

Immunohistochemical Evaluation of P53 and Epidermal Growth Factor Receptor Proteins Expression Levels in Gingival Tissue of Opium-dependent Patients

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Original Article

Abstract

Background: Although evidence indicates that tobacco use is one of the risk factors for oral cancer, the relationship between opium addiction and oral cancer has not yet been evaluated. The present study was performed aiming to evaluate P53 and epidermal growth factor receptor (EGFR) expression in the gingival tissue of opium-dependent patients.

Methods: 102 individuals (70 men and 32 women) were entered in the study. 63 and 39 individuals were included in the opium-dependent group and opium-independent group (control group), respectively. 1 gingival biopsy was taken from each individual. The biopsies were fixed in 10% buffered formaldehyde solution and embedded in paraffin at 56 °C. The slides were then stained with Hematoxylin-Eosin (H & E) and Immunohistochemistry (IHC) evaluation was performed with the antihuman antibodies of P53 and EGFR. The protein expression level was later assessed and data were analyzed statistically.

Findings: P53 expression was higher among the opium-dependent group, however the difference was not statistically significant ($P = 0.052$). EGFR expression was significantly higher among the opium-dependent group compared to the control group ($P = 0.006$).

Conclusion: Opium dependency significantly affects EGFR expression in gingival tissue, however it seems to have no significant effect on P53 expression.

Keywords: Opium; Oral cancer; P53 genes; EGFR genes

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Introduction

A total of 5% of all cancers occur in head and neck regions and almost half of them are observed in the oral cavity.¹ Esophageal squamous-cell carcinoma (ESCC) is the most upper respiratory and gastrointestinal tract (GI tract) cancers in oral mucosa, pharynx, and larynx with common risk factors.² Squamous cell carcinoma (SCC) accounts for 94% of all oral cavity malignancies.³

Oral cancer is one of common cancers and rates as the 12th most common cancer in the world.³ Oral squamous cell carcinoma (OSCC) have several risk factors, some of which include tobacco and alcohol use, some viruses, including herpes simplex virus (HSV) and human papillomavirus (HPV), poor diet and chronic trauma due to dental factors.² Many studies have been carried out on tobacco carcinogenicity and this substance has currently been accepted as a known risk factor for OSCC.^{2,4,5} However, there are few studies on other addictive drugs like opium and its derivatives and its relationship with SCC. Some studies have revealed the relationship between opium use and esophagus, larynx, and bladder cancers.⁶

Oral cavity is perpetually under the influence of a series of thermal, chemical, and mechanical inputs, which contribute to the development of oral cancer through affecting a series of genes, like p53 and epidermal growth factor receptor (EGFR).⁷

P53 is a tumor-suppressor protein, which is coded among humans by tumor protein TP53 genes on the short arm of chromosome 17. This protein adjusts cell cycle and thus is involved in cancer prevention as a tumor suppressor. P53 mutations cause an increase in cell proliferation, apoptosis loss, and increased genetic instability.^{8,9}

EGFR belongs to Erb-B receptor family. Studies have shown the relationship between mutations leading to overexpression of EGFR or excessive anemia activity, and a number of cancers, including lung cancer, anal cancer, and glioblastoma multiforme (GBM).^{10,11}

Opium has been considered as an effective factor in developing cancer and its effect on the gingival tissue has not been studied by immunohistochemistry (IHC). Therefore, this study was carried out aiming to investigate the expression of P53 and EGFR in the gingival tissue of opium-dependent individuals.

Methods

The present study was a controlled cross-sectional study. The study population included patients admitted to the Department of Periodontology, School of Dentistry, Kerman University of Medical Sciences, Kerman, Iran, for periodontal surgical treatments including flap surgery or crown lengthening procedure. The participants were divided into two groups, including opium-dependent and independent (control) groups. The sample size of this study was 102 patients (70 men and 32 women) with an age range of 20-70 years. Sampling was carried out using convenience sampling method.

There were a total of 63 individuals in the opium-dependent group (42 men and 21 women) with the age range of 20-70 years old. These individuals were selected based on the following criteria:

- 1) No history of systemic diseases affecting gingival conditions like diabetes mellitus (DM), acquired immune deficiency syndrome (AIDS), and corticosteroids
- 2) No history of cancer, chemotherapy, radiotherapy, and alcohol use
- 3) Opium dependence according to the Diagnostic and Statistical Manual of Mental Disorders-5th Edition (DSM-IV) criteria suggesting patients to have three or more of the following criteria:
 - A) Tolerance
 - B) Withdrawal
 - C) Large amounts over a long period
 - D) Unsuccessful efforts to cut down
 - E) Time spent in obtaining the substance replacement social activities
 - F) Continued use despite adverse consequences

The opium-dependent group smoking habits were also recorded and the effect of smoking was analyzed as a confounding variable. In this study, individuals who gave a positive response to the question "Do you currently smoke any tobacco products like cigarettes, cigars, or pipes?" were considered to be smokers. Optimum independent group (control) consisted of 39 patients (28 men and 11 women) with the age range of 20-70 years old; these patients were selected based on the following criteria:

- 1) No history of systemic diseases.
- 2) Lack of tobacco, alcohol, and opium use.

3) Having healthy gums (no bleeding on probing, absence of attachment loss in clinical examination, pink color, firm consistency, and normal size).

This study was conducted after obtaining the written consent from all patients and explaining the study steps to them. In addition, the participants were ensured that all their information in the study would remain confidential.

Gingival biopsies (gingival marginal or interdental papilla) with a size of 3 × 3 mm were obtained from all the individuals participating in the study using a scalpel No.15 under anesthesia with lidocaine 2% with epinephrine 1:80000. The biopsy samples were stored in 10% formalin until completion of IHC so that the sample size was almost 20 times bigger than the formalin volume. The biopsies were later transferred to the pathology laboratory and paraffin blocks were prepared from them using the tissue processor device (RH-12EP2 SAKURA Model, Japan) according to the manufacturer's instructions. Then, the IHC of proteins expressed by EGFR and P53 genes was carried out.

First, 3.5 micron slices of paraffin blocks prepared from the gingival biopsies of subjects were prepared using Microtome Device (ERMA, Tokyo, Japan) according to the manufacturer's instructions and were later placed on a slide. Slices were incubated in 10 mmol citrate buffer for 20 minutes in the microwave. Then, the slices were incubated for 15-20 minutes in 0.5% hydrogen peroxide. The slices were later covered using 2% bovine serum albumin (BSA) for 15 minutes. In the next step, slices underwent overnight incubation using specific P53 (Dako, Glostrup, Denmark) and EGFR antibodies (Abcam, Cambridge, UK) in accordance with the instructions of the manufacturers; the dilution rate of each antibody was determined according to the primary tests. In the following, slices were conjugated to the nearby biotin using secondary antibodies for 30 minutes. If required, slices were washed using PBS three times. Then, the slices were incubated for 5 to 10 minutes with diaminobenzidine (DAB) chromogen. Finally, slices were washed (3 times) with distilled water for 5 minutes and field staining was carried out with hematoxylin for 10 s. After being washed with distilled water, the slices were covered with a section adhesive for immunocytochemistry

(ICC) and were kept for imaging and data collection purposes.

It should be noted that to guarantee the quality and safety of markers, they were tested using positive and negative controls. Breast carcinoma and SCC were respectively considered as positive control of P53 and EGFR.

The proportion score was used to assess the IHC results. This score represents the estimated ratio of the stained cells to total cells. In this study, the average percentage of positive cells (stained cells) to the total number of cells (positive and negative cells) was used in at least 3 microscopic fields using an optical microscope with a magnification of ×400. Stained slides were later read by a pathologist who was blind to the studied groups.

The following criteria were used to score P53 expression:¹² Less than 25% staining of counted cells, 25%-50% staining of counted cells, 50%-75% staining of counted cells, and 75%-100% staining of counted cells. In addition, the following criteria were used for evaluating and grading EGFR staining:¹³ No staining of the related cells, less than 10% staining of the related cells, 10%-50% staining of the related cells, 51%-80% staining of the related cells, and 80% ≤ staining of the related cells.

To analyze the data, independent t-test, chi-square test, Fisher's exact test, one-way analysis of variance (ANOVA), and logistic regression analysis were used in SPSS (version 17, SPSS Inc., Chicago, IL, USA).

Results

The objective in the present study was to investigate the expression of EGFR and P53 proteins in gingival tissue of opium-dependent individuals compared to independent ones. The study included 102 patients (63 patients in opium-dependent and 39 in control groups, respectively). Most of the subjects aged between 30 and 40 years in both groups. Statistically, opium-dependent and control groups were homogenous in terms of age and gender (Table 1).

EGFR expression level in the opium-dependent group and control group was mainly between 10% and 50% and below 10%, respectively. The statistical tests showed a statistically significant difference between the two groups in terms of EGFR expression level. This meant that the EGFR expression in opium-

dependent group was significantly higher than the control group ($P < 0.050$) (Table 2).

Table 1. Demographic data of the participants both in opium-dependent and control groups

Demographic data	Opium dependent group rate	Control group rate	P
Sex [n (%)]			0.370
Man	42 (66.7)	28 (71.8)	
Woman	21 (33.3)	11 (28.2)	
Age (year) [n (%)]			0.330
20-30	10 (15.9)	9 (23.1)	
31-40	26 (41.2)	18 (46.2)	
41-50	22 (34.9)	7 (17.9)	
51-60	2 (3.2)	5 (12.8)	
61-70	3 (4.8)	0 (0)	

The main EGFR expression was between 10%-50% among men and women in the opium-dependent group and there was no statistical difference in terms of EGFR expression based on gender ($P > 0.050$) (Table 3).

The results also indicated that the majority of participants in both groups had a P53 expression of below 25%, and there was no statistically significant difference between the two groups (Table 2). There was also no significant difference in the opium-dependent group in terms of P53 expression based on gender and age ($P > 0.050$) (Table 3). Moreover, the results showed that smoking and years of opium consumption were not effective on the EGFR and P53 expression and there was not statistically significant difference between the two groups ($P > 0.050$).

Table 3. Expression levels of P53 and epidermal growth factor receptor (EGFR) proteins in the opium-dependent group based on gender, age, smoking and years of consumption of opium

Gene expression	Sex (%)		P	Age (year) (%)					P	Smoking (%)		P	Opium (%)		P
	Man	Woman		20-30	31-40	41-50	51-60	61-70		Yes	No		< 5 year	> 5 year	
EGFR			0.470						0.590		0.560			0.420	
< 10	45.1	42.3		20.6	44.1	23.5	8.8	2.9	33.3	50.0		50.0	32.3		
10-50	47.1	50.0		18.9	32.4	40.5	5.4	2.7	63.6	50.0		50.0	64.5		
51-79	5.9	0		33.3	66.7	0	0	0	3.0	0		0	3.2		
> 80	2.0	7.7		0	66.7	33.3	0	0	0	0		0	0		
P53			0.860						0.360		0.580		0.490		
< 25	91.8	88.0		20.5	41.1	30.8	3.8	3.8	85.7	88.2		85.0	89.5		
25-50	8.2	12.0		0	50.0	37.5	12.5	0	14.3	11.8		15.0	10.5		
50-75	0	0		0	0	0	0	0	0	0		0	0		
75-100	0	0		0	0	0	0	0	0	0		0	0		

EGFR: Epidermal growth factor receptor

Table 2. Expression levels of P53 and epidermal growth factor receptor (EGFR) proteins in gingival tissue of opium-dependent and control group and their comparison

Gene expression	Opium dependent group rate	Control group rate	P
EGFR (%)			0.006
< 10	38.8	55.2	
10-50	59.2	27.6	
51-79	2.0	6.9	
> 80	0	10.3	
P53 (%)			0.052
< 25	86.4	100	
25-50	13.6	0	
50-75	0	0	
75-100	0	0	

EGFR: Epidermal growth factor receptor

Discussion

The expression of P53 and EGFR proteins in gingival tissue of opium-dependents compared to independents was investigated in this study. According to previous studies, P53 and EGFR are two important genes evaluated by IHC in oral cancer.^{12,14}

The results showed that there was a statistically significant difference between opium-dependent and control groups in terms of EGFR expression, however there was no statistically significant difference in terms of P53 expression. Only 13.6% of the subjects had P53 expression level of above 25% in the opium-dependent group and 86.4% of them had P53 expression level of less than 25%.

In a study by Cruz et al., it was stated that in case of considering a cut-off value of 25% for the positive expression of P53, it could predict TP53 mutation in OSCC.¹² Therefore, considering this cut-off value in this study, P53 gene mutation was possible among 13.6% of opium-dependent subjects. However, it should be considered that IHC, which is used to detect the P53 protein expression level, does not always show the presence of mutation in P53 gene. EGFR overexpression has been estimated at 50%-98% of oral cancers.¹⁴ EGFR expression seems to be increased appropriately for grading of oral epithelial dysplasia.¹⁵ The results of the present study showed no statistical difference in EGFR protein expression in gingival epithelium of opium-dependent group compared with the control group to the extent that EGFR expression level was reported to be between 10%-50% among 59.2% of opium-dependent subjects. According to higher EGFR expression level in opium-dependent group, which was statistically significant, it can be concluded that opium use was a factor affecting the level of EGFR protein expression and can contribute to EGFR gene mutations and carcinogenicity.

In a study on rats, Pinto and Swann indicated that opium and morphine caused damage to DNA in esophagus cells and increased the risk of esophageal cancer through a mechanism similar to that of alcohol, i.e. through affecting the metabolism of N-nitrosodimethylamine (NDMA) and N-nitrosedimethylamine, hence the production of cancer-causing N-nitrosamine.¹⁶

Naghialhossaini et al. investigated levels of carcinoembryonic antigen (CEA) and Tissue polypeptide antigen (TPA) in the serum of opium users and showed that CEA level in serum of opium users was significantly higher than that in serum of smokers and non-smokers.¹⁷

In the present study, the highest EGFR staining rate was observed in the basal layer cells and this rate was reduced in the surface layers (spinous and horny layers). This indicates that EGFR synthesis occurred in the basal layer cells and was stopped after cell division.

EGFR expression level in healthy gums was 10% in 44.8% of healthy gum samples. This upregulation of EGFR expression in healthy gums is not clear yet. However, given that small foci of inflammatory cells can be seen in healthy gums

clinically, factors released from activated macrophages and lymphocytes can be involved in EGFR expression.¹⁸

Case-control studies accounted for most of previous studies on opium carcinogenicity, reporting the relationship between opium use and esophagus, larynx, and bladder cancers.¹⁹⁻²¹ There is only one case-control study on the investigation of the relationship between opium addiction and tobacco use and OSCC. In a study conducted by Fahmy et al. between 1962 and 1978 on 381 patients with oral cancer in Fars Province, Iran, it was shown that SCC accounted for 97% of these cancers. Addiction to opium and tobacco were more common in case groups than controls.²²

In a study, Jane et al. investigated the expression levels of (B-cell lymphoma 2) Bcl2, Bcl-2-associated X (Bax) and p53 proteins in tissue samples of 38 Indian patients with OSCC cancer and 17 patients with oral leukoplakia by IHC. All these patients used chewing tobacco. The results of this study suggested that p53 protein was overexpressed in OSCC, however none of leukoplakia lesion samples were positive for p53 staining.²³

Mizobuchi et al. examined the relationship between smoking and P53 mutations among 74 patients with ESCC. Histochemical results of this study showed that the p53 gene was one of the regions targeted by cigarette smoke and the extent of damage to these genes depended on the number of cigarettes used per day.²⁴ It was shown in the present study that smoking along with opium use has not considerable effect on the expression of P53 and EGFR in the gingiva compared to opium use alone.

The difference between this study and previous studies is that the present study investigated P53 and EGFR expression levels in the gingival tissue with no sign of malignancy in opium-dependent individuals. The final result of this study indicated overexpression of EGFR in the gingival tissue of opium-dependent individuals.

According to the case-control studies and the present study, it can be concluded that opium could be a carcinogenic agent, however further studies are necessary to be conducted in this regard.

Conclusion

Increased EGFR expression in the gingival tissue of opium-dependent individuals indicated the

role of opium in EGFR gene function and thus its related changes.

Conflict of Interests

The Authors have no conflict of interest.

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بررسی ایمونوهیستوشیمی میزان بیان پروتئین‌های P53 و گیرنده فاکتور رشد اپیدرمال در بافت لثه افراد وابسته به تریاک

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مقاله پژوهشی

چکیده

مقدمه: با وجودی که شواهدی مبنی بر این که تنباکو از عوامل خطر سرطان دهان است وجود ندارد، اما تاکنون ارتباط بین وابستگی به تریاک و سرطان دهان مورد ارزیابی قرار نگرفته است. هدف از انجام مطالعه حاضر، ارزیابی بیان P53 و گیرنده فاکتور رشد اپیدرمال (epidermal growth factor receptor یا EGFR) در بافت لثه بیماران وابسته به تریاک بود.

روش‌ها: نمونه‌های لثه از بافت سالم لثه پایلاری ۱۰۲ بیمار در حین جراحی‌های مختلف پریدونتال تهیه و در محلول بافر فرمالدئید ۱۰ درصد ثابت شد. سپس از آن‌ها بلوک‌های پارافینی در دمای ۵۶ درجه سانتی‌گراد تهیه گردید. برش‌ها با استفاده از هماتوکسیلین-ائوزین رنگ‌آمیزی شد و بررسی ایمونوهیستوشیمی با آنتی‌بادی انسانی P53 و EGFR صورت گرفت. در نهایت، میزان بیان پروتئین‌ها ارزیابی گردید و داده‌ها مورد تجزیه و تحلیل قرار گرفت.

یافته‌ها: بیان P53 در گروه وابسته به تریاک بالاتر بود، اما اختلاف معنی‌داری بین دو گروه وجود نداشت ($P = ۰/۰۵۲$). بیان EGFR در گروه وابسته به تریاک در مقایسه با گروه شاهد به طور قابل توجهی بیشتر بود ($P = ۰/۰۰۶$).

نتیجه‌گیری: وابستگی به تریاک به میزان چشمگیری بر بیان EGFR در بافت لثه تأثیر می‌گذارد، اما به نظر می‌رسد که بر بیان P53 اثر چندانی ندارد.

واژگان کلیدی: تریاک، سرطان دهان، ژن P53، ژن گیرنده فاکتور رشد اپیدرمال

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