CLINICAL STUDY

PROGNOSTIC IMPORTANCE OF HYPOXIA-INDUCIBLE FACTOR 1-ALPHA AND NUCLEAR FACTOR KAPPA B IN EARLY-STAGE GLOTTIC LARYNGEAL SQUAMOUS CELL CARCINOMA TREATED WITH RADIOTHERAPY

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SUMMARY

Objective: Early-stage glottic laryngeal squamous cell carcinoma has good mortality and morbidity rates with radiotherapy. However, recurrent cases are at risk of increased morbidity and mortality. Therefore, the identification of new prognostic markers is needed.

Material and Methods: Forty patients with early-stage glottic carcinoma managed with radiotherapy were included in this study. Immunohistochemical evaluation was performed for Nuclear factor-kappa B and Hypoxia-inducible factor 1-alpha from biopsy specimens. The relationship between overall survival and investigated parameters was analyzed by Kaplan-Meier and regression analysis.

Results: There were 11 recurrences and 20 deaths occurred during the follow-up period. There was no significant relationship between Nuclear factor-kappa B and Hypoxia-inducible factor 1-alpha staining and overall survival. There were no significant correlations between age, smoking status, differentiation, T stage, and anterior commissure involvement with Nuclear factor-kappa B and Hypoxia-inducible factor 1-alpha staining.

Conclusion: It seems pretreatment Nuclear factor-kappa B and Hypoxia-inducible factor 1-alpha expression levels do not correlate with overall survival in early-stage glottic carcinoma primarily managed with radiotherapy.

Keywords: Glottic; cancer; radiotherapy; survival; resistance

RADYOTERAPİ İLE TEDAVİ EDİLEN ERKEN EVRE GLOTTİK LARİNGEAL SKUAMÖZ HÜCRELİ KARSİNOMDA HİPOKSİ İLE İNDÜKLENEBİLİR FAKTÖR 1-ALFA VE NÜKLEER FAKTÖR KAPPA B'NIN PROGNOSTİK ÖNEMİ

ÖZET

Giriş: Erken evre glottik laringeal skuamöz hücreli karsinom, radyoterapi ile iyi mortalite ve morbidite oranlarına sahiptir. Bununla birlikte, nüks vakalar artmış morbidite ve mortalite riski altındadır. Bu nedenle yeni prognostik belirteçlerin tanımlanmasına ihtiyaç duyulmuştur.

Amaç ve Yöntemler: Bu çalışmaya radyoterapi ile tedavi edilen erken evre glottik karsinomlu kırk hasta dahil edildi. Biyopsi örneklerinden Nükleer faktör-kappa B ve Hipoksi ile indüklenebilir faktör 1-alfa için immünohistokimyasal değerlendirme yapıldı. Genel sağkalım ile araştırılan parametreler arasındaki ilişki Kaplan-Meier ve regresyon analizleri ile analiz edildi.

Bulgular: Takip süresince 11 nüks ve 20 ölüm meydana geldi. Nükleer faktör-kappa B ve Hipoksi ile indüklenebilir faktör 1-alfa boyama ve genel sağkalım arasında anlamlı bir ilişki yoktu. Yaş, sigara içme durumu, farklılaşma, T evresi ve ön komissür tutulumu ile Nükleer faktör-kappa B ve Hipoksi ile indüklenebilir faktör 1-alfa boyama arasında anlamlı bir ilişki yoktu.

Tartışma ve Sonuçlar: Primer olarak radyoterapi ile tedavi edilen erken evre glottik karsinomda tedavi öncesi Nükleer faktör-kappa B ve Hipoksi ile indüklenebilir faktör 1-alfa ekspresyon düzeylerinin genel sağkalım ile ilişkili olmadığı saptanmıştır.

Anahtar Sözcükler: Glottik; kanser; radyoterapi; sağkalım; direnç

INTRODUCTION

Early-stage laryngeal carcinoma is defined as a T1-T2 tumor without evidence of any clinical local and/or distant metastases. Laryngeal carcinoma arises most commonly from the glottic region. The prognosis is better because of the possibility of early diagnosis and poor lymphatic drainage. The treatment goal for early-stage glottic carcinoma is a single-treatment modality, either open or endoscopic surgery or radiotherapy. Satisfaction with survival and local control rates can be achieved by using each treatment method. Although salvage laryngectomy can be performed in cases of radiotherapy failure, patients are usually at risk with increased complication rates. Therefore, identifying prognostic factors that can

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estimate radioresistance in the pretreatment period is crucial.

Nuclear factor-kappa B (NF-κB) is a transcription factor normally located in the cytoplasm in an inactive form. NF-κB can be stimulated with hypoxia and chemical and physical stimulants. When activated, it translocates into the nucleus, where it activates genes related to angiogenesis, anti-apoptosis, and carcinogenesis[1]. Tumor hypoxia is a well-known factor that participates in treatment-resistant head and neck squamous cell carcinoma[2,3]. Hypoxia-inducible factor 1-alpha (HIF-1α) is another transcription factor that can be stimulated by hypoxia and other physical stimulants. HIF-1α also stimulates genes that participate in carcinogenesis. Therefore, both NF-κB and HIF-1α have been investigated in many tumors as prognostic factors[4]. Additionally, it has been shown that NF-κB may be responsible for radioresistance in laryngeal carcinoma[5,6].

In this study, we investigated the relationship between NF-κB and HIF-1α expression and overall survival in patients with early-stage glottic carcinoma managed primarily with radiotherapy.

MATERIAL and METHODS

Ethical approval for this study was obtained from the local ethics committee (approval number 2020/391) and was supported with the number 201111035 by Project Development and Support Unit of University. Patients diagnosed with early glottic laryngeal squamous cell carcinoma who underwent radiotherapy between November 2005 and January 2011 were included in the study. Patients with a history of surgery or radiotherapy in the head and neck region for any reason, and who were followed up for <1 year were excluded from the study. In total, 40 patients were eligible for inclusion in the study. Since our study is retrospective, informed consent was not obtained. The patients" clinical and pathologic parameters (e.g. age, smoking status, alcohol consumption, tumor differentiation, stage, anterior commissure involvement) and follow-up information (e.g. visit times, recurrence, or death) were recorded.

The anterior commissure involvement was managed during direct laryngoscopy and with radiologic imaging. The patients were followed up according to the European Laryngeal Society guidelines[7].

Radiotherapy was given 5 days per week and 2 Gy daily per fraction. A total of 66-70 Gy (median 68 Gy) radiotherapy was applied. The patients were treated with a 64 MV photon and the field size was 6x6 cm. Elective neck radiotherapy was not given to any patient.

Surgical specimens were obtained for pathological examination. The tissues were sectioned at 4 mm thick. Routine deparaffinization and rehydration were applied. Immunohistochemical staining was performed using a Benchmark staining platform (Ventana Medical Systems Inc.) with a Ventana Universal DAB detection kit. HIF-1a Ab-4 (H1alpha 67) Mouse Mab and NF-κB p65 (Rel A) RB were used as primary antibodies. After staining, sections were dehydrated and covered with coverslips.

For each marker, at least 1000 cells were counted under a light microscope. Scoring was made without knowledge of clinical data.

HIF 1-a staining was divided according to the positive staining cell ratio into five groups; group 1: 0%, group 2: 1-10%, group 3: 11-50%, group 4: 51-80%, and group 5: 81-100%. For the statistical evaluation, groups 1, 2, and 3 were accepted as negative, and 4 and 5 as positive[8]. HIF 1-a staining of more than 50% of cells is seen in Figure 1.

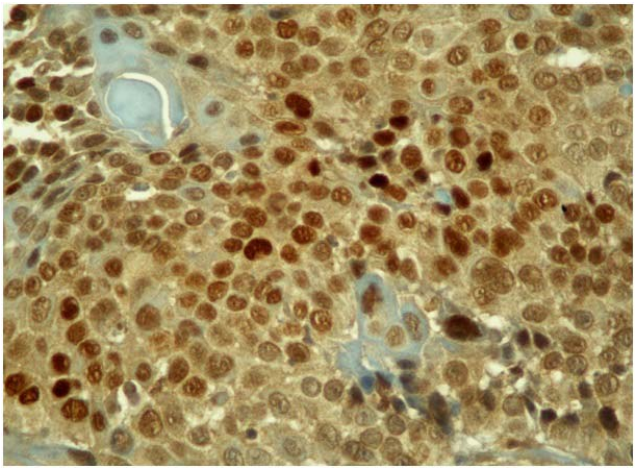


Figure 1: *HIF 1-a nuclear staining in more than 50% of tumor cells (H&E, x400).*

NF-κB staining was divided according to the nuclear and/or cytoplasmic staining cell ratio into three groups; group 1: <10% focal, group 2: 11-50% regional, and group 3: 51-100% diffuse. Also, three groups according to the staining intensity; group 1: weak, group 2: moderate, and group 3: strong. For the statistical evaluation, if one of the groups was 1, it was accepted as negative, if none of the groups were 1, it was accepted as positive[5]. The diffuse NF-κB staining is seen in Figure 2.

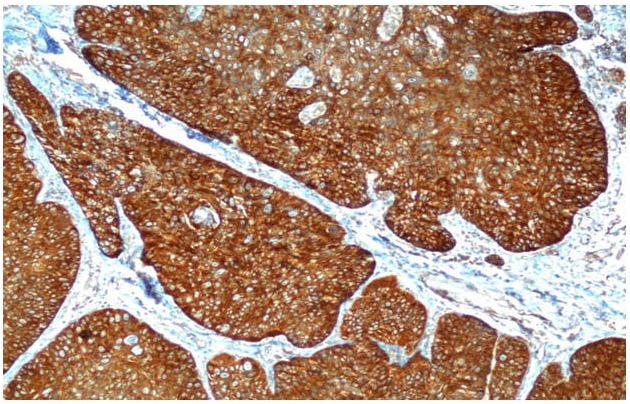


Figure 2: *Diffuse NF-κB cytoplasmic staining in tumor cells (H&E, x100).*

Statistical analysis

NCSS (Number Cruncher Statistical System, Kaysville, UT) 2007 software was used for the statistical analysis. Age, smoking, alcohol consumption, anterior commissure involvement, tumor stage, HIF-1a, and NF kappa B status considered for statistical evaluation. The relationship between HIF-1a and NF-κB status and age, smoking, alcohol consumption, anterior commissure involvement, and tumor stage was investigated using the Chi-square and Fisher's exact tests. The Brinkman Index [(number of cigarettes smoked per day) x (number of years smoked)] was used for the smoking status evaluation. Alcohol consumption was assessed as user and non-user. Logistic regression analysis was performed for univariate analysis. Overall survival [(OS); the time between the last date of radiotherapy to the date of the last follow-up or death from any cause) was calculated using the Kaplan-Meier analysis. In all analyzes, $p<0.05$ was considered to indicate statistical significance.

RESULTS

The study population comprised 39 (97.5%) men and 1 (2.5%) woman with an average age of 64.70±9.22 years. The mean follow-up period, recurrence time, and time from radiotherapy to death were 60.85±31.19 months, 48.43±39.73 months, and 58.30±38.93 months, respectively. During the follow-up period, 11 (27.5%) recurrences and 20 (50%) deaths [9 (22.5%) of which were due to recurrence] occurred. Among 11 recurrent cases, three underwent total laryngectomy with neck dissection, one patient underwent neck dissection only, three patients received chemoradiotherapy, and four patients were out of follow-up after recurrences. The clinicopathologic data of the patients are presented in Table 1.

Eighteen (45%) of the patients had NF-κB cytoplasmic staining, and 22 of the patients (55%) had both cytoplasmic and nuclear staining. According to the staining cell ratio; 2 (5%) patients were in the focal category, 17 (42.5%) were regional, and 21(52.5%) were in the diffuse category. According to the staining intensity, there were 12 (30%), 16 (40%), and 12 (30%) patients in groups 1, 2, and 3, respectively. Twelve (30%) patients were accepted as negative for NF-κB staining and 28 (70%) patients were accepted as positive.

For HIF-1α, according to the positive staining cell ratio, there were 5 (12.5%), 15 (37.5%), 7 (17.5%), 5 (12.5%), and 8 (20%) patients in groups 1, 2, 3, 4, and 5, respectively. Therefore, 27 (67.5%) patients were accepted as negative for HIF-1α staining and 13 (32.5%) patients were accepted as positive.

The relationship between clinicopathological parameters and HIF-1α and NF-κB expression status is presented in Table 2. There were no statistically significant relations ($p>0.05$).

Univariate analysis was performed for age ($p=0.077$), smoking status ($p=0.960$), alcohol consumption ($p=0.791$), tumor differentiation ($p=0.990$), anterior commissure involvement

(p=0.613), NF-κB status (p=0.620), HIF-1α status (p=0.726), and stage (p=0.814). We did not perform multivariate analysis because only one parameter showed a p-value less than 0.1.

Table 1: Clinicopathological characteristics of patients

		Number of cases n (%)	Recurrence n(%)	Exitus n(%)
Age	65>	24 (60)	9 (81)	9 (45)
	65≤	16 (40)	2 (19)	11 (55)
Brinkmann index	600>	16 (40)	4 (37)	8 (40)
	600≤	24 (60)	7 (63)	12 (60)
Alcohol consumption	(-)	30 (75)	9 (81)	15 (75)
	(+)	10 (25)	2 (19)	5 (25)
Differentiation	Well-differentiated	11 (27.5)	4 (37)	5 (25)
	Moderately differentiated	29 (72.5)	7 (63)	15 (75)
Anterior commissure involvement	(-)	29 (72.5)	9 (81)	14 (70)
	(+)	11 (27.5)	2 (19)	6 (30)
Tumor stage	1a	22 (55)	5 (44)	11 (55)
	1b	8 (20)	2 (19)	3 (15)
	2	10 (25)	4 (37)	6 (30)
HIF-1α status	Positive	13 (32.5)	2 (19)	6 (30)
	Negative	27 (67.5)	9 (81)	14 (70)
NF kappa B status	Positive	27 (67.5)	7 (63)	14 (70)
	Negative	13 (32.5)	4 (37)	6 (30)
Recurrence	(-)	29 (72.5)		11 (55)
	(+)	11 (27.5)		9 (45)
Exitus	(-)	20 (50)		
	(+)	20 (50)		

Table 2: Relationship between clinicopathological parameters with HIF-1α and NF kappa B immunostaining status.

			HIF-1α status		NF kappa B status	
			Negative	Positive	Negative	Positive
Age	65>	24 (60)	16	8	9	15
	65≤	16 (40)	11	5	4	12
Brinkmann index	600>	16 (40)	12	4	7	9
	600≤	24 (60)	15	9	6	18
Alcohol consumption	(-)	30 (75)	21	9	12	18
	(+)	10 (25)	6	4	1	9
Differentiation	Well-differentiated	11 (27.5)	9	2	5	6
	Moderately differentiated	29 (72.5)	18	11	8	21
Anterior commissure involvement	(-)	29 (72.5)	19	10	9	20
	(+)	11 (27.5)	7	4	4	7
Tumor stage	1a	22 (55)	15	7	5	17
	1b	8 (20)	6	2	4	4
	2	10 (25)	6	4	4	6

DISCUSSION

In this study, we investigated the relationship between the expression of NF-κB and HIF-1α and OS in early-stage glottic carcinoma primarily managed with radiotherapy, with more than 5 years of follow-up. We also analyzed the effect of the T stage, the anterior commissure involvement, and tumor

differentiation. We found no significant relationship between OS and NF-κB status, HIF-1α status, T stage, anterior commissure involvement, tumor differentiation, alcohol consumption, and smoking status. Also, there was no relationship between NF-κB and HIF-1α expressions with T stage, the anterior commissure involvement, tumor differentiation, alcohol consumption, and smoking status.

It is well known that genetic and environmental factors play a role in tumorigenesis. Many cytokines, transcription factors, and markers are involved in different parts of this complicated process. These factors can be stimulated by various types of stimulants, such as hypoxia, bacterial and viral agents, radiation, chemical carcinogens, and chemotherapeutics[9]. The NF-κB family, composed of p50, p52, RelB, c-Rel, and p65/Rel, is usually located in the cytoplasm in an inactivated form[9-11]. When activated, NF-κB dimers translocate into the nucleus and stimulate more than a hundred genes responsible for inflammatory processes, metastasis, cellular motility, angiogenesis, and apoptosis[9-11]. It has been shown that NF-κB plays a role in the carcinogenesis of laryngeal carcinoma[6]. Additionally, NF-κB may be enhanced by chemotherapeutics and radiation, which causes chemo-radioresistance[9,10]. HIF-1α is a transcription factor that translocates to the nucleus and promotes the production of several genes under hypoxic conditions. Although hypoxia is a major stimulant for HIF-1α, it can also be stimulated by other factors[12]. Stimulation of HIF-1α causes induction of angiogenesis, cell proliferation, and apoptosis[4]. Also, it is known that NF-κB and HIF-1α have extensive crosstalks[4].

Schrijvers et al. investigated the prognostic ability of HIF-1α in patients with T1-T2 glottic carcinoma treated primarily with radiotherapy. There was no significant relation between HIF-1α and T and N status. They found that high levels of HIF-1α were significantly correlated with worse local control and OS[13]. By contrast, in another study, 271 patients with early glottic carcinoma treated primarily with radiotherapy were analyzed and no significant relation between HIF-1α levels and local control, cancer-specific survival, and the stage was observed[14]. Additionally, there was no significant relationship between HIF-1α status and advanced-stage larynx and hypopharynx carcinoma treated with an organ preservation protocol[15]. HIF-1α was also investigated in supraglottic T1-T2 tumors and no statistical significance was observed between HIF-1α levels and local control rates[12]. In another study, HIF-1α was investigated in surgically treated supraglottic larynx carcinoma. Although HIF-1α expression was correlated with T status, there was no significant correlation with survival[16]. Kyzas et al. showed that HIF-1α expression had no significant relationship with age, sex, histological grade, clinical stage, and survival[17]. A recent meta-analysis showed that HIF-1α had no predictive value in laryngeal carcinoma primarily managed with radiotherapy[18]. Our study results were similar to the literature. We observed no significant relationship between HIF-1 α status and age, Brinkman Index, alcohol consumption, T stage, differentiation, and OS.

It has been shown that NF-κB plays role in laryngeal carcinogenesis, and in patients with head and neck carcinoma; NF-κB expression was significantly correlated with survival and local recurrence[6,11,19,20]. Yoshida et al. investigated NF-κB expression in early-stage laryngeal carcinoma primarily managed with radiotherapy. They reported that 77% of patients with radioresistance had positive NF- κB staining, which we have 70% in our study. They evaluated NF-κB expression in recurrent tumors and revealed that the positivity ratio was 90%. An increased expression ratio in recurrent tumors may indicate that NF-κB is one of the factors responsible for radioresistance. Also, they observed that positive NF-κB expression was correlated with poor local control and 5-year survival. In another study, Huang et al. investigated NF-κB expression in 78 patients with laryngeal carcinoma primarily managed with surgery[19]. They observed that NF-κB expression was significantly related to lymph node metastasis, T stage, and OS. Jiang et al.'s investigation included 89 patients with laryngeal carcinoma primarily managed with surgery, and they showed that NF-κB expression was significantly related to lymph node metastasis, T

stage, clinical stage, and OS[21]. In our study, we observed no significant relationship between NF-κB expression and T stage, tumor differentiation, age, Brinkman Index, and anterior commissure involvement. Also, there was no relationship with OS.

It has been thought that anterior commissure involvement plays a crucial role in laryngeal carcinoma. Although some authors state that the anterior commissure area is resistant to cancer invasion and therefore plays a protective role, most authors agree that this area is prone to invasion, and invasion of the anterior commissure may ease the invasion of critically important areas such as the pre-epiglottic region and cricothyroid membrane. A recently published meta-analysis included only patients with T1 glottic carcinoma and showed that the anterior commissure invasion negatively affected 5-year local control rates, regardless of the treatment modality[22]. Also, in another meta-analysis, Eskiizmir et al. analyzed factors causing radiotherapy failure in early-stage glottic carcinoma and found the anterior commissure invasion to be among them[23]. However, another review showed that in early-stage laryngeal carcinoma, the anterior commissure invasion did not affect local control when evaluated as the anterior commissure (+/-)[24]. These conflicting results may be due to the difficulty in evaluating the anterior commissure invasion. In our study, we found no significant relationship between the anterior commissure invasion and local recurrence, and OS.

This study had several limitations. Since our study design is retrospective, it may be a patient affected by selection bias. Also, our study population is relatively small.

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