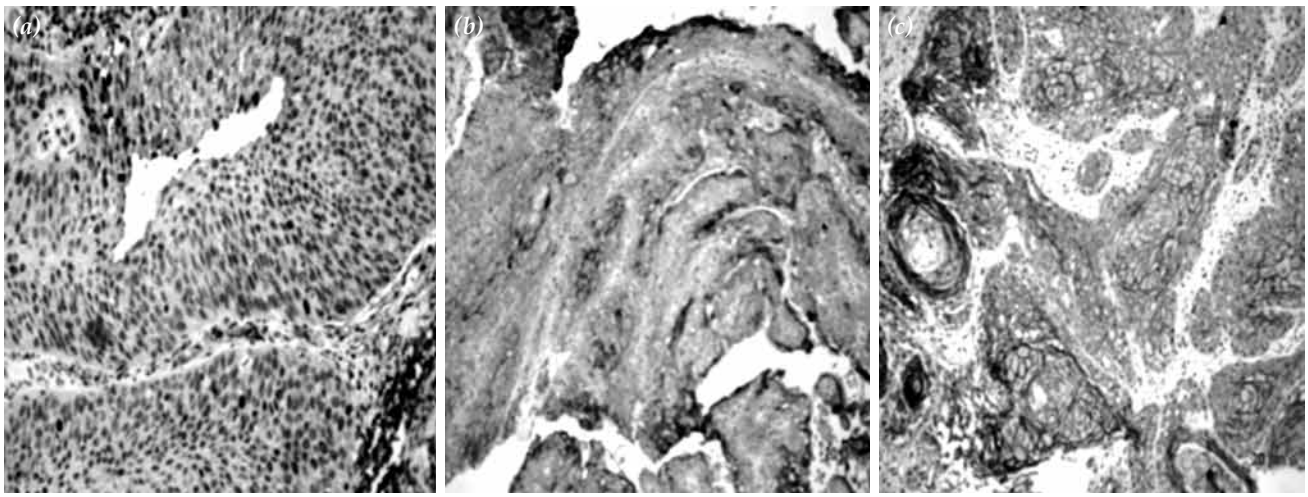

Figure 2. *Immunohistochemical staining of c-erbB-2 in islands of tumor.* ***(a)*** *(+) positive staining x200,* ***(b)*** *(+ +) positive staining x100,* ***(c)*** *(+ + +) positive staining x100.*

neck metastasis accepted as the most important prognostic factor.[1,7] In cases with cervical lymph node (LN) metastasis the five-year survival rate is reduced to 50%. However even similar histopathological diagnosis, grade and stage tumors exhibit different tumor behavior indicating a need to research different prognostic factors and complete new molecular studies. When determining the treatment for cases, understanding the recurrence factors gains importance. This study looked at the relationship between parameters such as E-cadherin, c-erbB-2 and Ki-67 levels and LN metastasis, recurrence rate, survival and death rates.

E-cadherin is an adhesion molecule that provides connections of intercellular areas. As E-cadherin release increases, the linkages between cells strengthen and the risk of tumor development reduces. However E-cadherin release reduction breaks the linkage between cells and creates an appropriate environment for tumor development and metastasis. E-cadherin expression loss starts in the early stage of laryngeal carcinogenesis.[3] A study of larynx cancers that evaluated less than 50% staining of E-cadherin as weak and more than 50% staining as strong, found that 62% of cases had strong and 38% of cases had weak staining.[2] In our study the same staining scale was used and 73.1% were strong while 26.9% were weakly stained.

Studies examining E-cadherin release and the anatomic location of the tumor have produced different results.[8,9] In our study both supraglottic and glottic tumors had higher levels of strong staining (87.5% and 64%, respectively). However there was no statistical difference between strong and weak E-cadherin staining and the anatomic location of the tumor.

Li et al.[10] believed that increasing T-stage would cause stronger staining of E-cadherin, but they did not find any statistically significant correlation. In our study, as the T-stage increased, the E-cadherin strong staining was observed to increase (E-cadherin strong staining at T_1 was 66%, at T_2 was 71% and at T_3 was 80%). However no correlation was observed between increasing T-stage and strong or weak E-cadherin staining.

In head and neck tumor cases, as E-cadherin release decreased, LN metastasis was shown to increase.[7,11] In our study, E-cadherin strongly stained in all cases with cervical LN metastasis, but no statistically significant correlation was found.

Studies on cases with poor differentiation have found weaker staining of E-cadherin.[8,12] In our study there was no statistically significant correlation between strong or weak staining of E-cadherin and tumor differentiation (E-cadherin weak staining in cases with poor differentiation was 34%, in moderately differentiated cases was 13%, and in well differentiated cases was 36%).

Kurtz et al.[13] studied head and neck SCCs and found a statistically significant correlation between perivascular invasion and weak

Table 2. Statistical data between C-erbB-2 and the clinicopathological values

C-erbB-2	Negative staining		Positive staining		
	n	%	n	%	*p*
Tumor location					
Supraglottic tumors	4	25	12	75	0.185
Glottic tumors	11	44	14	56	
Tumor, node, metastasis					
T_1	8	53.3	7	46.7	
T_2	6	28.5	15	71.5	0.098
T_3	1	20	4	80	
T_4	-	-	-	-	
Tumor differentiation					
Poor	5	55.5	4	44.5	0.859
Moderate	4	26.6	11	74.4	
Well	6	35.2	11	64.8	
Perivascular invasion					
Presence	-	-	3	100	0.244
Absence	15	39.4	23	60.6	
Perineural invasion					
Presence	1	20	4	80	0.387
Absence	14	38.8	22	61.2	
Pericapsular invasion					
Presence	1	50	1	50	0.687
Absence	14	35.8	25	64.2	
Lymph node					
Positive	2	28.5	5	71.5	0.623
Negative	13	38.2	21	61.8	
Recurrence					
Presence	3	60	2	40	0.249
Absence	12	33.3	24	66.7	
Ex					
Presence	2	50	2	50	0.467
Absence	13	35.1	24	64.9	

staining of E-cadherin; they did not find a statistically significant correlation between presence of perineural invasion and strong or weak staining of E-cadherin. In our study, in cases with perineural invasion strong staining of E-cadherin was 80% and it was 66% in cases with perivascular invasion. In both cases of pericapsular invasion (100%) E-cadherin strongly stained; however there was no statistically significant correlation between perineural, perivascular and pericapsular invasion and strong or weak E-cadherin staining.

In five cases with recurrence, four (80%) were observed to have strong E-cadherin staining, while of the four cases that died, all (100%) had strong staining. However there was no statistically significant correlation found between recurrence or death and strong or weak staining of E-cadherin. A variety of studies have found similar results.[10,12]

Excess expression of c-erbB-2 is observed in the development of many malignancies. As a result it is thought to play a role in cell transformation and tumor pathogenesis. Recent studies of

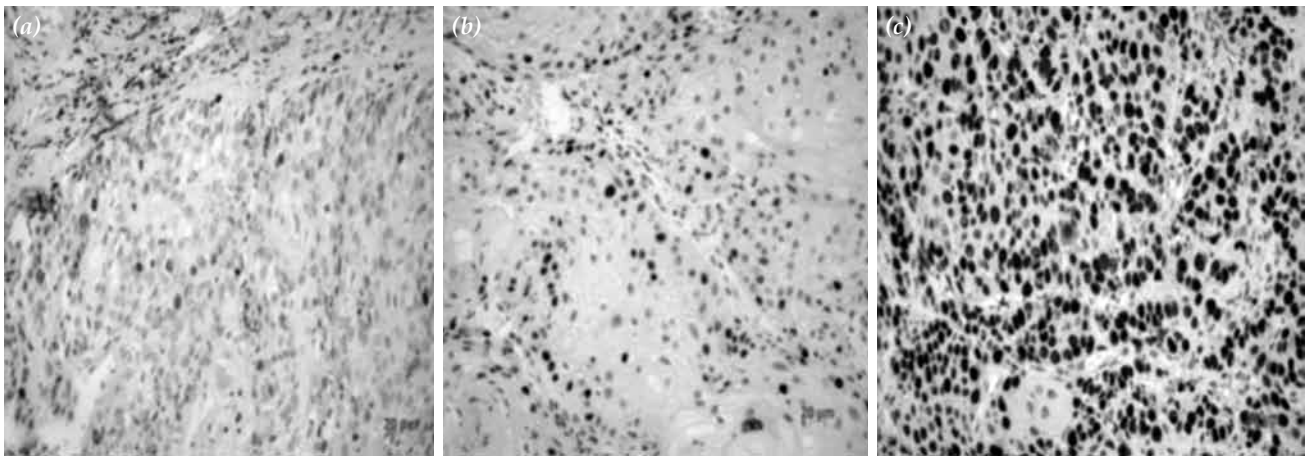

Figure 3. *Immunohistochemical staining of Ki-67 in islands of tumor.* ***(a)*** *(+) positive staining x200,* ***(b)*** *(+ +) positive staining x200,* ***(c)*** *(+ + +) positive staining x200.*

head and neck SCCs have identified that in tumor tissues, increased c-erbB-2 amplification plays an important role in cell proliferation, differentiation, adhesion, migration, tumor invasion and apoptosis.[14] In our study, positive staining for c-erbB-2 was 46% at T_1, 71% at T_2 and 80% at T_3. Similar to the literature there was no statistically significant correlation between positive or negative staining degree for c-erbB-2 and increase in T-stage.[15]

In the literature some studies did not find a statistically significant correlation between positive or negative c-erbB-2 staining and LN metastasis of larynx cancer cases, though some did find a statistically significant correlation.[16,17] In our study of seven cases with LN metastasis, five (71%) had positive staining for c-erbB-2 and two (29%) had negative staining. However in cases with or without cervical LN metastasis there was no statistically significant correlation found with positive or negative c-erbB-2 staining.

There has been no statistically significant correlation found between positive or negative c-erbB-2 staining and tumor differentiation.[18] In our study, c-erbB-2 positive staining was 55% in cases with poor differentiation, 73% in moderately differentiated cases and 65% in well differentiated cases. There was no significant statistical correlation between c-erbB-2 positive or negative staining and tumor differentiation.

A study of cases with perineural, perivascular and pericapsular invasion found more cases with negative c-erbB-2 staining than positive staining (negative staining was 71%, 100% and 73% respectively).[18] However there was no statistically significant correlation found between presence of perineural, perivascular or pericapsular invasion and positive or negative staining for c-erbB-2. In our study, of five cases with perineural invasion positive staining for c-erbB-2 was 80%, of three cases with perivascular invasion positive staining for c-erbB-2 was 100% and of two cases with pericapsular invasion one was positive for c-erbB-2 and one was negative. There was no statistically significant correlation found between presence of perineural, perivascular and pericapsular invasion and positive or negative c-erbB-2 staining.

Shiga et al.[19] found c-erbB-2 positivity statistically significant in cases with recurrence and recommended chemotherapy especially in situations with c-erbB-2 overexpression. Yu et al.[20] found c-erbB-2 positivity was significant in cases with recurrence and especially distant metastasis. A study by Krecicki et al.[15] did not find a statistically significant correlation between recurrence and positive or negative c-erbB-2 staining. In our study we did not find a statistically significant correlation between recurrence and c-erbB-2 positive or negative staining.

Another study found all four cases of death had negative c-erbB-2 staining (100%), while surviving cases had 56% positive staining for c-erbB-2 and 44% negative staining. However there was no statistically significant correlation

Table 3. Statistical data between Ki-67 (according to the first method) and the clinicopathological values

Ki-67	Negative staining		Positive staining		
	n	%	n	%	*p*
Tumor location					
Supraglottic tumors	-	-	16	100	0.268
Glottic tumors	3	12	22	88	
Tumor, node, metastasis					
T_1	2	13.3	13	86.7	
T_2	1	4.7	20	96.3	0.446
T_3	-	-	5	100	
T_4	-	-	-	-	
Tumor differentiation					
Poor	-	-	9	100	0.251
Moderate	2	13.3	13	86.7	
Well	1	5.8	16	94.2	
Perivascular invasion					
Presence	-	-	3	100	0.791
Absence	3	7.8	35	82.2	
Perineural invasion					
Presence	-	-	5	100	0.670
Absence	3	8.3	33	91.7	
Pericapsular invasion					
Presence	-	-	2	100	1.000
Absence	3	7.6	36	92.4	
Lymph node					
Positive	-	-	7	100	0.561
Negative	3	8.8	31	91.2	
Recurrence					
Presence	-	-	5	100	1.000
Absence	3	8.5	33	91.5	

found between positivity or negativity for c-erbB-2 and cases of death.[16] In our study of four cases of death, half were negative for c-erbB-2 and half were positive. Surviving cases had positive staining for c-erbB-2 in 65% and negative staining for 35% and similarly there was no statistically significant correlation between survival and positivity or negativity for c-erbB-2.

Ki-67 is a marker for increased proliferation thought to play an important role in tumor development and progression, frequently used for mitotic index and tumor grading. A study of 65 squamous cell larynx cancer cases classified by Ki-67 staining as 0, 1+, 2+, 3+ found in all cases a positive degree for Ki-67.[21] In our study we used a similar scale and all cases stained with a degree of positivity.

In the literature while different results are found, in our study both supraglottic and glottic tumors had a higher rate of positive staining for Ki-67 compared to negative staining (Positive staining for Ki-67 in supraglottic tumors was 100%, in glottic tumors was 88%).[22,23] However there was no statistically significant correlation between positive or negative staining for Ki-67 or Ki-67 proliferation index and anatomic location of the tumor. Similar to the literature as the degree of positive staining for Ki-67 increased there was no statistically significant correlation with increased T-stage.[22,23] There was no statistically

significant correlation found between tumor stage and Ki-67 proliferation index.

Studies have found a significant correlation between Ki-67 positivity and LN metastasis and increased LN staging and Ki-67 proliferation index.[23-25] In our study in all of seven cases of cervical LN metastasis (100%) Ki-67 stained positively; however there was no statistically significant difference between cervical LN metastasis and Ki-67 positivity and Ki-67 proliferation index.

In our study Ki-67 positive staining was 9% in well differentiated cases, 86% in moderately differentiated cases and 100% in poorly differentiated cases. However there was a statistically significant reverse correlation between Ki-67 positive or negative staining and tumor differentiation. In the literature, similarly, a reverse correlation was found between tumor differentiation and Ki-67 proliferation index.[26,27] However according to cut-off value there was no statistically significant correlation between Ki-67 positive or negative staining or Ki-67 proliferation index and tumor differentiation.

A study on perivascular or perineural invasion cases found positive staining for Ki-67 was higher but did not find a statistically significant correlation.[28] In our study, all five cases with perineural invasion, all three cases of perivascular invasion and all three cases of pericapsular invasion stained positive for Ki-67, however no statistically significant result was found.

In studies of cases with recurrence, Ki-67 proliferation index was much higher than in those without recurrence.[22,29] In our study all cases with recurrence stained positive for Ki-67 but no statistically significant result was found. Additionally, no significant statistical result was found between recurrence and Ki-67 proliferation index. Sarafoleanu et al.[26] found Ki-67 proliferation index was statistically significantly high in cases that died of squamous cell larynx cancer compared to surviving cases. In our study, of four cases that died, (100%) stained positive for Ki-67 but no statistically significant result was found.

In our study, there was a positive correlation between E-cadherin and Ki-67 proliferation index. It is known that loss of or reduction in E-cadherin expression has been associated with increased invasiveness, advanced T and N stages and unfavorable prognosis. Its expression was protected in well-differentiated and less invasive cancers, which maintain their cell-cell adhesions whereas it was reduced in undifferentiated cancers. This positive correlation between E-cadherin and Ki-67 proliferation shows us that histopathological differentiation of laryngeal cancers is maintained in spite of the high proliferation index.

In all statistical evaluations of E-cadherin, Ki-67 and c-erbB-2 and clinicopathologic variables in our larynx cancer cases, no significant result was found. Combined with the contradictory results in the literature, we are led to believe that these markers are not reliable prognostic factors.

To understand the molecular structure of the multi-stage process of carcinogenesis studies with larger numbers of cases (especially case groups at the same tumor stage, histological grade and undergoing the same surgical treatment should be chosen) completed using different techniques will be beneficial.

Declaration of conflicting interests

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