Study the association among human papilloma virus subtypes 16 and 18, codon 72 P53 gene polymorphism, and oral squamous cell carcinoma in Upper Egypt: a case–control study

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Objectives
To investigate the possible association among oral squamous cell carcinoma (OSCC), human papilloma virus (HPV) subtypes 16 and 18, and P53 codon 72 genotypes in Upper Egypt population.
Patients and methods
The present case–control study included patients presented to Maxillofacial Unit, General Surgery Department, Faculty of Medicine at Assiut University Hospital, Egypt. The biopsies were collected from patients with OSCC, patients with leukoplakia, and healthy oral mucosa as a control group. The P53c72 genotypes and gene expression for HPV subtypes 16 and 18 were determined using the real-time PCR method.
Results
The study was done on 69 patients: 45 cases (28 OSCC cases and 17 leukoplakia cases) and 24 patients as a control group. There was no statistically significant association between both OSCC and leukoplakia and P53 codon 72 gene polymorphism. The distribution of P53 genotypes in patients with OSCC was 21.4% were wild, 39.3% were mutant, and 39.3% were heterogeneous, whereas in leukoplakia, 23.6% were wild, 17.6% were mutant, and 58.8% were heterogeneous when compared with controls, where 45.8% were wild,12.5% were mutant, and 41.7% were heterogeneous.
Conclusion
No statistically significant correlation between HPV 16 and 18 genotypes and OSCC in the present study could be noted. Moreover, there is no association between codon 72 of P53 gene polymorphism and OSCC, so other environmental factors should be studied to detect the causal factors of oral carcinoma in our society.

Keywords:
human papilloma virus, leukoplakia, oral squamous cell carcinoma, P53 codon 72 single nucleotide polymorphism

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Introduction

Globally, more than 350 000 oral cancer cases are diagnosed every year; moreover, the prevalence differs according to geographic distribution [1]. In Egypt, ~4500 cases are diagnosed every year, with a 50% mortality rate [2]. Despite recent advances in surgical resection, adequate chemotherapy, and radiotherapy, the 5-year survival rate was less than 65% over the world [3]. Therefore, emphasis should be focused on prevention of oral cancer through the study of its etiology [4].

P53 is a tumor-suppressor gene which protects human cells from malignancy by repair damaged DNA and by inducing apoptosis causing cell cycle arrest [5]. Recently, there is great interest on the study of the association of P53 polymorphisms and cancer risks. There are 13 polymorphisms described in this gene [6]. The most frequently studied one is a single nucleotide polymorphism (SNP) at codon 72 in exon 4, which results in the replacement of arginine by proline in the trans-activating domain (rs1042522) [7].

For this SNP, the G allele encodes an arginine (CGC) at position 72 of the protein, where there is normally a proline (CCC), resulting in minor allele homozygote (C; C), called Pro/Pro genotype, or major allele homozygote (G; G), called Arg/Arg genotype, or heterozygote variant (G; C), called Arg/Pro genotype. Comparative sequence analyses indicate that the Pro allele is the ancestral form [8]. These

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variants exhibit different biochemical properties, resulting in differences in the ability of each variant to stimulate target gene expression [9].

Human papilloma virus (HPV), especially subtypes 16 and 18 and rarely 31 and 33 are other important risk factors in oral cancer [10]. Some studies found an association between HPV and P53 gene mutation, as HPV expresses two viral proteins (E6 and E7 proteins) that bind to the P53 gene, causing its mutation and malignant transformation [11].

Leukoplakia is the most frequent precancerous oral lesion [12]. It represents 85% of these precancerous lesions [13].

The rate of malignant transformation of leukoplakia depends on many parameters like DNA ploidy, P53 expression, and HPV subtypes [14].

To our knowledge, there is limited access to similar researches. So, the current study aims to investigate the association between oral squamous cell carcinoma (OSCC), precancerous lesions, and HPV (subtypes 16 and 18), codon 72 of P53 SNP as a tool for prevention and/or prediction of oral cancer.

Patients and methods

The current case–control study was done on patients presented to Maxillofacial Unit, General Surgery Department at Assiut University Hospital, Egypt. The ethical review board of Assiut Faculty of Medicine approved the study. The group of cases consisted of all patients with OSCC and leukoplakia registered from March 2016 to December 2018 in the area of the study. All cases are revised and approved by the pathologist. The control group is obtained from the healthy oral mucosa of patients presented to the trauma unit of Assiut University Hospital with soft tissue injury of the oral cavity.

Eligible participants

The study included patients with OSCC and oral leukoplakia who were diagnosed by a pathologist. Personal data such as age, sex, smoking status, and education level, of each participant were included in the study. The study excluded other pathological types of oral cancer, and other types of oral precancerous lesions were excluded from the study.

Intervention

Biopsy

All specimens were obtained from the participants by incisional biopsy from oral tissues where samples were immediately frozen in liquid nitrogen within 30 min of biopsy and stored at −80°C until use. Pathological diagnoses were reviewed by the pathologist (Figs 1 and 2).

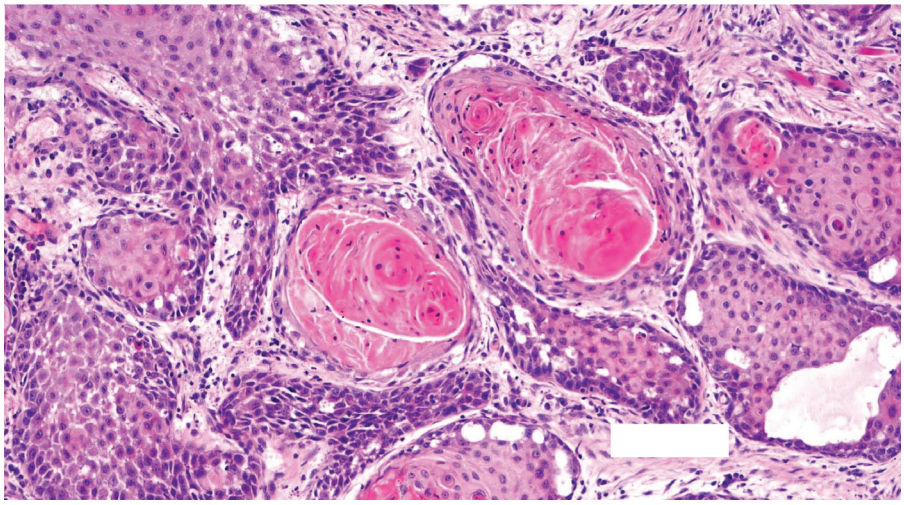
DNA extraction

DNA extraction from oral tissue was performed using Clamp DNA Mini Kit 50 Cat. no. 51304 (Qiagen) with all recommended by the manufacturer. DNA concentration is determined using Spectrostar Nano (BMG Lab Tech, Franc). The constant concentration is 20 ng.

P53 genotyping

Genotyping was performed using Real-Time PCR (Applied Biosystem, 7 Kingsland Grange-Woolston,

Figure 1



Hematoxylin and eosin-stained section from squamous cell carcinoma revealed the presence of malignant squamous cells arranged in nests with keratin pearls surrounded by desmoplastic stroma (×20).

Warrington, Cheshire, UK, Step One plus), and the thermal profile was 60° for 30s, 95° for 10 min, 95° for 15s, and 60° for 90 s, for SNP (rs1042522). NCBI shows the type of polymorphism (A/C/G). C is the ancestral allele, and G is the mutant allele. Thermo Fischer shows that C is Vic (allele 1) G is fam (allele 2).

Context Sequence [VIC/FAM]: AGGAGCTGCTG GTGCAGGGGCCACG[C/G]GGGGAGCAGC CTCTGGCATTCTGGG

PCR for human papilloma virus 16 and 18

DNA extraction from oral tissue was performed using Qlamp DNA Mini Kit 50 T, Cat. no. 51304 (Qiagen), according to the manufacturer instructions. The DNA concentration was determined using Spectrostar Nano (Lab Tech), at a constant concentration of 20 ng. Gene expression was performed using Applies Biosystem Step One Plus real-time PCR; the thermal profile was 60° for 30s, 95° for 10 min, 95° for 15s, and 60° for 90 s. The primer and probe sequences for HPV 16 and 18 are shown in Table 1.

Table 1 The probe-primer sequences for human papilloma virus 16 and 18 used in real-time PCR

Assay	Primer	Sequence (5′→3′)
HPV 16	HPV 16 E7-F	GAGGAGGAGGATGAAATAGATGGT
E7	HPV 16 E7-R	AGCGTAGAGTCACACTTGCAACA
	HPV 16 E7-P	CTCTGTCCGGTTCTGCTTGTCC
HPV 18 E6	HPV 18 E6-F	CTGGGCACTATAGAGGCCAGT
	HPV 18 E6-R	GTGTTTCTCTGCGTCGTTGG
	HPV 18 E6-P	TGCAACCGAGCACGACAGGAACGA

F, forward primer; HPV, human papilloma virus; P, probe; R, reverse primer.

Study outcomes

The primary outcomes of the current study were to know the percentage of patients with OSCC and oral leukoplakia having P53 codon 72 gene polymorphism to study the possible association between OSCC and P53 codon 72 genotypes. So, persons with genetic susceptibility can be identified and undergo close follow-up for early detection. The secondary outcome was to know the percentage of patients with squamous cell carcinoma (SCC) infected with HPV subtypes 16 and 18, so that vaccination could be developed against HPV if a positive association was detected, and early treatment of highly susceptible persons.

Statistical analysis

Data analysis was accomplished using the Statistical Package for the Social Sciences software program (SPSS Inc., USA), version 21. Statistical significance was acceptable to a level of P value less than 0.05. Quantitative data were represented by mean and SD, whereas qualitative data were represented by number and percentage. The study outcomes were analyzed using c^2 test, Fisher exact test, and independent samples t test.

Results

The study was done on 69 patients, comprising 28 (40.6%) OSCC cases, 17 (24.6%) cases of leukoplakia, and 24 (34.8%) samples as a control group.

Demographic data for the selected risk factors are shown in Table 2, in which 14 (50.0%) cases of OSCC were males and 14 (50.0%) cases were females, with no statistically significant difference

Table 2 Demographic details for selected data for oral squamous cell carcinoma, leukoplakia, and control

Variables	SCC (N=28) [n (%)]	Leukoplakia (N=17) [n (%)]	Control (N=24) [n (%)]	P value[a]	P value[b]	P value[c]
Sex				0.002*	0.548	0.014*
Male	14 (50.0)	16 (94.1)	14 (58.3)			
Female	14 (50.0)	1 (5.9)	10 (41.7)			
Age				0.004*	0.000*	0.476
<50	6 (21.4)	11 (64.7)	19 (79.2)			
≥50	22 (78.6)	6 (35.3)	5 (20.8)			
Mean±SD	58.14±12.45	45.18±10.67	38.88±12.14	0.001*	0.000*	0.076
Education				0.384	0.521	0.184
Literate	8 (28.6)	7 (41.2)	5 (20.8)			
Illiterate	20 (71.4)	10 (58.8)	19 (79.2)			
Smoking				0.001*	0.054	0.000*
Smoker	10 (35.7)	15 (88.2)	3 (12.5)			
Nonsmoker	18 (64.3)	2 (11.8)	21 (87.5)			

[a]Comparison between squamous cell carcinoma and leukoplakia. [b]Comparison between squamous cell carcinoma and control. [c]Comparison between leukoplakia and control. *P* value less than 0.05 was acceptable statistically significant. *Means that the result is statistically significant.

Table 3 Distribution of P53 genotypes in patients with oral squamous cell carcinoma, patients with leukoplakia, and controls

Codon 72 P53 SNP	SCC (*N*=28) [*n* (%)]	Leukoplakia (*N*=17) [*n* (%)]	Control (*N*=24) [*n* (%)]	*P* value[a]	*P* value[b]	*P* value[c]
Mutant	11 (39.3)	3 (17.6)	3 (12.5)	0.290	0.055	0.344
Wild	6 (21.4)	4 (23.5)	11 (45.8)			
Heterogeneous	11 (39.3)	10 (58.8)	10 (41.7)			

SCC, squamous cell carcinoma; SNP, single nucleotide polymorphism. [a]Comparison between SCC and leukoplakia. [b]Comparison between SCC and control. [c]Comparison between leukoplakia and control. *P* value less than 0.05 was acceptable statistically significant.

Table 4 Human papilloma virus subtypes 16 and 18 in patients with oral squamous cell carcinoma, patients with leukoplakia, and controls

	SCC (*N*=28) [*n* (%)]	Leukoplakia (*N*=17) [*n* (%)]	Control (*N*=24) [*n* (%)]	*P* value[a]	*P* value[b]	*P* value[c]
HPV 16				0.547	1.000	0.166
Positive	1 (3.6)	2 (11.8)	0 (0.0)			
Negative	27 (96.4)	15 (88.2)	24 (100.0)			

HPV, human papilloma virus; SCC, squamous cell carcinoma. [a]Comparison between SCC and leukoplakia. [b]Comparison between SCC and control. [c]Comparison between leukoplakia and control. *P* value less than 0.05 was acceptable statistically significant.

when compared with control group (*P*=0.548). However, in leukoplakia, 16 (94.1%) cases were males and one (5.9%) case were female, with a statistically significant difference when compared with the control group (*P*=0.014).

The present study showed that the frequency of OSCC was higher in nonsmokers (64.3%), whereas in leukoplakia, there were a significant association between leukoplakia and smoking, as 15 (88.2%) cases were smokers and two (11.8%) cases were nonsmokers, with statistically significant difference when compared with the control group (*P*=0.000).

As shown in Tables 3 and 4, distribution of P53 genotypes and HPV 16 and 18 in patients with OSCC, patients with leukoplakia, and controls, it was observed that three (4.3%) cases were positive for HPV 16 (one (1.4%) case of OSCC, and two (2.9%) cases of leukoplakia), whereas all cases were negative for HPV 18 compared with controls, with negative results for both HPV 16 and 18. It was observed that there was no significant association between HPV 16 and OSCC or leukoplakia when compared with the control group (*P*=1.00 and 0.166, respectively).

We also observed that there was no statistically significant association between OSCC or leukoplakia and P53 codon 72 gene polymorphism.

As shown in Table 5, we also stratified the associations between codon 72 P53 SNP genotypes and age, sex, smoking, education level, site of lesion, staging, grading of OSCC, grading of leukoplakia, and HPV 16 to study the correlations between codon 72 P53 SNP genotyping and the clinicopathological features of both OSCC and leukoplakia.

On studying the interaction of all previous factors, there was no statistically significant association between them and codon 72 P53 genotypes.

By classifying education level into two groups (literate and illiterate), we observed that the high frequency of mutant genotypes was found in the illiterate group, with a statistically significant difference compared with the literate group (*P*=0.047).

By using the TNM staging system for OSCC, it was observed that stage II OSCC had the highest frequency in both mutant and heterogeneous genotypes, with a statistically significant difference compared with other stages (*P*=0.000).

It was observed that moderate differentiated OSCC had the highest frequency in both mutant and heterogeneous genotypes, with a statistically significant difference compared with other grades (*P*=0.002) (Fig. 2).

Discussion

OC etiology is multifactorial [15]. Numerous risk factors contribute to its etiology, which includes genetic variation and environmental factors such as smoking, alcohol drinking, and viral infections [16,17]. Previous reports show that TP53 mutation may contribute to OC tumorigenesis [18]. On the contrary, other studies implied that mutation of TP53 gene has been supposed to be an uncommon event in carcinogenesis of oral carcinoma [19,20].

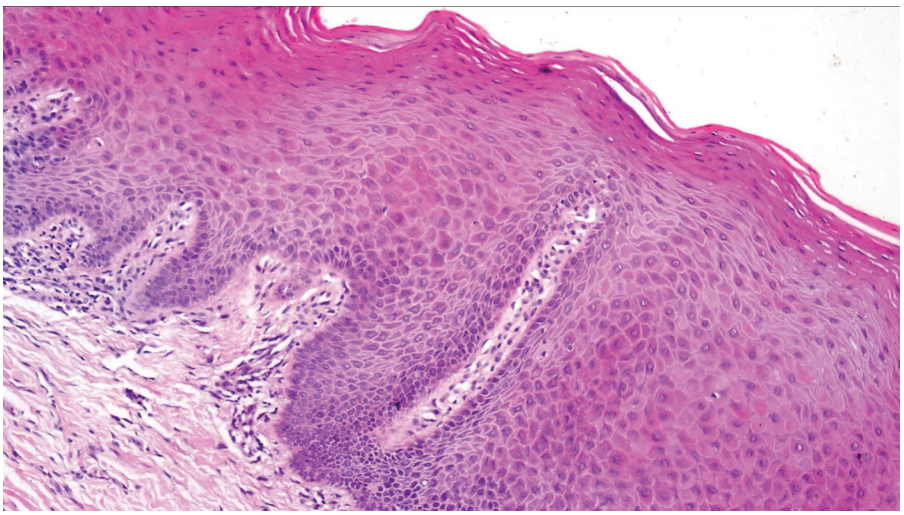
In the present study, there was no significant association between codon 72 of the P53 gene polymorphism and OSCC. These results were

Table 5 The clinicopathological features of the patients with oral squamous cell carcinoma according to codon 72 P53 single nucleotide polymorphism genotyping (*N*=69)

Variables	*N*	Wild	Mutant	Heterogeneous	*P* value
Age (years)					
≥50	33	10	11	10	0.079
<50	36	11	6	21	
Gender					
Male	44	11	11	22	0.391
Female	25	10	6	9	
Smoking					
Smoker	28	6	9	13	0.288
Nonsmoker	41	15	8	16	
Education level					
Literate	20	4	3	13	0.047
Illiterate	49	17	14	18	
Site of lesion					
Tongue	25	10	3	12	0.485
Gingiva	10	3	2	5	
Cheek	22	7	6	9	
Lip mucosa	9	1	4	4	
Floor of the mouth	1	0	1	1	
Retro-molar	2	0	1	0	
Staging of SCC					
Stage I	3	1	1	1	0.000
Stage II	18	2	7	9	
Stage III	6	2	3	1	
Stage IV	1	0	0	1	
Grading of SCC					
Well differentiated	7	1	4	2	0.002
Moderate differentiation	18	4	6	8	
Poorly differentiation	3	1	0	2	
Grading of leukoplakia					
No dysplasia	4	2	1	1	0.080
Mild dysplasia	10	1	0	9	
Moderate dysplasia	3	1	2	0	
HPV 16					
Positive	3	1	0	2	0.574
Negative	66	20	17	29	

HPV, human papilloma virus; SCC, squamous cell carcinoma. *P* value less than 0.05 was acceptable statistically significant.

Figure 2



Hematoxylin and eosin-stained section revealed the presence of leukoplakia with mild dysplasia (×10).

similar to the results gained by Shen *et al*. [21] on head and neck SCC, as well as the study in northern Iran [22].

In the previous studies on codon 72 of P53 SNP, Arg variant has been believed to increase susceptibility to gastric [23] and breast carcinoma [24]. The Arg72 homozygosity was associated with cervical cancer [25]. Conversely, Pro homozygosity was associated with the lung [26] and hepatocellular cancer [27]. The heterozygous genotype has been accused to increase the risk of bladder cancer [28].

In studies done in Taiwan [29] and Germany population [30], a higher risk of OC development in the presence of Arg/Arg compared with those with Pro/Pro was reported. Another study done in South India suggested a strong association of both Arg/Pro and Pro/Pro of codon 72 of the P53 polymorphism with tumor progression [31].

The previous variations might be attributable to other environmental factors, which may contribute to the carcinogenic mechanisms (OC) [32].

The current study results showed no association between both HPV 16 and 18 genotypes and OSCC or leukoplakia. These negative results might be owing to uncommon sexual behavior as a route of transmission in our country. This behavior is owing to religious and social considerations. Moreover, the negative results might be owing to the limited sample size.

We observed that frequency of SCC was higher in the age group more than or equal 50 years (78.6%). The frequency of leukoplakia was higher in age group less than 50 years (64.7%), with a statistically significant difference when SCC and leukoplakia were compared with the control group.

In the present study, the mean age of patients with OSCC was 58.14±12.45 years. The mean age of male patients with OSCC was 60±14.48 years and female patients with OSCC was 55.86±10.04 years. These results were similar to the study done in north-eastern Nigeria [33] but different from the studies done in India [34] and the Netherlands [35], in which the mean age of female patients with OSCC was more than males. On the contrary, in patients with leukoplakia, the mean age was 45.18±10.67 years, whereas the mean age of male patients with leukoplakia was 42.15±8.94 years and female patients with OSCC was 42 years. This observation was similar to the prevalence of leukoplakia in the world [36] but different than the study done in India, in which oral leukoplakia was predominant in older female patients [37]. This may be owing to the widespread use of tobacco and pipe among males in Upper Egypt.Moreover, the current study showed no association between OSCC and smoking, which is against other studies, which confirmed the association between tobacco use and risk for OSCC [38]. This might be owing to the presence of other environmental factors accused in the etiology of OSCC. Moreover, this explained the high percentage of OSCC among females (50%), despite none of the females were smokers.

In leukoplakia, we observed a significant association between leukoplakia and smoking, which explained the high frequency of oral leukoplakia in males, which might be owing to the higher use of tobacco and pipe among males than females in the Egyptian community.

Regarding the anatomical sites of lesions, tongue was the commonest site of SCC (46.4%), which is similar to other studies done in Netherlands [35], Iran [39], and Basque country [40]. This observation was different than other studies, in which the lip was the commonest site [41]. However, in the study done in Zimbabwe, the gingiva was the most common site for OC [42]. The high frequency of tongue SCC might be owing to poor dentition and sharp teeth causing chronic irritation of the tongue.

Up to our knowledge, there was no previous study on the association between codon 72 P53 gene polymorphism and OSCC in Egypt. On the contrary, the limited fund resources restricted the sample size in the current study, hoping to repeat it in a large population in the future.

Conclusion

There is no association between codon 72 of P53 gene polymorphism and OSCC; other studies on a large sample size may be needed to confirm these results. On the contrary, HPV 16 and 18 are not associated with an increased incidence of OSCC in our study, so other environmental factors should be studied to detect the causal factors of oral carcinoma in our society.

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Conflicts of interest
There are no conflicts of interest.

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