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The relation of immunohistochemical p53 and p63 expression with histopathological parameters in laryngeal carcinomas

Larinks karsinomlarında immünhistokimyasal p53 ve p63 ekspresyonunun histopatolojik parametreler ile ilişkisi

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ABSTRACT

The aim of this study is to investigate whether there is a correlation between overexpression of p53 and p63 and the histopathological features of laryngeal squamous cell carcinomas (LSCC) and to reveal any possible prognostic value. For these purposes, 43 paraffin sections of laryngeal carcinomas were studied using immunohistochemical staining for p53 and p63. We detected p53 expression in 97.7% of the cases. Overexpression of p53 was significantly associated with histologic grade (p=0.001) and vascular invasion (p=0.028) but, there was no any statistically significant correlation with the other histopathological parameters. In our study 100% of laryngeal carcinomas showed positive p63 immunostaining. We did not detect any statistically significant association between expression of p63 protein and histopathological parameters. As the tumors with an aggressive phenotype showed more intense p53 expression, we suggest that p53 expression in LSCC can predict poor prognosis. However, according to the staining pattern of p63, its expression may reflect the immaturity of the tumor of the cell lineage. We speculate that p63 expression may give an idea about the differentiation of LSCC rather than its prognosis.

Key words: Larynx, squamous cell carcinoma, p53, p63, immunohistochemistry

ÖZET

Bu çalışmanın amacı larinks skuamöz hücreli karsinomlarının (LSHK) histopatolojik özellikleri ile p53 ve p63 aşırı ekspresyonu arasındaki ilişkiyi araştırmak ve olası prognostik değerini ortaya koymaktır. Bu amaçla 43 larinks karsinomu parafin kesitleri bu proteinler ile immunohistokimyasal olarak boyanarak çalışılmıştır. Olguların %97,7'sinde p53 ekspresyonu saptanmıştır. p53 aşırı ekspresyonu histolojik grade (p=0.001) ve vasküler invazyon (p=0.028) ile anlamlı şekilde ilişkiliyken, diğer histopatolojik parametreler ile arasında istatistiksel olarak anlamlı ilişki saptanmamıştır. Çalışmamızda larinks karsinomlarının %100'ü p63 ile pozitif boyanma göstermiş, histopatolojik parametreler ile p63 ekspresyonu arasında istatistiksel olarak anlamlı ilişki saptanmamıştır. Agresif fenotipe sahip tümörlerin daha yoğun p53 ekspresyonu göstermesi nedeniyle LSHK'sinde p53 ekspresyonunun kötü prognoz göstergesi olabileceğini ileri sürülebilir. Bununla birlikte boyanma paternine göre, p63 ekspresyonu tümör hücrelerinin immatüritesini yansıtabilir. p63 ekspresyonunun LSHK'nin prognozundan ziyade diferansiyasyonu hakkında fikir verebileceği kanısındayız.

Anahtar sözcükler: Larinks, skuamöz hücreli karsinom, p53, p63, immünhistokimya

INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) is one of the most frequent malignant tumors of head and neck (1). Clinical factors such as the patient's age, sex, alcohol consumption and smoking habits, clinical stage, size and location of the tumor and histopathological parameters such as the presence of metastatic cervical lymph nodes, and grades of differentiation were investigated as prognostic indicators of

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survival in patients with LSCC (2-4). As tumors with similar clinical and histomorphologic features may have different clinical outcomes, other variables should be considered to make more precise prognostication of laryngeal carcinomas (2).

One of the most commonly observed gene alterations in human cancer is the mutation of the p53 tumor suppressor gene (5). Mutations or gene losses result in altered protein expression which is associated with serious cell cycle disturbances. The p53 gene acts as 'guardian of the genome' and may force the altered cell into apoptosis. Apoptosis is induced if the cell is unable to repair its DNA damage after p53-induced cell cycle arrest (5).

In many studies overexpression or mutation of the related gene in p53 protein has been found to be linked with impaired survival (6,7), however some other studies have failed to establish a significant correlation between p53 expression and prognosis in LSCC (8,9). The p63 gene, a recently defined member of the p53 gene family, is located on chromosome 3q27-29 (10). It exhibits a high sequence and structural homology to p53. However, there are significant differences between p53 and p63 proteins. The p63 gene contains six different isoforms: three transactivating isoforms (TA-p63α, TA-p63β, TA-p63γ) encode proteins with transactivation domain and they are capable of transactivating p53 target genes and induce apoptosis or cell-cycle block, whereas three other isoforms lack the N-terminale domain necessary for transactivation (ΔNp63α, ΔNp63β, ΔNp63γ) and act as a dominant negative agent, inhibiting the transactivation activity of both TA-p63 and p53 (10,11). As transactivating and truncated p63 isoforms show opposite effects in regulating proapoptotic and differentiating genes, such as p21 and bax, this data suggested that the process of epithelial differentiation depends on a dynamic balance between TA-p63 and ΔNp63 isoforms (12).

In recent studies p63 expression has been demonstrated in the nuclei of basal cells of the multilayered epithelia including skin, cervical and vaginal mucosa, urothelium, respiratory tract, myoepithelial/basal cells of the prostate, breast and sweat glands (13,14). Data from p63-deficient mice have made it apparent that p63 plays a key role in regulating epithelial proliferation and differentiation programs (15).

A number of studies have investigated the role of p63 in neoplastic transformation and tumor progression (13,14). The expression of p63 has been demonstrated in squamous cell carcinomas of multiple regions, like digestive tract, head and neck, cervix, lung, and also in urothelial carcinomas, some thymomas and non-Hodgkin lymphomas (16). The expression of p63 has also been documented in basal cell carcinomas of the skin (13). Although the functions of p63 in normal and neoplastic tissues still continue to be largely elusive, it has been demonstrated that this protein may induce growth suppression and death in tumor cell lines, and terminal differentiation in human keratinocytes or skeletal cells (17).

In our present study, we examined the expression of p53 and p63 proteins in LSCCs in an attempt to investigate their involvement in laryngeal carcinogenesis and to find out whether a correlation exists between overexpression of these proteins and histopathological parameters.

MATERIALS and METHODS

Patients: Our study was performed on 43 patients with LSCC diagnosed and treated in Suleyman Demirel University Medical Faculty between 1998 and 2005. Patient and tumor data were obtained from patient records. Hematoxylin-eosin sections were re-evaluated to confirm the histopathological diagnosis and grading of the tumors. Histopathological differentiation was graded in three categories according to the World Health Organization (WHO) classification. The most representative slides were chosen for the immunohistochemical staining.

All patients had previously undergone laryngectomies, and lymph node dissection. There was no clinical evidence of distant metastasis in any of the patients at the time of operation. Tumors were staged according to the 1992 American Joint Committee on Cancer Staging Classification System. Clinical stage distribution at the time of diagnosis was as follows: stage I, 5 cases (11.6%), stage II, 5 cases (11.6%), stage III, 10 cases (23.3%), and stage IV, 23 cases (53.5%).

Immunohistochemical staining: Immunohistochemical analysis for p53 and p63 were performed on formalin-fixed, paraffin-embedded archival tissue using the streptavidin-biotin-peroxidase technique. For all cases, a 4µm histological section was deparaffinized in xylene and rehydrated in descending dilutions of ethanol. For the antigen retrieval, slides were treated by microwave heating in citrate buffer (pH 6.0) for 10 minutes. Endogenous peroxidase activity was blocked by 20 minutes of incubation with 0.3% hydrogen peroxidase. Slides were incubated with mouse monoclonal anti-p53 antibody (1:100, Clone Do-7+BP53-12, Neomarkers) and mouse monoclonal anti-p63 antibody (1:100, Clone 4A4+Y4A3, Neomarkers). Sections were incubated with a streptavidin-biotin-peroxidase kit (Ultra Vision Large Volume Detection System Anti-Polyvalent, HRP, LabVision, USA), and after incubation the reaction product was detected using diaminobenzidine (DAB). Finally, the sections were counterstained with Mayer's hematoxylin, and mounted with mounting medium. Only nuclear p53 and p63 expressions were accepted as specific reactions. Appropriate positive and negative controls were stained for each antibody.

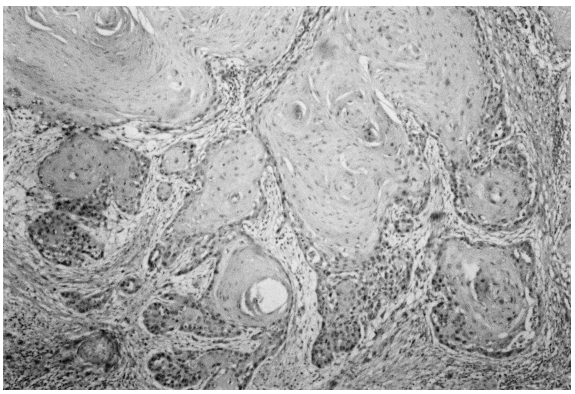
In microscopic analysis, the percentage of positive nuclei in 1000 consecutive cells of most evenly stained areas of the tumor were counted. Expressions of p53 and p63 proteins were scored on a semi-quantitative scale as negative, 1+, 2+, and 3 + (-, +, ++, +++) by calculating the percentage of stained nuclei. Expression of each protein was graded as -, negative no staining of neoplastic cells; +, 1-9% positivity of neoplastic cells; ++, 10-50% positivity of neoplastic cells; +++, 51-100% positivity of neoplastic cells respectively.

Statistical analysis: Associations between expressions of p53 or p63 protein and histopathological findings were analyzed by Pearson chi-square test, Kruskal-Wallis test, and Mann-Whitney U test. The association between p53 and p63 protein expression was analyzed by Spearman's rank correlation coefficient test. $P<0.05$ was considered significant.

RESULTS

The study population consisted of 42 (97.7%) men and 1 (2.3%) woman. The mean age was 57.9 (range 27-80) years. The distribution of tumors according to the anatomic location was as follows: 26 glottic (60.5%), 16 supraglottic (37.2%), and 1 infraglottic (2.3%) cases. The mean tumor size was 3.02 cm (range 0.5-6.2 cm). Among 43 LSCCs studied, 18 (41. 9%) well differentiated, 21 (48.8%) moderately differentiated, and 4 (9.3%) poorly differentiated carcinomas were diagnosed. Cervical lymph node metastases (n,14; 32.6%), cartilage involvement (n, 17; 39.5%), perineural (n, 7; 16.3%) and vascular invasions (n, 6; 14%) were detected.

Of 43 laryngeal carcinomas, 42 (97.7%) were immunohistochemically positive for p53. Specimens demonstrated +, ++, and +++ positive staining for p53 in 11 (25.6%), 4 (9.3%), and 27 (62.8%) cases, respectively. One had no staining. Although overexpression of p53 was significantly associated with the histological grade ($p=0.001$) and vascular invasion ($p=0.028$). But, there was no any statistically significant correlation with clinical stage, size and location of tumor, lymph node metastasis, perineural invasion and cartilage involvement. Figure 1 displays a representative immunohistochemical analyses for p53 in well, moderately and poorly differen-



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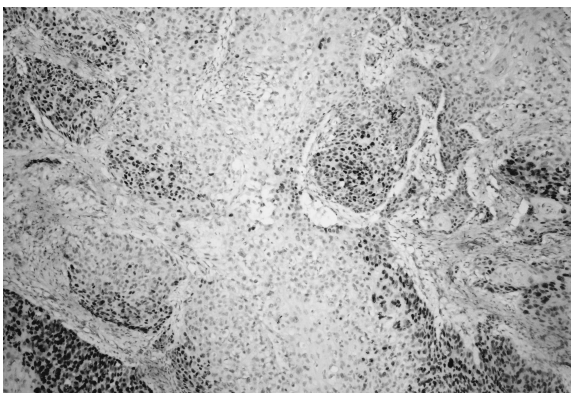

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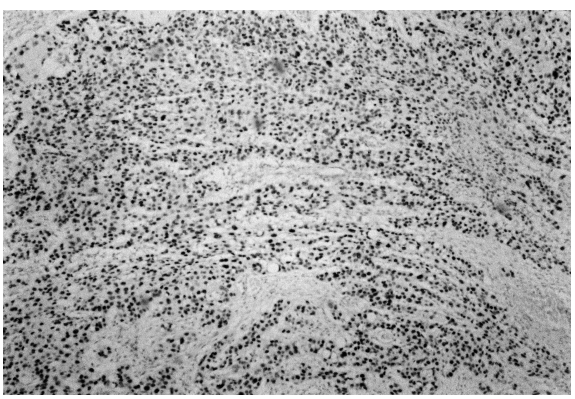

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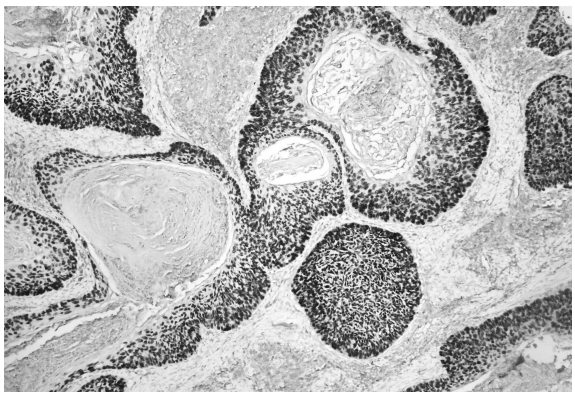
Figure 1. Representative immunostaining for p53 in (a) well (x40), (b) moderately (x40), and (c) poorly differentiated LSCCs (x40).



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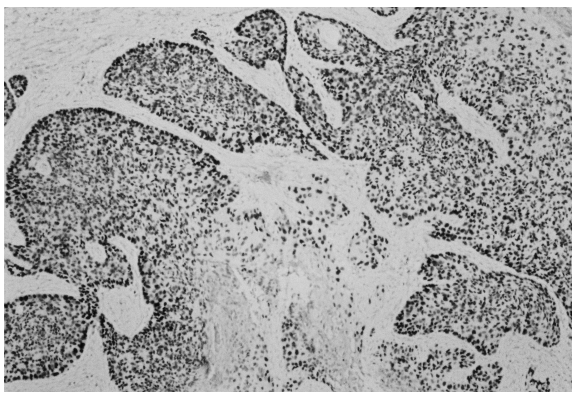

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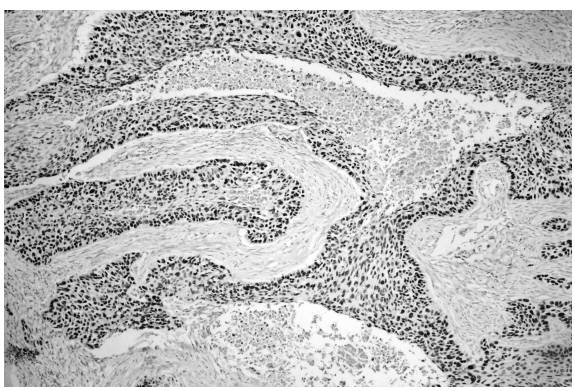

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Figure 2. p63 expression in (a) well (x40), (b) moderately (x40), and (c) poorly differentiated LSCCs (x40).

tiated LSCCs.

In our study all of laryngeal carcinomas showed positive p63 immunostaining. While 1(2.3%) specimen showed ++ expression, 42 (97.7%) specimens demonstrated +++ expression. There was no statistically significant association between expression of p63 protein, size and location, clinical stage, histological grade of the tumor, vascular invasion, presence of metastatic lymph node, perineural invasion, and carti-

Table 1. Correlation between expressions of p53 and p63 proteins and histopathological parameters in LSCC.

Parameters	No. of patients n (%)	p53 n (%)					p63 n (%)			
		(-)	(+)	(++)	(+++)	p	(-/+)	(++)	(+++)	p
Tumor size										
<3cm	22 (51.2)	1 (2.3)	6 (14)	2 (4.6)	13 (30.2)		-	1 (2.3)	21 (48.8)	
≥3cm	21 (48.8)	-	5 (11.6)	2 (4.6)	14 (32.6)	0.776	-	-	21 (48.8)	0.512
Tumor location										
Glottic	26 (60.5)	1 (2.3)	8 (18.6)	1 (2.3)	16 (37.2)		-	1 (2.3)	25 (58.1)	
Supraglottic	16 (37.2)	-	2 (4.6)	3 (7)	11 (25.6)	0.338	-	-	16 (37.2)	
Infraglottic	1 (2.3)	-	1 (2.3)	-	-		-	-	1 (2.3)	0.716
Histological grade										
G1	18 (41.9)	-	8 (18.6)	4 (9.3)	6 (14)		-	1 (2.3)	17 (39.5)	
G2	21 (48.8)	1 (2.3)	3 (7)	-	17 (39.5)	0.001	-	-	21 (48.8)	
G3	4 (9.3)	-	-	-	4 (9.3)		-	-	4 (9.3)	0.491
Clinical stage										
Early (I+II)	10 (23.3)	1 (2.3)	3 (7)	2 (4.6)	4 (9.3)		-	-	10 (23.3)	
Advanced (III+IV)	33 (76.7)	-	8 (18.6)	2 (4.6)	23 (53.5)	0.211	-	1 (2.3)	32 (74.4)	0.828
Vascular invasion										
(+)	6 (14)	1 (2.3)	-	-	5 (11.6)		-	-	6 (14)	
(-)	37 (86)	-	11 (25.6)	4 (9.3)	22 (51.1)	0.028	-	1 (2.3)	36 (83.7)	0.860
Lymph node metastasis										
(+)	14 (32.6)	-	4 (9.3)	-	10 (23.3)		-	-	14 (32.6)	
(-)	29 (67.4)	1 (2.3)	7 (16.3)	4 (9.3)	17 (39.5)	0.435	-	1 (2.3)	28 (65.1)	0.674
Perineural invasion										
(+)	7 (16.3)	-	1 (2.3)	1 (2.3)	5 (11.6)		-	-	7 (16.3)	
(-)	36 (83.7)	1 (2.3)	10 (23.3)	3 (7)	22 (51.1)	0.817	-	1 (2.3)	35 (81.4)	0.837
Cartilage involvement										
(+)	17 (39.5)	-	3 (7)	1 (2.3)	13 (30.2)		-	1 (2.3)	16 (37.2)	
(-)	26 (60.5)	1 (2.3)	8 (18.6)	3 (7)	14 (32.6)	0.469	-	-	26 (60.5)	0.395

lage involvement. Figure 2 displays p63 expression in well, moderately and poorly differentiated LSCCs. The immunostaining results and histopathological parameters are summarized in Table 1. Also there was no statistically significant correlation between expressions of p53 and p63 proteins.

DISCUSSION

In our study, we examined the expression of p53 and p63 proteins in LSCCs with the aim of establishing a relationship between overexpression of these proteins and histopathological parameters. Overexpression p53 protein has been reported in LSCCs at a frequency ranging from 38% to 79% of the tumor samples analyzed (7,18,19). In agreement with recent studies, we found a higher incidence (97.7%) of p53 overexpression in patients with LSCC.

In previous reports Golusinski et al. (20) found statistically significant correlation between p53 expression T and N stages, the degree of histological malignancy and survival time in LSCC. Khademi et al. (21) emphasized the presence of a correlation between p53 expression and nodal involvement however; p53 expression did not show any correlation with histological grades in their cases. Similar to Morawski et al (22) we found no correlation between p53 expression and N-stage. However, overexpression of p53 was significantly associated with the histological grade and vascular invasion in our group. Therefore, p53 expression correlates with aggressiveness of the tumor. The relation between p53 expression, histological grade and

vascular invasion suggests the role of p53 in the progression and metastatic invasions of laryngeal carcinomas.

The role of p63 in oncogenesis is still being investigated, but the complex nature of this molecule with its various isoforms that appear to have opposing actions, make this investigation particularly challenging (10,11). The accumulation of p63 immunoreactive cells in laryngeal intraepithelial neoplasias allows for the speculation that just like p53 gene disorders, p63 gene abnormalities may be involved in the very early stages of laryngeal cancer development, in a similar manner as for p53 (23). Since the 3q27-29 chromosomal region where p63 is located, is the most frequently overrepresented genomic locus in head and neck cancer, it was suggested that an abnormal status and expression of p63 gene might play a pivotal role in the multiple stage model of laryngeal tumorigenesis (24). Pruneri et al. (23) documented TA-p63 and îNp63 expression in all samples of normal laryngeal mucosa, where it was restricted to the basal and suprabasal epithelial cell layers and reported that a downregulation of the antiproliferative agent TAp63 favors an aggressive behavior in SCC of the larynx.

In the study of Sniezek et al. (25) Western blot analysis indicated that ΔNp63α is the predominant isoform identified in the head and neck SCC specimens and implied an overexpression in the tumors when compared with the adjacent nonmalignant tissue. They suggested that p63 plays an antidifferentiating and antiapoptotic role in the mucosal epithelium of the head and neck, possibly leading to tumor formation (25).

Pruneri et al. (23) detected p63 immunopositivity of the neoplastic cells of all the LSCCs analyzed with a percentage of immunoreactive cells ranging from 10% to 98%. We found a very high prevalence of p63 immunoreactivity in LSCCs. In our study we used a monoclonal antibody test which recognized all p63 isoforms without distinguishing between truncated and transactivating isoforms. Truncated isoforms, however, are preferentially expressed in the basal cell compartment of a variety of normal epithelial tissues found in skin, cervical and vaginal mucosa, urothelium, respiratory tract, and the prostate, as well as in several types of neoplasms, including lung, and head and neck carcinomas (16).

To our knowledge, although the expression of p63 in laryngeal cancer has not been studied extensively unlike intense investigations in prostate, breast and lung, the lack of prognostic significance of p63 expression has been reported in laryngeal cancer (23). In our study, there was no statistically significant correlation between expression of p63 and p53 protein. Studies in other tumor types, such as breast, bladder, laryngeal and nonsmall cell lung cancer have also noted a lack of correlation between p63 and p53 expression or mutational status (23, 26-28).

In our study group p53 and p63 overexpression showed no statistically significant differences with regard to the size location, and clinical stage of the tumor, presence of metastatic lymph nodes, cartilage involvement, and perineural invasion. While overexpression of p53 was significantly associated with the histological grade and the presence of vascular invasion, there was no statistically significant correlation between p63 overexpression and tumor histological grade of the tumor, and p63 immunoreactivity seemed to be affected by the degree of differentiation in our tumor samples. Poorly differentiated LSCCs showed diffuse p63 staining throughout tumoral tissue, whereas well differentiated LSCCs had immunoreactive cells around the periphery, but the central zones lacked staining.

Parsa et al (29) and De Laurenzi et al. (30) demonstrated that p63 expression gradually reduced from the basal cells to the terminally differentiated keratinocytes in accordance with studies on human normal skin, where p63 staining was confined to almost all the basal and suprabasal cells, and gradually decreased in the midd-

le layer of epidermis, with no expression in superficial epidermal layers where terminal differentiation occurred. In our study basal and parabasal pattern of p63 expression was detected in normal mucosa adjacent to the tumor. Nuclear labeling was detected with nuclei showing an intense staining, stronger in the basal layer with respect to the parabasal layer. The staining pattern was similar to normal skin in well differentiated LSCCs. In tumors with lower grades, the cells become more undifferentiated and liken to basal cells. Staining for p63 was not detected in keratin-pearl areas in tumors. Intense and diffuse p63 expression was found in the less-differentiated cells situated at the periphery of the tumor islands.

Tumors having an aggressive phenotype such as less differentiation and vascular invasion, showed more intense p53 expression in our tumor samples. With these findings we have concluded that p53 expression in LSCCs can predict poor prognosis. Also we have found no significant relationship between overexpression of p63 and the histopathological parameters. However, according to the staining pattern, p63 expression seems to be linked to cell maturation in squamous epithelium of larynx and p63 staining might reflect the immaturity immature state of the tumor of the cell lineage. We speculate that p63 expression can give an idea about differentiation of LSCC rather than prognosis of these tumors.

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