Investigation of HPV Infection and P53 Expression in Laryngeal Verrucous Carcinoma

Larengeal Verrüköz Karsinomlarda HPV Enfeksiyonu ve P53 Ekspresyonu

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ABSTRACT Objective: Verrucous carcinoma is an uncommon, well-differentiated variant of squamous cell carcinoma that is regarded as a low grade, locally invasive malignant tumor with rare potential for distant metastasis. Although the pathogenesis of verrucous carcinoma is unknown, many possible causes have been proposed. The leading theories include human papillomavirus (HPV) infection and chemical carcinogenesis. The aim of this study is to analyse the status of the p53 gene and human papillomavirus (HPV) infection in verrucous carcinoma of larynx, and to investigate the correlation of p53 expression and HPV infection in verrucous carcinoma of the larynx. **Material and Methods:** A retrospective study has been performed between the years of 1994 and 2004, comprising 21 consecutive patients with verrucous carcinoma of larynx. Detailed clinical parameters have also been reviewed. HPV identification was accomplished with polymerase chain reaction. p53 immunohistochemistry was performed on 21 verrucous carcinoma using polyclonal serum CM-1. **Results:** The HPV infection has been detected in in all of the twenty-one cases of verrucous carcinomas. p53 immunohistochemistry was performed on 21 HPV containing verrucous carcinomas and showed the expression of p53 in 14 cases. No statistically significant differences were found between p53 expression and stage. **Conclusion:** The data indicate that HPV and the increased expression of p53 can coexist in verrucous carcinomas. Our study suggests that verrucous carcinomas of the larynx may arise through a still unknown mechanism which causes p53 inactivation or HPV infection.We think that p53 overexpression and oncogenic HPV infection may cooperate in the establishment of malignant cells in verrucous carcinoma of the larynx. The interaction between the HPV infection and certain cellular oncogenes such as p53 needs further studies.

Key Words: Carcinoma, verrucous; larynx; p53 protein, human; papillomavirus infections

ÖZET Amaç: Nadir metastaz potansiyeli bulunan düşük dereceli lokal invaziv malign tümör olarak bilinen verrüköz karsinom, skuamöz hücreli karsinomun iyi diferansiye bir varyantıdır. Verrüköz karsinomun patogenezi bilinmese de bazı olası nedenler öne sürülmüştür. Bunların başında human papilloma virus enfeksiyonu ve kimyasal karsinogenez gelmektedir. Çalışmamızın amacı larinksin verrüköz karsinomlarında p53 gen ve human papilloma virus enfeksiyonunun analizi ve p53 ekspresyonu ile human papilloma virus arasındaki ilişkinin değerlendirilmesidir. **Gereç ve Yöntemler:** Retrospektif çalışma 1994-2004 yılları arasında tanı almış 21 larengeal verrüköz karsinomlu olguda yapılmıştır. Detaylı klinik parametreler gözden geçirilmiştir. Human papilloma virus identifikasyonu polimeraz zincir reaksiyonu ile yapılmıştır. P53 immun boyası, 21 larengeal verrüköz karsinomlu olguda polyclonal CM-1 serumu kullanılarak uygulanmıştır. **Bulgular:** Human papilloma virus enfeksiyonu 21 larengeal verrüköz karsinomlu olgunun 21'inde (%100) saptanmıştır. Artmış p53 expresyonu ise 21 larengeal verrüköz karsinomlu olgunun 14'ünde tespit edilmiştir. P53 ekspresyonu ile evre arasında istatistiksel olarak anlamlı bir ilişki saptanmamıştır. **Sonuç:** Çalışmamızda HPV enfeksiyonunun ve artmış p53 ekspresyonunun verrüköz karsinomlarda birarada bulunabileceği gösterilmiştir. Çalışmamız larengeal verrüköz karsinomunun p53 inaktivasyonuna veya human papilloma virus enfeksiyonuna neden olan henüz bilinmeyen bir mekanizma sonucu geliştiğini destekler niteliktedir. Sonuç olarak; elde ettiğimiz veriler human papilloma virus enfeksiyonu ile p53 overekspresyonunun larengeal verrüköz karsinomlu olgularda malign hücre gelişiminde birlikte hareket ettiğini düşündürmektedir. Human papilloma virus enfeksiyonu ile p53 gibi belli başlı onkogenler arasındaki ilişkinin ortaya konması için daha fazla sayıda çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: Verrüköz karsinom; larenks; insan p53 proteini; papilloma virus enfeksiyonu

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Verrucous carcinoma (VC) which comprises 1-3% of all laryngeal neoplasms is an uncommon variant of squamous cell carcinoma with low malignant potential. It was first described as a distinct clinicopathological entity by Ackerman in 1948. He noted that the characteristic morphologic appearance, specific clinical behaviour and the excellent prognosis with proper treatment should separate VC from other epidermoid carcinomas. This lesion has a predilection for mucous membranes of the head and neck region, and is most commonly found in the oral cavity followed by the larynx. The preferred location of VC in of the larynx is supraglottic region.[1-3]

Etiology and carcinogenetic mechanism of VC of the larynx are largely unknown. Oncogenic HPV infection has been implicated in the pathogenesis of VC.[4] Another important factor in the development of the carcinoma appears to be the presence of alterations in p53 gene activity.[5] p53 is an important tumor suppressor gene involved in the regulation of cell growth, DNA maintenance and apoptosis. In addiation, experimental evidence suggests that the p53 protein is related to cell aggressiveness and tumor metastasis.[6] Several studies from different countries reported significant association between p53 polymorphism and human cancer. In addition, the Arg isoform of the p53 protein was shown to be more susceptible to degradation by the human papillomavirus (HPV) E6 protein than the proline one, and homozygosity for the arginine allele was found at higher frequency in the germlines of individuals affected by HPV-linked cancer.[7] The multistep process from HPV infection to carcinogenesis is not yet completely understood. HPV genetic sequences have been observed to be integrated into the host genome just as the cell develops invasive properties.[8] It is known that the E6 protein produced by high-risk HPV types 16 and 18 can combine with the p53 protein and cause the same functional consequence as a p53 gene mutation.[9,10] In contrast, the E6 protein expression from the low-risk HPV-6 does not produce such an effect. The E7 protein of HPV-16 was also shown to bind to the p105-RB protein encoded by the retinoblastoma gene (RB1).[11]

The aim of the study was to investigate the expression of p53 oncogene and its relation with HPV infection in VC of the larynx.

MATERIAL AND METHODS:

In a retrospective study, among 561 larynx carcinoma patients, 21 cases of VC of the larynx were collected and reviewed from the files of the Department of Pathology, Izmir Atatürk Research Training Hospital between 1994 and 2004. This study was approved by the local ethics committee and informed consents were retrieved from all the patients before the procedure. Histological slides from the tumors were re-examined to determine whether the corresponding paraffin blocks were suitable for the immunohistochemical study and in situ hybridization. Four-micrometer-thick sections were cut from the selected paraffin blocks. Sections were deparaffinized with xylene and rehydrated through graded concentrations of alcohol. The sections were then treated with microwave irradiation at 95°C in 10 mmol/L citrate buffer for 9 minutes. They were washed in water, rinsed with Tris-buffered saline (TBS), and treated with 3% hydrogen peroxide in methanol for 10 minutes at room temperature to block the intrinsic peroxidase activity. The sections were again washed with water and rinsed with TBS. Ten percent normal rabbit serum was then added at room temperature and incubated for 10 minutes. Afterwards, primary mouse monoclonal anti-p53 antibody was added and incubated in a moist chamber overnight at 4 °C. The slides were again washed 3 times in TBS for 3 minutes. Rabbit anti-mouse biotinylated IgG and preincubated avidin-biotin complex were added for 30 minutes at 37°C. The slides were washed in TBS as before and then developed in freshly prepared diaminobenzidin-hydrogen peroxide solution for 10 minutes at room temperature. The sections were then washed in water, counterstained with Mayer's haematoxylin. p53 immunostaining was evaluated at X 400 high-power field using standard light microscope. The brown nuclear stain was regarded as positive. The level of p53 accumulation was graded semiquantitatively into 4 categories (0, 1+, 2+, 3+) as described by Franchi et al (0 indicates negative; 1+, less than 10% of the

tumor cells were positive; 2+, 11% to less than 60% of the tumor cells were positive; and 3+, more than 60% of the tumor cells were positive).[12]

The polymerase chain reaction was performed on paraffin embedded tissue from 21 patients with VC of the larynx. Synthetic consensus and type–specific primaries were used to determine the HPV type from both VCs via real-time PCR. Amplifications were performed with a Nucleospin Tissue Extraction Kit (Macharey Nagel, 100 test) according to manufacturer's instructions. 5-6 μm thick histological sections of formalin-fixed paraffin-embedded tissues were cut for PCR analysis. The sections were deparaffinized in xylene, washed with absolute alcohol, and air dried. Deoxyribonucleic acid (DNA) was isolated from cells by digestion with proteinase K and extraction with phenol-chloroform followed by ethanol precipitation. The DNA was resuspended in Tris-EDTA (TE) buffer and quantified at the spectrophotometer. Extracted DNA was subjected to 35 cycles of PCR at 94, 64, 72°C for 45,45 seconds and 1 minutes, respectively, for denaturation, annealing, and elongation, with a pair of primaries: forward primary, 52-ATC AAC TTC GAC TGG CCC TTC-3', and backward primary, 5'- CCG TAC ATG TCG ATG TTC ACC -3'. The amplified PCR products yielded a positive band of approximately 179 base pairs. For typing HPV DNA, the PCR products were electrophoresed in 2% agarose gel (Sigma E8751), and were visualized with ethidium bromide staining. Finally, the tissue sections were counterstained and examined by transmitted light microscopy.

Continuous variables were described as median ± standard deviation and categorical data were presented as number and percentage. To compare continuous variables, we used the Chi-square test. It was performed for the comparison of categorical data. The statistical analysis was carried out by using Statistical Package of Social Science (SPSS; v10.0, Chicago, IL, USA) and a p value of <0.05 was considered as statistically significant.

RESULTS

A total of 21 patients were identified as having VC of the larynx over the ten year period between 1994 and 2004. All of them were males. The median age was 56 years (range 35-75 years). One of the cases died because of myocardial infarction in the pre-operative period. The most frequent localization of tumors was the glottic region (66%). The HPV infection was detected in all of the 21 cases. However, HPV-subtyping could be performed in only two cases, and both of these subtypes were HPV type11 (Figure 1) (Table 1). p53 immunostaining was performed in only 18 cases, due to lack of paraffin blocks in the other three cases. p53 immunohistochemistry performed on 18 HPV infections containing VC cases that showed elevated p53 levels in 14 cases (Figure 2a, b) (Table 1). There was no statistical relationship between the p53 overexpression and stage (Table 2).

DISCUSSION

VC, or Ackerman's tumor is a variant of well-differentiated squamous cell carcinoma which must be regarded as a specific tumor entity owing to its characteristic morphologic appearance and specific clinical behaviour with excellent prognosis.[1,13,14] The etiology of VC remains speculative and includes the use of tobacco products.[15] All of the subjects in our study were chronic tobacco users.

Viral induction may be a factor in the development of VC.[16,17] However, there are conflicting data in the literature on the identification of HPV DNA by ISH techniques. Some investigators either failed to identify HPV DNA in none of oral cavity VC,[18] or identified HPV DNA in a very limited number of cases.[19-21] Perhaps these discrepancies relate to the difficulties in the interpretation of ISH studies. This may be complicated by problems of non-specific staining or sensitivity of detection and it is unclear that VC has been separated reliably

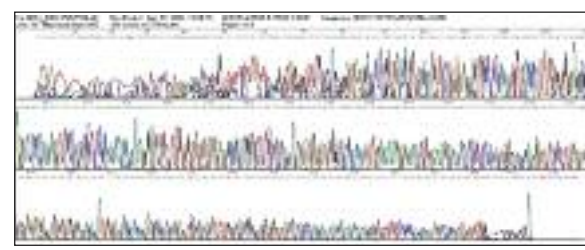

FIGURE 1: HPV 11 detected by PCR in two cases.

TABLE 1: Clinicopathologic features of laryngeal verrucous carcinomas. (During our analysıs we have utlızed Internatınal Unıon Agaınst Cancer – TNM stagıng system. Since we had no reported deaths among the patıents which we are able to obtain information from, we were not able to apply any survival analysis on our dataset.

Case	Age	Localization	Tobacco use	Stage (TNM)	Treatment	HPV DNA	Follow-up and (the median survival time was 16.09±5.68, range= 9-28 months) and survival	P53 %	Surgery date
Case1	60/M	Left vokal cord	(+)	E1 (T1N0M0)	Left vertical hemilarengectomy+ left functional neck dissection	(+)	12 month alive	25(+)%	Apr. 2002
Case2	71/M	Epiglotis	(+)	E1 (T1N0M0)	Pre –op ex	(+)	Pre –op ex	15(+)%	Dec. 2001
Case3	48/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	9 month alive	7(+)%	May 2004
Case4	55/M	Right vocal cord + anterior comissur	(+)	E2 (T2N0M0)	Right frontolateral larengectomy	(+)	12 month alive	0%	June 1996
Case5	75/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	16 month FTR	0%	May 1999
Case6	56/M	Subglottic	(+)	E2 (T2N0M0)	Total larengectomy+bilateral functional neck dissection	(+)	22 month alive	15(+)%	Jan. 2001
Case7	42/M	Left vocal cord	(+)	E1 (T1N0M0)	Left cordectomy	(+)	16 month alive	12(+)%	May.1998
Case8	45/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	19 month FTR	0%	Aug.2000
Case9	51/M	Left vocal cord	(+)	E1 (T1N0M0)	Left cordectomy	(+)	15 month alive	20(+)%	Jan. 2003
Case10	53/M	Epiglottis +right false cord + arytenoid	(+)	E3 (T3N0M0)(*)	Total larengectomy+ bilateral lateral neck dissection	(+)	11 month alive	22(+)%	Feb. 1999
Case11	48/M	Epiglottis +right + false vocal cord	(+)	E3 (T3N0M0) (*)	Total larengectomy+ right functional neck dissection	(+)	9 month alive	30(+)%	Apr. 2001
Case12	65/M	Epiglottis +left false+ vocal cord	(+)	E3 (T3N0M0) (*)	Total larengectomy+ left functional neck dissection	(+)	9 month alive	40(+)%	Feb. 2003
Case13	68/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	26 month FTR	0%	Jan. 2003
Case14	40/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	23 month alive	18(+)%	Aug. 2004
Case15	73/M	Epiglottis +left false vocal cord	(+)	E3 (T3N0M0) (*)	Total larengectomy+ left functional neck dissection	(+)	11 month FTR	0%	Sep. 1999
Case16	35/M	Left vocal cord	(+)	E1 (T1N0M0)	Left cordectomy	(+)	28 month alive	35(+)%	Nov. 1999
Case17	50/M	Subglottic	(+)	E2 (T2N0M0)	Total larengectomy+ bilateral functional neck dissection	(+)	22 month FTR	10(+)%	May. 1999
Case18	43/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	19 month alive	14(+)%	Mar. 2001
Case19	65/M	Left vocal cord	(+)	E1 (T1N0M0)	Left cordectomy	(+)	12 month FTR	0%	Oct. 2002
Case20	58/M	Left vocal cord	(+)	E1 (T1N0M0)	Left cordectomy	(+)	19 month FTR	8(+)%	Nov. 1994
Case21	63/M	Right vocal cord	(+)	E1 (T1N0M0)	Right cordectomy	(+)	14 month FTR	0%	Feb. 2001

FTR:Fail to reach; (*): Cord vocal fixation.

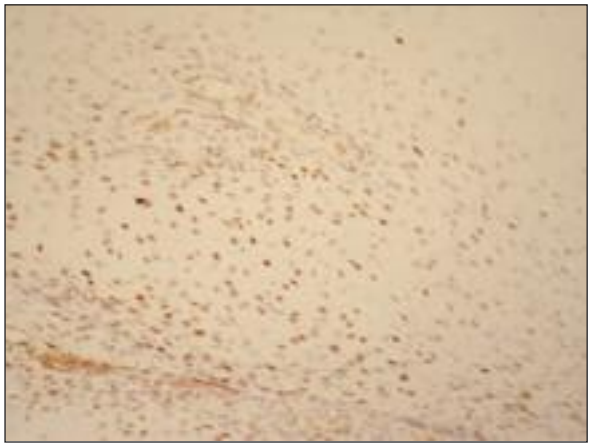

FIGURE 2a: Photomicrograph showing cells staining strongly positive for the p53 (Peroxidase stain; original magnification ×100).

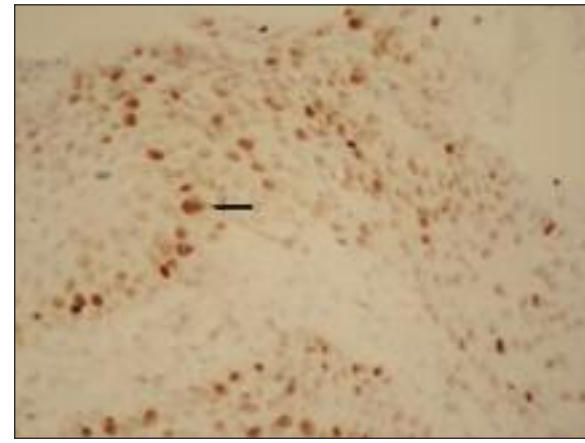

FIGURE 2b: The arrow points to the tumor cell positive for nuclear p53 staining (Peroxidase stain; original magnification ×200).

TABLE 2: Relation between P53 and stage (Pearson Chi-square Test).

	STAGE								p
	1		2		3		Total		
p53 %	N	%	N	%	N	%	N	%	
1-10	2	22.2	1	50	0	0	3	21.4	0.408
11-60	7	77.8	1	50	3	100	11	78.6	
Total	9	100	2	100	3	100	14	100	

from conventional squamous cell carcinoma. In comparison, other studies which failed to demonstrate the presence of HPV genome by ISH were able to detect HPV-DNA by PCR. PCR analysis for the presence of HPV in VCs has confirmed the presence of HPV DNA.[4,22] These studies suggest a direct pathogenetic role of HPV, rather than that of an innocent bystander in the development of VC. HPV may act as a promoter in the multistep process of carcinogenesis in squamous cells of the upper aerodigestive tract. Dyson et al have shown that a protein product of HPV can bind to the retinoblastoma gene product, thereby removing the regulatory block on cell cycle progression from G1 to S-phase.[23]

The p53 and pRb proteins participate in the activity at the G1-S cell-cycle checkpoint that normally causes cells with DNA damage to undergo either cellular arrest at G1 or apoptosis.[24] The E6 and E7 oncoproteins produced by HPV-16 cause decreases in p53 and pRb proteins, respectively, undetermining this cell-cycle checkpoint.[25,26] The alteration of the G1-S checkpoint leads to the inappropriate survival of genetically damaged cells and, thus, may be a level in the development of a malignancy. Various cofactors are probably necessary for carcinogenesis to progress completely.

Many authors have tried to demonstrate the presence and role of HPV infection in the promotion and development of VC due to its particular growth pattern and morphologic characteristics.[4,5,17,22,27] Szentirmay et al concluded that epidemiologic and extensive laboratory evidence supports the association between HPV and a subset of cancers of the oral cavity, orapharynx, and larynx.[28] In addition, they pointed out that HPV- positive cancers of the head and neck region were more likely to have verrucous histological features.[28] Our findings are in agreement with previous reports of the presence of HPV DNA in VC.[4,17,22,29,30]

There are no large case control studies comparing the risk factors or other characteristics of HPV-positive and HPV-negative tumors, and current literature is conflicting about the role of HPV in VC of the larynx.[4,17,29] Many types of HPV have been isolated in the VC of the larynx.[4,17,22,29,30] Numerous epidemiologic and molecular studies have demonstrated that high risk types of HPV are the agents of the overwhelming majority of cases of VC. Kasperbauer et al demonstrated a variety of HPV types in 20 VC of the larynx using HPV DNA insitu hybridization and PCR.[22] In their article, HPV DNA was detected in 17 (85%) of 20 tissue samples by PCR; and none of the 20 samples were positive for the seven genotypes tested by in situ hybridization.[22] A previous study by Fliss et al documented the presence of HPV DNA in 29 VC of larynx using PCR.[4] They found HPV DNA in 45% of cases. Type 18 was present in 14% of the tissue specimens, whereas a mixture of type 16 and 18 was identified in 17% of the cases.[4] Brandsma et al analyzed 6 cases of laryngeal VC by Southern and DNA dot blot hybridization for HPV DNA.[17] They did not identify the presence of subtypes 16 or 11 in any of the specimens. Surprisingly, Shroyer et al. detected HPV type 6 in two cases, type 11 in four cases, and both types 6 and 11 in one case of oral verrucous carcinoma.[31] They concluded that low-risk HPV types 6 and 11 are frequently present in cases of oral verrucous carcinoma.[31] Similarly, Lubbe et al demonstrated HPV 11 (a low-risk virus) in an early stage biopsy specimen of oral VC.[32] In our study, HPV 11 was detected in two cases of the HPV DNA-positive VC, but none of the other viral types were found. The tumor suppressor gene p53 has been claimed to play an essential role in the pathogenesis of squamous cell carcinoma of the head and neck (SCCHN). An abnormal accumulation of p53 was found in cases of laryngeal squamous cell carcinoma.[33-35] As such, immunostaining for p53, an indicator of an abnormal accumulation of mutated p53, has been detected in the nuclei of VC.[36-38] Fujita et al detected p53 expression in 16 cases of oral VC (70%) and demonstrated the inverse correlation between the HPV infection and p53 expression.[39] They concluded that inactivation of p53 was suggested as crucial in the tumorigenesis of oral VC.[39]

The coexpression of p53 and HPV in VC of the larynx has been previously demonstrated only in one study.[5] Lopez-Amado et al. observed p53 expression in four of 10 tissue samples (40%).[5] In their study, three cases had both p53 oncoprotein and HPV antigen.[5] In the current study, all the HPV-positive cases were positive for p53.

The results of our study demonstrate that HPV DNA is constantly present in VC of the larynx, which supports the role of HPV in the development of verrucous laryngeal carcinomas. However, further studies are still recommended to confirm this concept. Given the size of the study, a larger cohort is required in order to understand in more detail the effect of high risk HPV types in VC of the larynx. The interaction between HPV sequences and certain cellular oncogenes such as p53 needs further clarification.

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