The expression of p53, p16 proteins and prevalence of apoptosis in oral squamous cell carcinoma

Correlation with mode of invasion grading system

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ABSTRACT

Objectives: Inactivation of p53 and p16 tumor suppressor genes, and apoptosis which is crucial in carcinogenesis have commonly been studied in oral squamous cell carcinoma (OSCC). However, their prognostic value has not yet been clearly established.

Methods: This study was conducted in the Department of Oral Pathology, Faculty of Dentistry, Gazi University, Ankara, Turkey during the period 2002 to 2003 on formalin-fixed paraffin embedded tissue specimens of 12 lip and 18 intraoral primary squamous cell carcinoma cases. The expression of p53 and p16 proteins were studied by immunohistochemistry, and the apoptosis by TdT-mediated dUTP-biotin nick end-labeling (TUNEL) methods. The possible prognostic value of p53, p16 expression and apoptotic index (AI) value in OSCC were examined on the basis of their correlation with mode of invasion (MI) grading system.

Results: Seven lip (58%) and 9 intraoral cancer (50%) cases showed p53 positivity; where 5 lip (42%) and 15 intraoral cancer (83%) cases showed loss of p16 protein. P53 positive cases increased parallel to MI grade where the AI value decreased. There was not any correlation either between p16 expression and MI grade or AI value. The mean AI value was found as 1,884. Apoptotic index values were higher in invasive site of tumors, and it was statistically significant in MI grade 2 OSCC cases. Apoptotic index value of both central and invasive sites were lowest in MI grade 4 cases.

Conclusion: The present findings revealed that p53 mutations alone, may play a role in pathogenesis of lip cancers but not in intra OSCC. P16 may have a greater role in the development of intra OSCC. P53 positivity and low AI value may be a predictor of poor prognosis in OSCC.

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Despite the therapeutic and diagnostic progress during the last decades, the prognosis of oral squamous cell carcinoma (OSCC) remains poor. Prognostic evaluation, and thus planning of treatment of OSCC is mainly based on clinical TNM (T-tumor size, N-regional lymph node involvement, M-metastases) classification.[1] However, there is still need an evaluation of other cellular and molecular characteristics, which can supplement TNM staging. In this study, the histological malignancy grading system[1,2] and mode of invasion grading system[3] have been used in an

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attempt to predict the clinical behavior of OSCC. In these grading systems, features of the cells at the invasive tumor margins are accepted to be of greater importance for prognostic evaluation than other parts of the tumor.[1-4] Recent studies have suggested that mode of invasion is one of the best grading system for evaluating the invasive and metastatic potential of the tumor and has a prognostic value in OSCC.[3] By the use of novel molecular biologic methods, some of the tumor suppressor genes and oncogenes can be assessed and are proposed as excellent candidates for biomarkers in oral carcinogenesis.[5] In studies of oral cancer with specific reference to p53 and p16, it has been reported that alterations affecting the p53 and p16 tumor suppressor genes are the most common genetic abnormality detected in human cancers, including OSCC. The protein products of these genes play a critical role in the regulation of cell cycle checkpoints, and mutation of these sequences permit the multiple aberration of the cells in the process of OSCC development.[6] The p53 tumor suppressor gene is crucial in the cellular response to a variety of injuries resulting in either cell cycle arrest or apoptosis.[7] Loss of heterozygosity and mutation at the p53 gene are frequent event in OSCC.[8,9] Gene mutations prolong the half life of p53, facilitating its detection by immunohistochemistry and by using this method the p53 mutation has been reported in 34-100% of both head-neck squamous cell carcinoma (HNSCC) and OSCC.[10,11] Another tumor suppressor gene p16, mapped on the chromosome 9p21 region, encodes an inhibitor of the cyclin-dependent kinase 4/cyclin D complex, which controls the cell cycle progression in the G1/S border.[12] Inactivation of p16 is frequently found in HNSCC and OSCC and is thought to be caused by several different mechanisms including homozygous deletion, DNA methylation and point mutations.[13-16] Inactivation of p16 gene is evaluated by immunohistochemistry which is proposed as an accurate method for detection the loss of p16 gene product.[17,18] The growth of malignant tumors is dependent on balance between cell proliferation and cell death.[7] In recent years, the role of program cell death, called apoptosis, and its associated proteins have been widely investigated in the pathogenesis of OSCC.[7,19,20] Although the apoptotic index (AI) value of a tumor has been shown to be of biological and clinical relevance,[21] the results of the studies are still debatable.

In the present study, we evaluated the expression of p53 and p16 proteins and examined the incidence of apoptotic cells in OSCC. The goal of the current study was to investigate correlations among the expression of p53, p16 proteins, AI and mode of invasion grading system, and to determine their possible prognostic value as a biomarker.

Methods.

Tissue specimens. The study was conducted on formalin-fixed paraffin embedded tissue specimens of 12 lip and 18 intraoral primary squamous cell carcinoma cases, which were obtained from the archives of the Department of Pathology, Faculty of Medicine and Department of Oral Pathology, Faculty of Dentistry, Gazi University, Ankara, Turkey between the years 2002 and 2003. Tissue specimens were obtained from lips (12 lesions), tongue (8), gingiva (4), retromolar region (2), alveolar mucosa (1), buccal mucosa (1), palate (1) and floor of the mouth (1). The tumors were initially classified as well (n=21), moderate (n=6) and poorly (n=3) differentiated. The mode of tumor cell invasion (MI) was graded by the method of Yamamoto et al[3] as follows: grade 1 (MI 1), a well-defined borderline; grade 2 (MI 2), cordless, marked borderline; grade 3 (MI 3), groups of cells, no distinct borderline; grade 4 (MI 4), diffuse invasion (4c, cordlike type; 4d, wide spread type).

Immunohistochemical staining. Five-micrometer thick sections of formalin fixed and paraffin embedded biopsy samples were processed by the avidin-biotin-peroxidase complex (ABC) method. Deparaffinization and rehydration of the sections were followed by the blocking of endogenous peroxidase activity with incubating the sections in 3% H_2O_2 for 10 minutes. After rinsing with PBS, the sections were treated in microwave by antigen retrieval solution (Biogenex HK087-5K, United States of America [USA]) for 15 minutes and then the slides were left in cool at room temperature for 30 minutes. Non-specific binding was reduced with protein blocking serum (Biogenex HK112-9K, USA) for 20 minutes. Sections were incubated with p53 primary mouse monoclonal antibody (p53 mouse monoclonal antibody, Biogenex DO7, USA), p16 primary mouse monoclonal antibody (p16 mouse monoclonal antibody, Neomarker Ab-4, USA) at room temperature for 120 minutes. After rinsing thoroughly with PBS, the slides were incubated with biotinlayed secondary antibody (Multilink Biogenex HK340-9K) for 30 minutes. The sections were washed with PBS; the slides were incubated with label (Label Biogenex HK330-9K, USA) for 30 minutes. The diaminobenzidine tetrachloride (DAB, Lipshow Immunon, USA) was used as a chromogen for visualization of the antibody binding. Finally, the sections were counter stained with hematoxylin, cleared and mounted.

Colon carcinoma and cervix carcinoma specimens were used as positive control tissues for p53 and p16 immunohistochemical staining.

TdT-mediated dUTP-biotin nick end-labeling (TUNEL). Apoptotic cells were detected by TUNEL method with the Apop Tag Plus Peroxidase in situ detection kit (Intergen, S7101, Oxford, United Kingdom [UK]) following the manufacturer's instructions. Briefly,

deparaffinization and rehydration of the sections was followed by incubation with 20 ml/ml proteinase K (Intergen, S7101, Oxford, UK) for 15 minutes at room temperature. The slides were washed in 2 changes of distilled water in a coplin jar for 2 minutes and incubated in 3% hydrogen peroxide for 5 minutes at room temperature to block endogenous peroxidase activity. After 2 rinsing steps with phosphate buffer solution (PBS, Ph 7.4) the slides were covered with 75 ml of equilibration buffer for at least 10 seconds at room temperature and incubated with terminal deoxynucleotidyl-transferase (TdT) at 37˚C for one hour. Working strength stop/wash buffer was applied for 10 minutes at room temperature and then the slides were washed in 3 changes of PBS for one minute and incubated with anti-digoxigenin peroxidase conjugate for 30 minutes at room temperature. Subsequently, the slides were rinsed 4 times in PBS and peroxidase substrate was applied for 5 minutes at room temperature. The slides were then transferred to double distilled water, washed 3 times and counterstained with 0.5% methyl green. After 3 further washing steps using water, the slides were immersed in 3 changes of 100% N-Butanol, dehydrated in xylene and mounted with cover slips.

Evaluation of sections and statistical analyses. Immunohistochemical slides were examined for positive staining by light microscopy. The immunoreactive cells were semiquantitatively estimated. The sections were graded according to the following scale: (-) means <5% of tumor cells were positive, (+) means 5-20% of tumor cells were positive, (++) means 20-50% of tumor cells were positive and (+++) means >50% of tumor cells were positive. The apoptotic cells were counted according to a modification of method of AI reported by King et al.[22] Four fields at x 400 magnifications were counted (2 fields at invasion area, 2 were central region: upper 1/2 of the space between stratum basale of surface epithelium and invasive border). An index of apoptotic staining was calculated as the number of positive cells per 100 cells in cancerous epithelium.

All data were grouped as cancer of lip and oral cavity and the statistical analysis were performed based on these groups. Chi-Square tests, Kruskall-Wallis test and Mann-Whitney U test were used to analyze differences in various data sets.

Results. A total of 30 OSCC specimens (12 lip, 18 intraoral carcinoma) from 30 patients were utilized in the study, with the results displayed in **Table 1**. The age range of the 30 patients was from 30-80 years, with a median of 54.9 years. Male to female ratio was 3:1. Invasion grading of the cases were as follows: MI 1 = 7 cases, MI 2 = 10 cases, MI 3 = 6 cases, MI 4 = 7 cases. Seven lip (58%) and 9 intraoral carcinoma (50%) cases showed positive nuclear staining in neoplastic cells for p53. In well-differentiated carcinoma cases, p53 protein expression is detected in the cells that are located at the basal and supra basal layer of the tumor islands whereas all cells of tumor parenchyma had positive staining in poorly differentiated carcinoma cases. At the invasive margins of the tumor, cells of basal layer in tumor islands showed single or double layers of positive staining in 14 of 16 immunohistochemically p53 positive cases (**Figure 1**). The number of p53 positive cases increased parallel to the degree of mode of invasion. However, a significant correlation was not found between p53 expressions and mode of invasion grading system. **Table 2** shows the distribution of p53 immunohistochemical staining according to mode of invasion. The majority of the p53 positive cases (11 of 16 cases) contained p53 positive cells at frequency of more than 50%. No difference was found between the degrees of MI based on the frequency of p53 positive cells range (LR=2.421, p=0.490). Five lip (42%) and 15 intraoral carcinoma (83%) cases did not show any immunohistochemical staining with p16, while 10 of 30 cases (33.3%) showed positive nuclear and cytoplasmic staining with p16 in neoplastic cells. Most of the p16 positive stained cases (6 of 10, 60%) were seen in well-differentiated carcinomas with the frequency of p16-positive cells ranging from 5-60% maximum, and the most common pattern of staining was as scattered or mosaic pattern among tumor cells (**Figure 2**). The data summarized in **Table 2** indicates that the number of p16 negative cases were higher than p16 positive cases in MI 1, MI 2 and MI 3, whereas in MI 4, p16 positive cases were higher. However, no significant difference was observed between the positive and negative p16 cases according to mode of invasion grading system (LR=3.655 p=0.301). Two lip and 9 intraoral carcinoma cases showed both p53 and p16 alterations while 3 lip and 6 intraoral carcinoma cases did not show alterations in either p53 or p16. There is no any intraoral cancer case that shows only p53 mutations whereas 5 of lip cancers did. P16 gene product loss apart was observed in 3 lip and 6 intraoral carcinoma subjects.

Apoptotic cells were detected by TUNEL method as stained nuclei or nuclear fragments. Except one case, apoptotic cells were visualized in all cases enzymatically. The mean AI value was 1,884. As shown in **Table 3**, in central sites the AI was lowest in MI 4 OSCC cases and there were significant differences between both MI 1 and MI 4, and MI 3 and MI 4 OSCC cases ($p<0.05$). At the invasion site, the lowest apoptotic indices value was also found at MI 4 OSCC cases, however no significant correlation was found between the MI degrees and AI value. Apoptotic index values were higher at the

Table 1 - The results of immunohistochemistry and TUNEL analysis of oral squamous cell carcinoma.

Case	Age	Gender	Site	Grade	MI	AI	P53	P16
1	63	Female	Tongue	Well	3	3.947	+++	-
2	35	Male	Lip	Modarate	4D	1.084	++	+
3	50	Male	Lip	Well	2	1.780	-	-
4	30	Male	Lip	Poor	1	1.4	-	++
5	80	Female	Gingiva	Poor	3	6.533	+++	-
6	58	Male	Lip	Well	4C	0.30	++	++
7	52	Male	Lip	Well	2	6.904	+	+++
8	61	Male	Lip	Well	1	2.105	-	-
9	70	Female	Tongue	Moderate	2	1.923	++	-
10	66	Male	Lip	Well	3	0.833	-	-
11	52	Male	Tongue	Well	1	1.428	+	-
12	75	Male	Lip	Well	1	2.258	+++	-
13	43	Male	Tongue	Well	4D	0.705	+++	-
14	35	Male	Gingiva	Well	2	5.894	-	-
15	50	Male	Lip	Well	3	1.166	+++	-
16	53	Male	Gingiva	Well	2	1.294	-	+
17	45	Female	Floor m.	Modorate	2	0.461	-	-
18	35	Male	Lip	Modorate	3	1.222	-	+
19	64	Male	Gingiva	Modorate	2	2.5	-	-
20	40	Female	Tongue	Well	1	1.428	-	-
21	67	Female	Alv.muc.	Well	3	3.214	+++	-
22	53	Male	Lip	Poor	4C	3.050	+++	+
23	54	Male	Retromolar	Well	4C	-	+++	-
24	60	Female	Tongue	Well	1	2.103	-	++
25	60	Female	Bucc.muc	Modorate	4D	1.684	-	-
26	58	Male	Palate	Well	4C	0.615	-	+
27	45	Male	Lip	Well	1	0.6	+++	+
28	62	Female	Tongue	Well	2	1.521	+++	-
29	66	Female	Tongue	Well	2	1.75	+++	-
30	65	Male	Retromolar	Well	2	0.847	-	-

TUNEL - TdT-mediated dUTP-biotin nick end-labeling MI - mode of invasion degree, AI - apoptotic index, LN - lymph node resection material, NA - not available, (-) means <5% of tumor cells were positive, (+) means 5-20% of tumor cells were positive, (++) means 20-50% of tumor cells were positive and (+++) means >50% of tumor cells were positive.

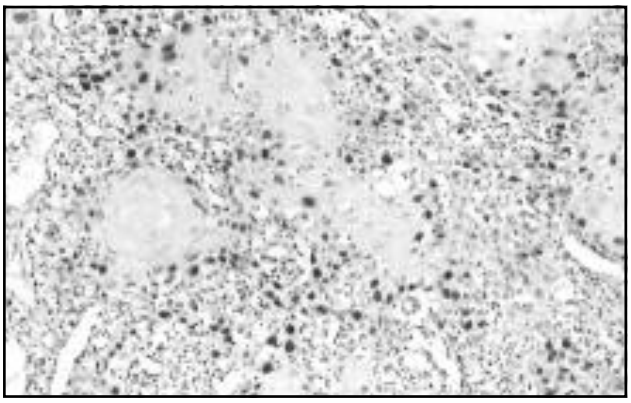


Figure 1 - Immunohistochemical reactivity for p53 in the invasive margins (avidin-biotin-peroxidase complex x100).

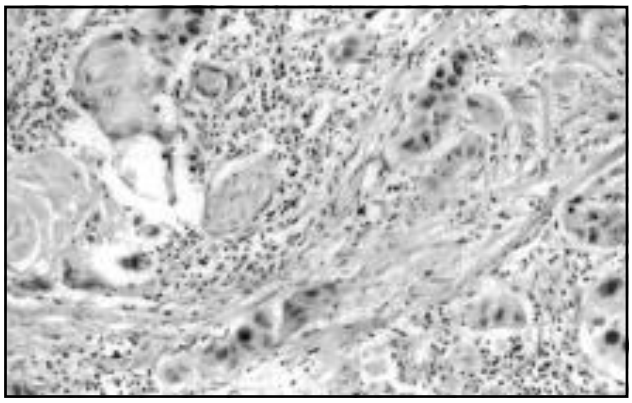


Figure 2 - Immunohistochemical reactivity for p16 in the invasive margins (avidin-biotin-peroxidase complex x100)

Table 2 - The distribution of p53 and p16 immunohistochemical staining according to MI grading system.

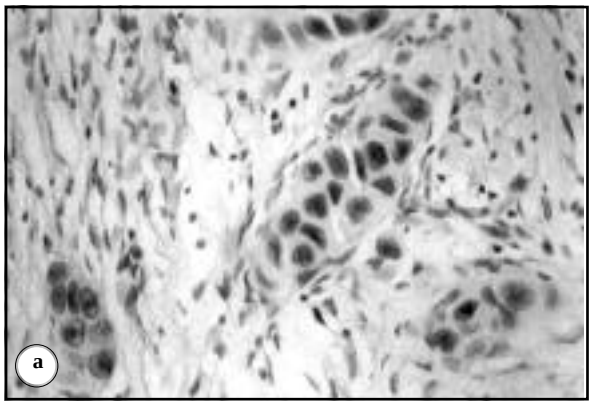
MI N=30	P53 - N=14 (46.7%)	P53 + N=2 (6.6%)	P53 ++ N=3 (10%)	P53 +++ N=11 (36.7%)	P16 - N=20 (66.7%)	P16 + N=6 (20%)	P16 ++ N=3 (10%)	P16 +++ N=1 (3.3%)
MI 1 (n=7)	4	1	0	2	4	1	2	0
MI 2 (n=10)	6	1	1	2	8	1	0	1
MI 3 (n=6)	2	0	0	4	5	1	0	0
MI 4 (n=7)	2	0	2	3	3	3	1	0

MI - mode of invasion degree, (-) means <5% of tumor cells were positive, (+) means 5-20% of tumor cells were positive, (++) means 20-50% of tumor cells were positive and (+++) means >50% of tumor cells were positive.

Table 3 - Statistical results for apoptotic index by MI grading and sites

Apoptotic index and measure	M1 (n=7)	M2 (n=10)	M3 (n=6)	M4 (n=7)	KW Results
Central site					
Mean	1.636	2.066	2.831	0.700	
Standard deviation	0.583	4.000	2.078	0.817	x^2=8.030
Median	1.818	0.871	2.250	0.312	p=0.045
Minimum	0.666	0.000	0.769	0.000	
Maximum	2.307	13.333	5.806	2.068	
Invasion site					
Mean	2.810	3.417	2.839	1.372	
Standard deviation	2.147	3.268	3.240	1.498	x^2=4.635
Median	1.935	2.872	1.305	0.666	p=0.201
Minimum	1.000	0.750	0.000	0.000	
Maximum	6.666	12.000	8.125	4.000	
Total					
Mean	1.167	2.488	2.822	1.062	
Standard deviation	0.583	2.150	2.213	1.031	x^2=4.816
Median	1.428	1.765	2.218	0.705	p=0.186
Minimum	0.600	0.461	0.833	0.000	
Maximum	2.258	6.904	6.533	3.050	
For central and invasion					
U*	18.0	22.5	15.0	17.0	
p value	0.456	0.035	0.699	0.383	

MI - mode of invasion degree, M1 - grade 1, M2 - grade 2, M3 - grade 3, M4 - grade 4, KW - Kruskall Wallis test



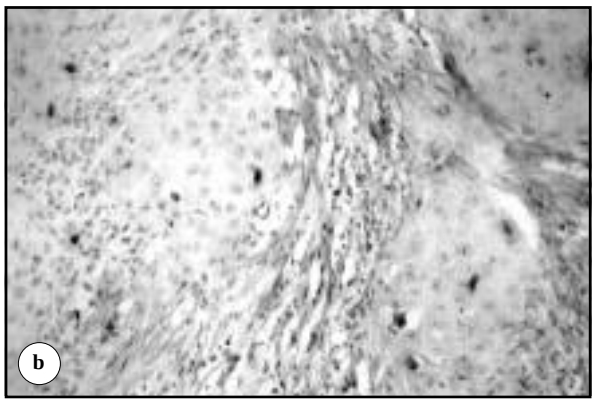


Figure 3 - The TUNEL reaction in a) invasive site of oral squamous cell carcinoma (x 400) and b) in central site of oral squamous cell carcinoma (x 200). TUNEL - TdT-mediated dUTP-biotin nick end-labeling.

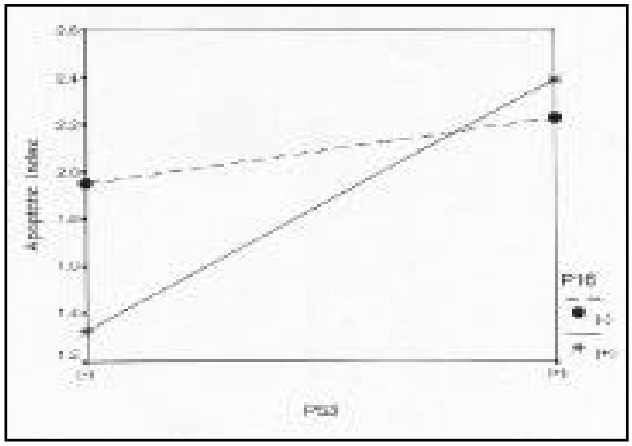


Figure 4 - Relationship among apoptotic index, p53 and p16.

Table 4 - Statistical results for apoptotic index by p53 and p16 immunohistochemical staining.

Apoptotic indices	Mean	Median	SD	Minimum	Maximum
p53 (-)	1.72	1.41	1.33	0.46	5.89
p53 (+)	2.40	1.08	2.04	0.00	6.90
p16 (-)	2.09	1.69	1.67	0.00	6.53
p16 (+)	1.85	1.25	1.94	0.30	6.90

invasive sites of tumors compared to central sites (**Figure 3a & 3b)**. However, significant difference was only seen in MI 2 OSCC cases ($p<0.05$).

Apoptotic index value in p53 positive cases was higher than AI value of p53 negative cases. On the other hand, lower AI values were observed in p16 positive cases compared to p16 negative cases (**Table 4**). There was no correlation between either AI and p16 or AI and p53 expression. As shown in **Figure 4**, highest mean AI values were detected in both p53 and p16 positive OSCC cases (2.387), whereas the lowest values were found in p53 negative and p16 positive cases (1.326). There was no statistical significance between cancer of the lip and oral cavity groups in any of the analyzed data sets.

Discussion. The unpredictable clinical behavior of oral cancer has led many investigators to search for biological factors that may be used as a " prognostic index".[23] Indeed, it is unlikely that a single parameter will ever be identified as the ideal prognostic or predictive marker. Thus, the pursuit of a panel of factors rather than a single one may be more fruitful. In this study, 2 important cell cycle proteins p16 and p53, were studied in OSCC by immunohistochemistry and apoptosis by TUNEL method to evaluate them as biomarkers by their correlation with a mode of invasion grading system. Additionally their possible role in pathogenesis of OSCC was considered and discussed. Alterations in p53 gene have commonly been found in head and neck cancers, but their prognostic value has not yet been clearly established.[24] The proportion of p53 positive tumors (53.3%) found in the current study was in the range reported in previous studies.[17,25,26] The p53 mutations alone were observed in 42% of lip cancer while none of intraoral cancer showed p53 positivity apart. This finding suggested that p53 mutations alone may have a role in lip cancers but not in intraoral cancers.

In the present study, the patients with p53 positive tumors showed higher AI value than those with p53 negative tumors, nevertheless the

difference was not statistically significant. The higher AI value in p53 positive tumors in the current study may be related to the fact that p53 is not always required in triggering apoptosis. Many cells have so called death receptors on their membrane and binding of appropriate ligand such as Fas, will lead to apoptosis through a pathway independent of p53.[27] Moreover, the induction of apoptosis or growth arrest requires activation of distinct sets of target genes.[28] Recent studies have reported that p53 mutations or p53 protein accumulation is associated with a high rate of disease recurrence and shorter overall survival,[29-31] whereas other studies have found no prognostic significance with regard to overall survival.[24,29,32-36] In the present study, no significant correlation was found between p53 expression and MI grade, which was used as prognostic marker. Nevertheless, the number of p53 positive cases increased parallel to MI grade, where AI value decreased. This data is in concurrence with the knowledge that p53 positivity is a predictor of poor prognosis in OSCC, and tumors with intact p53 are more susceptible to apoptosis. Alteration in p16 gene has been frequently found in many human cancers including head and neck SCC.[12,16,37-39] Reported loss of p16 expression determined by immunohistochemical analysis has ranged from 54%[40] to 82%[41] and these alterations were associated with decreased survival.[41] In the present study, 20 of 30 cases (66.7%) showed loss of p16 expression. This ratio was higher than that of p53 positive cases (16/30; 53.3%). In the same way, the number of p16 negative cases (20/30; 66%) and a number of other cases that showed alterations in both proteins (11/30; 36%) were much higher than that of other studies. Of interest, 83% of intraoral cancers showed lack of p16 gene product, which suggested a possible role for this gene in pathogenesis of intraoral cancers rather than lip cancers. Kannan et al,[37] Saito et al[42] and Warnakulasuriya et al,[17] who studied both p53 and p16 in OSCC, have found more frequent mutations in p53 than p16. For p53 they reported the mutational ratio as 21%, 50%, 41% and for p16, 9%, 11% and 25%.

Many investigators including Kannan et al[37] found a reciprocal relationship of p53 and p16 mutations. They found only one sample, which has mutations at both genes and they concluded that the reciprocal relation in mutations of these genes supports the view that these genes may share a common genetic pathway in their tumor suppressor activity. Kannan et al[37] and Saito et al,[42] who have found infrequent mutations in p16 in OSCC, have concluded that mutational inactivation of p16 may not play a major role in the genesis of OSCC. On the contrary, the results of the present study revealed that p16 might have a role in the development of intra oral SCC. Moreover, contrary to previous studies, our results did not show any inverse relation between p53 and p16 inactivation immunohistochemically. This finding arouses suspicion of the view that these genes may share a common genetic pathway in their tumor suppressor activity. Over expression of p16 has been observed by immunohistochemical analysis in SCC of the cervix[43] and tonsillar carcinoma,[44] which were associated with HPV. These observations were in line with the hypothesis that viral oncoprotein E7 blocks the Rb protein, which would otherwise inhibit p16 transcription. The lack of p16 in 66.7% of OSCC in this study from Turkey, may be elucidated by lack of HPV in the genesis of OSCC and this suggestion is in line with our unpublished manuscript's data, where we have found rather low HPV incidence in 40 OSCC cases by polymerase chain reaction technique. Significant correlation was found neither between p16 expression and MI grade nor AI value. Also, there was not any correlation between p16 and histological grading system, which suggested that p16, may not be worthy as a prognostic biomarker. However, the role of p16 has to be taken into consideration in the development of OSCC.

In many studies, the AI value of tumors has been shown to be of biological and clinical relevance. In oral (45), breast (46), bladder (21), renal cell (47), ovarian (48), prostatic (49), cervical (50) and hepatocellular (51) carcinomas a higher AI value has been proven to be in relevance with prognosis in different ways. Nevertheless, in other studies low AI value has been shown as a predictive factor of poor prognosis.[7,52] In the present study, AI value of both central and invasive sites was lowest in MI 4 OSCC. Although no significant correlation was found between AI values of MI degrees in invasive sites, there was significant differences between the AI values of MI 1- MI 4 and MI 3 - MI 4 OSCC cases ($p<0.05$) in central sites. This data supports the studies, which interpreted that low AI value is a predictive factor of poor prognosis. On the other hand, the higher AI value in invasive site of tumors compared to central sites, might indicate that the cells, which have more invasion potential, may have also acquired greater number of genetic aberrations, which trigger apoptosis. In that case, AI value might not be accepted as an independent factor to elucidate the biological and clinical behavior of a cancer. Indeed these contradictory findings are in accordance with the complexity of tumor heterogeneity and progression.

In conclusion, p53 mutations alone, may play role in pathogenesis of lip cancer, but not in intra OSCC. P16 may have a greater role in the development of intra OSCC. P53 positivity and low AI value may be a predictor of poor prognosis in

OSCC that needs to be investigated further and in addition may prove to be a promising future study.

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References

1. Bryne M. Prognostic value of various molecular and cellular features in oral squamous cell carcinomas: a review. ***J Oral Pathol Med*** 1991: 20: 413-420.
2. Anneroth G, Hansen LS. A methodologic study of histologic classification and grading of malignancy in oral squamous cell carcinoma. ***Scand J Dent Res*** 1987: 92: 448-468.
3. Yamamoto E, Miyakawa A, Kohama G. Mode of invasion and lymph node metastasis in squamous cell carcinoma of the oral cavity. ***Head Neck Surg*** 1984: 6: 938-947.
4. Bryne M, Koppang H, Lilleng R, Kjaerheim A. Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. ***J Pathol*** 1992; 166: 375-381.
5. Girod SC, Pfeiffer P, Reis J, Pape HD. Proliferative activity and loss of function of tumor suppressor genes as biomarkers in diagnosis and prognosis of benign and preneoplastic oral lesions and oral squamous cell carcinoma. ***Brit J Oral Max Surg*** 1998: 36: 252-260.
6. Partridge M, Kiguwa S, Emilion G, Pateromickelakis, Hern RA. New insights into P53 protein stabilisation in oral squamous cell carcinoma. ***Oral Oncology*** 1999: 35: 45-55
7. Bandoh N, Hayashi T, Kishibe K, Takahara M, Imada M, Nonaka S et al. Prognostic value of p53 mutations, bax and spontaneous apoptosis in maxillary sinus squamous cell carcinoma. ***Cancer*** 2002: 94: 1968-1980.
8. Boyle JO, Hakim J, Koch W, Ruppert JM, Sidransky D. The incidence of p53 mutations increases with progression of head and neck cancer. ***Cancer Res*** 1993: 53: 4477-4480.
9. Van Der Riet P, Nawroz H, Hruban RH, Cario R, Tokino K, Koch W. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. ***Cancer Res*** 1994: 54: 1156-1158.
10. Langdon JD, Partridge M. Expression of the tumor suppressor gene P53 in oral cancer. ***Brit J Oral Max Surg*** 1992: 30: 214-220.
11. Ahomadegbe JC, Barrois M, Fogel S, Le Bihan ML, Douc-Rasy S, Duvillard P et al. High incidence of P53 alterations in head and neck primary tumor and metastasis; absence of correlation with clinical outcome. Frequent protein overexpression in epithelium and in early noninvasive lesions. ***Oncogene*** 1995: 10: 1217-1227.
12. Akanuma D, Uzawa N, Yoshida MA, Negishi A, Amagasa T, Ikeuchi T. Inactivation patterns of the p16 gene in oral squamous cell carcinoma cell lines. ***Oral Oncol*** 1999: 35: 476-483.
13. Okamoto A, Demetric DJ, Spillare EA, Hagiwara K, Hussain SP, Bennet WP et al. Mutations and altered expression of p16INK4 in human cancer. ***Proc Natl Acad Sci U S A*** 1994: 91: 11045-11049.
14. Merlo A, Herman JG, Mao L, lee D, Gabrielson E, Burger PC et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. ***Nat Med*** 1995: 1: 686-692.
15. Cairns P, Polascik TJ, Eby Y, Tokino K, Galifano J, Merlo A. Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. ***Nat Genet*** 2001: 11: 210-212.
16. Shintani S, Nakahara Y, Mihara M, Ueyama Y, Matsumura T. Inactivation of the p14 ARF, p15 INK4B and p16 INK4A genes is a frequent event in human oral squamous cell carcinomas. ***Oral Oncol*** 2001: 37: 498-504.
17. Warnakulasuriya K, Tavassoli M, Jhonson NW. Relationship of P53 overexpression to other cell cycle regulatory proteins in oral squamous cell carcinoma. ***J Oral Pathol Med*** 1998; 27: 376-381.
18. Reed AL, Califano J, Cairns P, Wertra WH, Jones RM, Koch W et al. High frequency of p16 inactivation in head and neck squamous cell carcinoma. ***Cancer Res*** 1996; 56: 3630-3633.
19. Schoelch ML, Le QT, Silverman S, McMillan A, Dekker NP, FU KK et al. Apoptosis associated proteins and the development of oral squamous cell carcinoma. ***Oral Oncol*** 1999: 35: 77-85.
20. Itakura M, Mori S, Park NH, Bonavida B. Both HPV and carcinogen contribute to the development of resistance to apoptosis during oral carcinogenesis. ***Int J Oncol*** 2000; 16: 591-597.
21. Lipponen PK, Aaltomoa S. Apoptosis in bladder cancer as related to to standard prognostic factors and prognosis. ***J Pathol*** 1994: 173: 333-339.
22. King ED, Matteson J, Jacobs SC, Kyprianou N. Incidence of apoptosis, cell proliferation and bcl-2 expression in transitional cell carcinoma of the bladder: association with tumor progression. ***J Urol*** 1996; 155: 316-320.
23. Guerry M, Vabre L, Talbot M, Mamelle G, Lendant AM, Hill C et al. Prognostic value of histological and biological markers in pharyngeal squamous cell carcinoma: a case control study. ***Br J Cancer*** 1998: 77: 32-36.
24. Kapranos N, Stathopoulos GP, Monolopuolos L, Kokka E, Papadimitrıou CON, Bibas A et al. P53, p21 and p27 protein expression in head and neck cancer and their prognostic value. ***Anticancer Res*** 2001; 21: 521-528.
25. Koontongkaew S, Chareonkitkajorn L, Chanvitan A, Leelakriangsak M, Amornphimoltham P. Alterations of P53, Rb, cyclin D1 and cdk4 in human oral and pharyngeal squamous cell carcinomas. ***Oral Oncol*** 2000; 36: 334-339.
26. Ng IOL, Lam KY, Ng M, Regezi JA. Expression of p21/ waf1 in oral squamous cell carcinoma- correction with P53 and mdm2 and cellular proliferation index. ***Oral Oncol Europ J Cancer*** 1999; 35B: 63-69.
27. White D, Broton CE, Shilitoe EJ. The unexplained survival of cells in oral cancer: what is the role of p53. ***J Oral Pathol Med*** 2002; 31: 125-133.
28. Sionov RV, Haupt Y. Apotosis by P53 mechanism, regulation and clinical implication. ***Springer Semin Immunpathol*** 1998; 19: 345-362.
29. Nylander K, Dabalsteen E, Hall PA. The p53 molecule and its prognostic role in squamous cell carcinomas of head and neck. ***J Oral Pathol Med*** 2000; 29: 413-425.
30. Shin DM, Lee JS, Lippman SM, Lee JU, Tu ZN, Choi G, et al. p53 expression: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. ***J Natl Cancer Inst*** 1996; 88: 519-529.
31. Narayama A, Vaughan AT, Gunaratne S, Kathuria S, Walter SA, Reddy SP. Is P53 an independent prognostic factor in patients with laryngeal carcinoma? ***Cancer*** 1998; 82: 286-291.
32. Gapany M, Pavelic ZP, Gapany SR, Pavelic L, Li YG, Crawen JM et al. Relationship between immunohistochemically detectable p53 protein and prognostic factors in head and neck tumors. ***Cancer Detect Prev*** 1993; 17: 379-386.
33. Nylander K, Schildt EB, Eriksson M, Roos G. PCNA, Ki67, p53, bcl-2 and prognosis in intraoral squamous carcinoma of the head and neck. ***Analyt Cell Pathol*** 1997; 14: 101-110.
34. Piffko J, Bankfalvi A, Tory K, Fuzesi L, Bryne M, Ofner D et al. Molecular assessment of p53 abnormalities at the invasive front of oral squamous cell carcinoma. ***Head Neck*** 1998: 20: 8-15.

35. Koch VM, Brennan JA, Zahurak M, Goodman SN, Westra WH, Schwab D et al. p53 mutations and locoregional treatment failure in head and neck squamous cell carcinoma. ***J Natl Cancer Inst*** 1996; 88: 1580-1586.
36. Homma E, Furuta Y, Oridate N, Nakona Y, Kohashi G, Yagi K et al. Prognostic significance of clinical parameters and biological markers in a patient with squamous cell carcinoma of the head and neck treated with concurrent chemoradiotherapy. ***Clin Cancer Res*** 1999; 5: 801-806.
37. Kannan K, Munirajan AK, Krıshnamurty J. The p16/p19 gene mutations are infrequent and are mutually exclusive to P53 mutations in Indian oral squamous cell carcinoma. ***Int J Oncol*** 2000; 16: 585-590.
38. Heinzel PA, Balaram P, Bernard HU. Mutations and polymorphism in the p53, p21 and p16 genes in oral carcinomas of Indian betel qoud chewers. ***Int J Cancer*** 1996; 68: 420-423.
39. Gruttgen A, Reichenzeller M, Junger M, Schlien S, Affolter A, Bosch FX. Detailed gene expression analysis but no microsatellite marker analysis of 9p21 reveals differential defects in the INK4A gene locus in the majority of the head and neck cancers. ***J Pathol*** 2001; 194: 311-317.
40. Ambosch P, Schlott T, Hilmes D, Ruschenburg I. p16 alterations and retinoblastoma protein expression in squamous cell carcinoma and neighboring dysplasia from the upper aerodigestive tract. ***Virchows Arch*** 2001: 438: 343-349.
41. Caldas C, Hahn SA, Da Costa LT, Redston MS, Schutte M, Seymour AB et al. Frequent somatic mutations and homozygot deletions of p16 gene in pancreatic adenocarcinoma. ***Net Genet*** 1994; 8: 27-32.
42. Saito T, Nakajima T, Mogi K. Immunohistochemical analysis of cell cycle associated proteins p16, pRb, p53, p27 and Ki 67 in oral cancer and precancer with special reference to verrucous carcinomas. ***J Oral Pathol Med*** 1999; 28: 226-232.
43. Klaes R, Friedrich T, Spitkowsky D, Ridder R, Rudy W, Petry U et al. Overexpression of p16 (INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of cervix uteri. ***Int J Cancer*** 2001; 92: 276-284.
44. Klussmann JP, Gultekin SE, Weissenborn SJ, Weiland U, Dries V, Dienes HP et al. Expression of p16 protein ıdentifies a distinct entity of tonsillar carcinomas associated with human papilloma virus. ***Am J Pathol*** 2003; 162: 747-773.
45. Naresh KN, Lakshminarayanan K, Pai SA, Borges AM. Apoptosis index is a predictor of metastatic phenotype in patients with early stage squamous carcinoma of the tongue. ***Cancer*** 2001; 91: 578-584.
46. Lipponen P, Aaltomaa S, Kosma VM, Syrjanen K. Apoptosis in breast cancer as related to histopathological characteristics and prognosis. ***Eur J Cancer*** 1994; 30A: 2068-2073.
47. Hindermann W, Berndt A, Wunderlich H, Katenkamp D, Kosmehl H. Quantitative evaluation of apoptosis and proliferation in renal cell carcinoma. Correlation to tumor subtype, cytological grade according to thionase-classification and the occurrence of metastasis. ***Pathol Res Pract*** 1991; 193: 1-7.
48. Mc Menamin ME, O'Neill AJ, Gaffney EF. Extent of apoptosis in overian serous carcinoma : relation to mitotic and proliferative indices, p53 expression and survival. ***Mol Pathol*** 1997: 50: 242-246.
49. Aihara M, Scardino PT, Truong LD, Wheeler TM, Goad JR, Yang G et al. The frequency of apoptosis correlates with the prognosis of Gleason grade 3 adenocarcinoma of the prostate. ***Cancer*** 1995: 75: 522-529.
50. Levine EL, Renehan A, Gossiel R, Davidson SE, Roberts SA, Chadwick C et al. Apoptosis, intrinsic radiosensitivity and prediction of radiotherapy response in cervical carcinoma. ***Radiother Oncol*** 1995; 37: 1-9.
51. Ito Y, Matsuura N, Sakon M, Takedo T, Umeshita K, Nagano H et al. Both cell proliferation and apoptosis significantly predict shortened disease-free survival in hepatocellular carcinoma. ***Br J Cancer*** 1999; 81: 747-751.
52. Xie X, Clausen OP, De Angelis P, Boysen M. The prognostic value of spontaneous apoptosis, bax, bcl-2 and p53 in oral squamous cell carcinoma of the tongue. ***Cancer*** 1999; 86: 913-920.