

Immunoexpression of p53 mutant-type in Iranian patients with primary and recurrence oral squamous cell carcinoma

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Abstract

Mutations in tumor suppressor p53 protein can occur at different phases of malignant transformation and affect the patient's prognosis. This study aimed to evaluate the expression of mutant p53 protein in Iranian patients with the primary and recurrence oral squamous cell carcinoma (OSCC). This retrospective cross-sectional study conducted on a group of patients with the primary OSCC (n=122) and the control subjects with oral noncancerous reactive lesions (n=80). Immunohistochemistry was performed with the DO-7 monoclonal antibody against p53 protein, and samples with $\geq 10\%$ immunostaining were considered positive. Statistical analyses were carried out using SPSS. Positive staining for p53 was observed in none of the control subjects and 57.4% (70 of 122) of the primary OSCC patients ($p < 0.0001$, OR=107.69, 95%CI=6.49-179.0). The p53 immunopositivity had no significant differences between males and females (54.2% vs. 62%, $p = 0.390$), but significantly different between those aged below and over 50 years ($p < 0.0001$, OR=4.52, 95%CI=1.07-12.05). During follow-up, OSCC recurrence occurred in 104 patients, but the phenotype of the mutant p53 protein in patients who relapsed was the same as in matched primary tumors ($p = 0.763$). Risk of recurrence had no significant differences between p53-positive and p53-negative cases ($p = 0.953$), males and females ($p = 0.263$), and age below and over 50 years ($p = 0.223$). Despite its confirmed diagnostic value, the immunoexpression of the p53 mutant protein in OSCC in cancer recurrence was the same as in the primary tumor. However, further studies with a larger sample size and longer follow-up are needed to confirm or change our conclusions.

Key Words: Immunohistochemistry (IHC); oral squamous cell carcinoma (OSCC); p53; primary tumor; recurrence.

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Head and neck squamous cell carcinoma (HNSCC) is one of the most aggressive and heterogeneous human malignancies, with approximately 890,000 new cases and 450,000 deaths annually. They are primarily not hereditary, but typically developed in the sixth to seventh decade of life. Oral squamous cell carcinoma (OSCC) originate from the epithelial lining of the oral cavity, pharynx, larynx, and nasal cavity and is the most common tumor of the head and neck, encompassing about 90% of malignant lesions in the oral cavity.^{1,2} Due to the painless presentation, the initial wrong diagnosis, or the ignorance from the patient, OSCC cases is often diagnosed at an advanced stages accompanied by

metastasis. Although such patients are managed with aggressive multimodal treatment, cases lead to a poor prognosis and cause significant morbidity and mortality. Indeed, postoperative tumor recurrence is an important prognostic factor in patients with OSCC that affected the 5-year survival rate. Poor oral hygiene, alcohol abuse, tobacco, and immunodeficiency have been proposed to be responsible for the development of OSCCs.³⁻⁷ Those carcinogens may predispose individuals to develop tumor, but a large proportion of cases still develops OSCC in absence of carcinogen exposure. Previous studies have described multiple chromosomal abnormalities and genetic instability in

patients with HNSCCs, which may be required for tumorigenesis and full transformation to invasive form. Inactivation or deletion of the tumor suppressor genes such as *TP53* as well as overexpression of the epidermal growth factor receptor (*EGFR*) are the predominant pathogenetic events in HNSCCs. They not only predispose cells to initiate the tumorigenesis cascade but are also associated with poor clinical response and outcome.⁸⁻¹¹ The p53 protein, encoded by the *TP53* gene regulates the expression of a vast array of genes involved in cell cycle arrest, cellular senescence, deoxyribonucleic acid (DNA) repair, metabolism, differentiation, and apoptosis. Due to maintenance of genome stability, p53 is known as the guardian of the genome. The majority of human cancer cells exhibit the inactivation of the p53 pathway through mutations, dysregulation of endogenous regulators, and by the Human Papilloma Virus E6 protein. Emerging evidence suggests that p53 is activated in response to many stress stimuli such as DNA damage, hypoxia, high Reactive Oxygen Species (ROS) levels, heat shock, overexpression of oncogenes, and nutrient deficiency. However, these responses appear to depend on type and severity of the stressors.¹²⁻¹⁵ Three different *TP53* status/p53 function including wild-type (p53wt), mutant-type (p53mt), and deletion (null-type or p53null) can be found in cancer cells. Clinically, wild-type (wt) *TP53*-carrying subjects have shown a trend toward a better response to radiotherapy and chemotherapy; however, loss of wild-type p53 function has a dominant-negative effect on the remaining wild-type p53 and are significantly associated with resistance to treatments and short survival time. Besides, p53mt proteins exert additional oncogenic functions that promote tumorigenesis, progression, and metastasis.^{11,16,17} Although non-mutated tumor suppressor p53 protein plays an important role in the prevention of malignancies, mutations in the *TP53* gene are the most common molecular defects in OSCC. In Iranian population, OSCC is a common cancer; however, a few studies with limited scopes have investigated the correlation of p53 protein expression with development and progression of OSCCs in Iranian patients.¹⁸⁻²² Besides, and to the best of our knowledge, there isn't any published study regarding the possible changes in the expression of mutant p53 protein between OSCC patients with the primary and recurrence tumors. Therefore, this study for the first time, aimed to compare the pattern of p53mt protein expression in Iranian patients with primary and recurrence OSCC.

Materials and Methods

Ethics

The protocol of this study was approved by local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (Approval ID: IR.SUMS.DENTAL.REC.1401.017). Human participation was in accordance with the Ethical Standards of the Institutional and/or National

Research Committee as well as the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from each participants or his/her parent or guardians after explaining the study protocol and objectives. In this retrospective cross-sectional study, a total of 122 Iranian patients with a pathologically confirmed diagnosis of the primary OSCC were enrolled, without any limitation in age and gender. All patients were referred to Namazi Hospital, Khalili Hospital, or Madar Va Kodak Hospital, all affiliated with the Shiraz University of Medical Sciences, Shiraz, Iran between March 2012 and 2022 (over a period of 10 years). Medical records were obtained from the patients' database. Patients with a history of systemic or inflammatory diseases or a history of radio/chemotherapy prior to the first surgical treatment as well as those with evidence of tumors, except OSCC were excluded. All patients were routinely followed-up according to the institutional guidelines. The mean follow-up duration of the enrolled patients was 36 months. The control group of this study (n=80) was selected from those without a history of cancer, who were referred to Shiraz Dentistry School for evaluating different types of noncancerous reactive lesions of the oral cavity. They fulfilled all exclusion criteria mentioned above, and were matched by age and gender to each case.

Sample collection

The paraffin-embedded tissue blocks were retrieved from the archives of the Pathology Department of School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran. The serial sections of 5 µm thicknesses were cut using a microtome (DID SABZ Co., Iran), and stained with Hematoxylin and Eosin (H & E). Slides were re-evaluated by two independent oral and maxillofacial pathologists using a double-headed microscope (Olympus BX51) to confirm the initial diagnosis of OSCC. The clinical staging of patients was determined according to the Tumor, Node, and Metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC). The histopathological grade of OSCC was determined based on World Health Organization (WHO) criteria,²³ and Broder's classification for histopathologic grading.²⁴ Finally, the paraffin-embedded tissue blocks with adequate tissue and definite diagnosis were selected.

Immunohistochemical staining

The sections with 4 µm thickness were made and mounted on Poly-L-lysine-coated glass slides for Immunohistochemistry (IHC) analysis, according to the instructions provided by the manufacturers. After deparaffinization in xylene and dehydration in alcohol, the activity of endogenous peroxidase was blocked using 0.3% hydrogen peroxide solution in methanol for half an hour. Antigen retrieval was performed using 0.1 M sodium citrate buffer (PH=6.0) and then, slides were

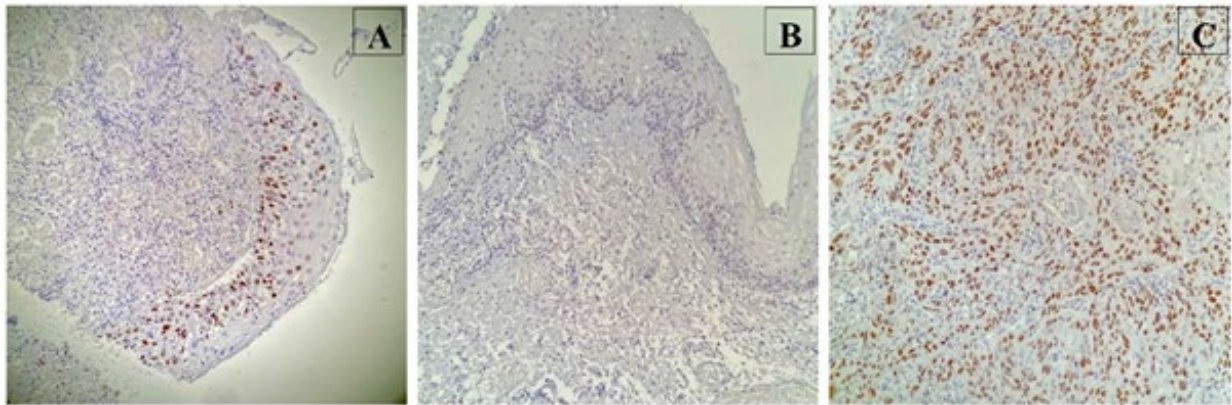


Fig 1. The immunoexpression of p53 protein in OSCC tissue. A) Photomicrograph showing the p53 immunoexpression in dysplastic squamous epithelium at the vicinity of invasive oral squamous cell carcinoma (IHC, ×250); B) The p53 negative oral squamous cell carcinoma (IHC, ×250); C) The p53 positive oral squamous cell carcinoma (IHC, ×400)

microwaved twice for 15 minutes. The sections were incubated for 30 minutes at room temperature with the primary monoclonal antibody against p53 (DO-7, Dako SA, Glostrup, Denmark) using dilution of 1:50. Subsequently, the sections were incubated with avidin-biotin-peroxidase complex followed by immersion in diaminobenzidine-H₂O₂ substrate (5 minutes, room temperature) for chromogen development. Finally, the sections were counterstained with Mayer's haematoxylin. The slides were rinsed under running water, dried, and covered with a cover slip. Samples of the control group were also stained with the same amount of antibody used for staining of tumoral tissue. The negative control sample underwent all the staining procedure, but incubated with phosphate-buffered saline instead of the primary antibody. The stained slides were evaluated by two oral and maxillofacial pathologists blinded to the clinical data, and possible disagreements were resolved by consensus. The labeling index (LI) for p53 was calculated by assessing the nuclear immunopositivity in 1000 neoplastic cells in 10 HPFs. Samples with $\geq 10\%$ immunostaining were considered p53-positive.²⁵

Statistical analysis

All statistical analyses were carried out using SPSS version 22.0 (IBM, Armonk, NY, USA). Variables were tested for normal dispersion by Kolmogorov-Smirnov test and continuous data were expressed as a mean \pm SD. Comparisons between the study groups were made by one-way analysis of variance (ANOVA), with a post-hoc Tukey test. Chi-square test and odds ratio (OR) were used to compare the mutant p53 levels in the case and control groups. The cox's proportional hazard regression and hazard ratio (HR) were used to determine the effect of p53mt and other categorical variables on the probability of OSCC recurrence. All reported

probabilities (p value) were two-sided, and considered statistically significant if less than 0.05.

Results

The OSCC group (n=122) consisted of 72 (59%) males and 50 (41%) females with a mean age of 64.6 ± 12.6 years (range of 31 to 78 years). The participants in the control group (n=80) were matched for age and sex distribution with the patients group; therefore, there were no statistically significant differences between the study groups ($p > 0.05$). The mutant p53 protein was detected by IHC in 57.4% (70 out of 122) of patients with the primary OSCC; however, none of the samples was positive in the control group ($p < 0.0001$, OR=107.69, 95%CI=6.49-179.0). In patients with the primary OSCC, the rate of positive p53mt protein had no statistically significant differences between males and females (54.2% vs. 62%, $p = 0.390$, OR=0.72, 95%CI=0.35-1.51); however, there was a significant difference in the p53 immunopositivity between those below and over 50 years, as it was detected in 81.3% (26 out of 32) of patients aged below 50 years in comparison to 48.9% (44 out of 90) of patients aged over 50 years ($p < 0.0001$, OR=4.52, 95%CI=1.07-12.05). The immunoexpression of p53mt protein is presented in Figure 1.

Discussion

OSCC is one of the most common causes of cancer-related deaths worldwide and its global burden is expected to grow rapidly due to the population growth and aging. Early diagnosis of OSCCs is a priority health objective, lead to less damages and a better prognosis; hence, it is crucial to identify potential biomarkers to improve diagnosis, prognosis, and the treatment outcome of such patients.^{4,6} Loss of the p53 tumor suppressor activity is an important step in the development and progression of human malignancies. A subset of mutations occurring in the wild-type TP53

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gene causes it to lose its tumor suppressor activity. The overexpression of a mutant p53 protein, which can accumulate in the nuclei of tumor cells is associated with more aggressive behavior in almost every type of cancers. An increased p53 protein immunoreactivity is found commonly in more than 50% of all human cancers; therefore, considerable research effort has been focused on elucidating the key mechanisms of action of p53 and its prognostic significance in cancer patients.^{16,26-29}

The findings of this study showed a significant difference in expression of p53mt protein in Iranian OSCC patients and the control group (57.4% vs. 0%, $p < 0.0001$).

Our observations are similar to the results obtained from previous studies in Iran. Fakhrijou A, Toutouchi SJ (2012)¹⁸ performed a study on two groups of OSCC lesions and the buccal mucosa of normal subjects, 20 per each group, and showed a higher rate of p53 positivity in OSCC subjects in comparison to the control group (100% vs. 40%). A case-control study conducted on 25 cases of well-differentiated OSCC and 22 samples from patients with erosive oral lichen planus (OLP) also presented a significant difference in the expression of p53 protein (49.6±29.6% in OSCC vs. 30.86±28.26% in OLP).¹⁹ Etemad-Moghadam et al. found the immunoexpression of p53 protein in the primary tumors of 28.57% of Iranian OSCC patients (8 out of 28 cases).²⁰ The concentration of p53 in unstimulated whole saliva of patients with OSCC was significantly higher than patients suffering from OLP or healthy participant (5.36±1.08 U/ml, 0.94±0.31 U/ml, and 0.41±0.04 U/ml, respectively).²¹ It was also observed by immunohistochemical analysis in 28.8% (17 of 59), 52.7% (48 of 91), 61.6% (40 of 65), 63.3% (19 of 30), and 100% (70 cases with low expression and 42 cases with high expression) of patients with the primary OSCC in Japan,³⁰ Spain,^{31,22} Brazil,³³ and Taiwan,³⁴ respectively. Although in all studies, the expression of p53 protein in OSCC group was higher than the control group, possible reasons for the discrepancy between the results may include the study population, sample size, type of the tumor sample (biopsy vs. surgery), size and location of the tumor, and the follow-up periods, which reduces the accuracy of statistical analysis.

According to the findings of present study, most of the OSCC patients were poorly differentiated and no significant association was observed between the expression of mutant p53 protein and the tumor grade. Mutations in the TP53 gene are the most common molecular defects in OSCC and it is essential for the initial phase of oral cancers.¹⁸⁻²² The lack of correlation between the OSCC tumor grade and the p53 expression in the current study can be attributed to their poorly differentiation.

However, patients in previous studies were predominantly well and moderately differentiated.³⁰⁻³⁴ Younger patients in this study (aged below 50 years) are

4.52-times more likely to express the mutant p53 protein than the older ones (aged over 50 years). OSCC occasionally occurs in young patients with more aggressive behavior and poor prognosis, and is likely to be distinct from OSCC in older patients.^{35, 36} Since the p53 is a genome guardian protein and plays an important role in the prevention of malignancies, the higher rate of p53 immunopositivity in younger patients can explain the more aggressive types of OSCCs.

Patients with OSCCs have a high incidence of recurrences, and it is an important prognostic factor in these patients.⁷ In the present study, the pattern of p53mt protein expression in cases of OSCC recurrence was the same as in the matched primary tumors. To the best of our knowledge the current study is the first investigation regarding the possible changes in the expression of mutant p53 protein between Iranian OSCC patients with the primary and recurrence tumors. Although we did not evaluate the prognosis of OSCC patients in this study, a considerable number of former investigations performed on patients with HNSCCs,^{37, 38} oral and oropharyngeal SCC,³⁹ and hypopharyngeal SCC,⁴⁰ showed no significant correlation between the expression of p53 protein and the patients' survival. In contrast, some reports in literature stated a negative prognostic value of p53 protein expression in laryngeal SCC, OSCC, and HNSCCs.^{30,41,42} The discrepancy between the results may be due to the differences in surgical techniques, types of adjuvant therapy, method of IHC staining, and other interference factors.

Mutations are likely to affect protein synthesis/function, depending on their context and location. Immunohistochemistry is a cheap and simple method to detect the inactivated p53 protein, either directly as a result of mutations in TP53 gene or indirectly through binding to viral proteins and cellular oncogenes. However, the p53 alterations was not evaluated in previous studies.³⁰⁻³⁴ Although the vast majority of p53 mutations in cancers are missense mutations that able to produce the full-length p53mt protein with single amino acid substitutions within the DNA binding domain (only one amino acid difference from p53wt protein), nonsense mutations or loss of both alleles can lead to a lack of p53 protein production/detection.^{30,43-45} Therefore, determining the type of inactivated p53 protein is so important and could clarify the limited prognostic significance of p53 immunoexpression in some human cancers.

This study also has some limitations due to the methodological flaws and potential unmeasured confounding, which can be addressed in future studies. Firstly, it is possible that imbalance between OSCC and control participants could lead to residual confounding, which can explain our findings.

The second limitation was small sample size of patients in different stages of OSCC, which lead to no correlation between the p53 expression and tumor grading. Therefore, further studies with larger sample

size, much cases in each tumor grade, and longer follow-ups are highly recommendable. Finally, the unification of clinical laboratory methods and the standardization of detection techniques might be beneficial to reduce the differences between the laboratories in analysis of the IHC results.

In conclusion, this study showed a significant difference in the expression of mutant p53 protein between OSCC patients and the control. Hence, monitoring of individuals with higher risk of OSCC development, especially younger patients, can be performed by this valuable marker to prevent malignant transformation and distant metastasis, i.e., for timely intervention. Despite in our study the immunoeexpression of the p53 mutant had no prognostic value for relapses, further in-depth studies could provide useful information in this domain.

List of acronyms

95%CI - 95% Confidence Interval
AJCC - American Joint Committee on Cancer
DNA - Deoxyribonucleic Acid
EGFR - Epidermal Growth Factor Receptor
H & E - Hematoxylin and Eosin
HNSCC - Head and Neck Squamous Cell Carcinoma
HR - Hazard Regression and Hazard Ratio
IHC - Immunohistochemistry
OLP - Oral Lichen Planus
OR - Odds Ratio
OSCC - Oral Squamous Cell Carcinoma
p53mt - p53 mutant-type
p53null - p53 null-type
p53wt - p53 wild-type
ROS - Reactive Oxygen Species
TNM - Tumor, Node, and Metastasis
TP53 – Tumor-suppressor Protein 53
WHO - World Health Organization

Contributions of Authors

All authors have read and approved the final edited typescript.

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Conflict of Interest

The authors declare no financial, personal, or other conflicts of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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