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HUMAN PAPILLOMA VIRUSES 16 AND 18 IN SUDANESE ORAL SQUAMOUS TISSUES

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ABSTRACT

Human papilloma viruses 16 and 18 (HPV-16 and HPV-18) are well known to play some roles in cervical and oral squamous cell carcinomas. The aim of this study was to investigate the expression of HPV-16 and HPV-18 in normal and cancerous oral squamous tissues using the immunohistochemistry (IHC) and polymerase chain reaction (PCR) techniques. Fifty one oral squamous carcinoma tissues and 49 normal oral squamous tissues were investigated for HPV-16 and HPV-18 detection. The immunohistochemistry results showed that the HPV-16 was positive in 2.04% of the normal tissues and in 9.8% of the cancerous tissues while the HPV-18 was positive in 8.2% and 17.6% of the normal and cancerous tissues, respectively. There was insignificant variation between the IHC results of the normal and cancerous tissues. 4.08% and 5.9% of the normal and cancerous tissues were positive for HPV-16 PCR, respectively. Concerning the PCR results of the HPV-18, all the normal and cancerous tissues were negative for HPV-18. There was insignificant variation between the HPV-16 detection by IHC and PCR while the HPV-18 detection by IHC was significantly sensitive compared to the PCR. The odd ratios of IHC for HPV-16 and HPV-18 showed that being positive was more likely to be OSCC 5.22 and 2.42 times, respectively compared to being negative for them. There was an insignificant variation between the expression of HPV-16 and HPV-18 in the normal and cancerous oral squamous tissues of the Sudanese subjects. The IHC was significantly sensitive for the detection of HPV-18 than the PCR.

KEYWORDS: OSCC, IHC, PCR, HPV-16, HPV-18.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) occurs on the floor of the mouth, lips and tongue. It can be preceded by erythroplakia, lump, red or white lesions and leukoplakia. Worldwide, the incidence of OSCC in males and females are 6.6 and 2.9 in every 100000 population, respectively. The mortality rate of the OSCC in males and females are 3.1 and 1.4 per 100000 population, respectively.[1]

Human papilloma viruses are double stranded DNA viruses without envelope and they belong to the Papillomaviridae family. There are more than 170 type of HPV affecting the mucosa of the upper respiratory system, epithelia of the genital tract and the skin.[2]

Five human papilloma viruses are associated with most of the genital tract and oropharyngeal cancers in males and females. The five human papilloma viruses associated with cancer incidence are HPV-16, HPV-18, HPV-31, HPV-33 and HPV-35.[3]

This article investigated the expression of HPV-16 and HPV-18 in oral normal squamous and cancerous squamous tissues from Sudanese participants. The human papilloma viruses expression were investigated using two techniques IHC and PCR so as to compare between the two techniques in the diagnosis of HPV infection.

MATERIALS AND METHODS

Study design

This research was designed as a descriptive case control study.

Study community, sampling and ethical license

Fifty one oral squamous cell carcinoma (OSCC) Sudanese patients and forty nine healthy Sudanese subjects were involved in this study after an informed consent was obtained. An academic and ethical approval was obtained from the faculty of medicine-University of Gezira-Wad Madani-Sudan.

Confirmation of the cancerous smears

The hematoxylin and eosin staining (H and E stain) was used for the diagnosis of the OSCC. The sections were washed with distilled water, and the nuclei were stained with the alum hematoxylin for 3 to 5 minutes. The color was differentiated by 3% acid alcohol for few seconds and the sample was rinsed again in tap water. Finally the tissue was stained with eosin for 2 minutes, dehydrated, cleared, mounted with Di-n-butyl phthalate in xylene (DPX) and finally, examined by two pathologists.

Detection of HPV-16 and HPV-18 by IHC

Sections were prepared from paraffin wax blocks by heating in oven for 30 minutes at 60℃ for the purpose of wax removal and the HPV-16 and HPV-18 were retrieved in the tissues through heating in water bath for 10 minutes at 90℃ as follows: Firstly, the sections were treated with hydrogen peroxide for 20 minutes under water. Secondly, the sections were washed in tap water and phosphate buffered saline for 5 minutes for 10 minutes (five minutes each). Thirdly, the sections were treated with primary antiserum for 30 minutes and washed using phosphate buffered saline for 5 minutes. Fourthly, the primary antiserum was treated with secondary antiserum for 30 minutes and rinsed in phosphate buffered saline. Fifthly, the sections were treated with Diaminobenzidine (DAB) for 15 minutes and washed using tap water. Finally, the nuclei were stained using Mayer's hematoxylin for 10 min, washed with tap water to obtain the blue color, dehydrated, cleared, mounted and examined by two expert histopathologists.

DNA-PCR of HPV-16 and HPV-18

Total cellular DNA (100 ng/µl) was amplified by PCR. Primers directed towards E7 and E6 open reading frame of HPV were used for the detection of HPV-16 and HPV-18, respectively. One microlitre (100 ng/µl) of DNA was mixed with 50 µl PCR mix [125 mM dNTPs and 0.5 units of Red Hot Taq polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$ and 200 mg/ml bovine serum albumin (BSA)]. The PCR was initiated by hot start at 95°C for 5 min, then 30 cycles (denaturation 95°C/40 s; annealing 50°C/60 s; and extension 72°C/90 s). Then one last step for extension at 72°C for 10 min .Ten microlitres of the PCR product was mixed with two loading solutions in 2% agarose gel electrophoresis and run for 60 min, then stained by ethidium bromide and photographed by a gel documentation system (Gel mega, digital camera and software in a computer).

Statistical analysis

The t-test percent of the MedCalc statistical software was used for the comparison between the IHC and PCR results of the normal and cancerous oral squamous tissues. The odd ratio, sensitivity and specificity of HPV-16 and HPV-18 to the OSCC was calculated manually.

RESULTS

General

This study was composed of 49 healthy Sudanese humans (normal) and 51 oral squamous cell carcinoma patients. The normal participants involved 23 females and 26 males while the OSCC patients contained 23 females and 28 males. Age wise, the study population was divided to three groups (20-40), (41-60) and (≥ 61). The first age group (20-40) involved 15 normal subjects and 8 OSCC patients, the second age group (41-60) contained 16 normal participants and 28 OSCC patients and the last age group; (≥ 61) was composed of 18 normal subjects and 15 OSCC patients (**Table 1**).

Results of IHC

The IHC results of HPV-16 showed that one normal sample (2.04%) was positive for HPV-16 compared to five positive cancerous oral tissues (9.8%). The difference between the HPV-16 percentages of the positive normal and OSCC was insignificant (*p*-value = 0.1) (**Table 2**) and [Fig 1].

Having a positive HPV-16 result had 5.22 higher to be OSCC compared to negative HPV-16 (**Table 3**). The sensitivity of HPV-16 for the detection of OSCC was 83.3% and a negative HPV-16 result dictated that the tissue was 50% normal tissue (**Table 4**).

The staining of the HPV-18 showed that 4 normal tissues (8.2%) and 9 cancerous oral tissues (17.6%) were positive and the difference between the two percentages was insignificant (*p*-value = 0.16) (**Table 3**)and [Fig 2]. The sensitivity and specificity of HPV-18 using IHC was 69.2% and 51.7%, respectively (**Table 4**).

Results of PCR

The PCR results of HPV-16 showed that two normal sample (4.08%) was positive for HPV-16 compared to three positive cancerous oral tissues (5.9%). The difference between the HPV-16 percentages of the positive normal and OSCC was insignificant (*p*-value = 0.68) (**Table 2**). Having a positive HPV-16 PCR result had 1.48 higher to be OSCC compared to negative HPV-16 (**Table 3**). The sensitivity of HPV-16 PCR for the detection of OSCC was 60% and a negative HPV-16 result dictated that the smear was 49.5% normal tissue (**Table 4**).

The PCR result of the HPV-18 showed that there were no positive tissues within the normal and cancerous tissues (**Table 3**). If the PCR for HPV-18 was negative, it was 49% a normal tissue (**Table 4**).

Comparison between the IHC and the PCR results: There was insignificant differences between the percentages of positive tissues using the IHC and PCR to detect the HPV-16 in the oral normal and cancerous tissues (*p*-value = 0.56 and *p*-value = 0.47, respectively) (**Table 2**). The IHC was significantly sensitive for the detection of HPV-18 in the normal and cancerous tissues compared to the PCR (*p*-value = 0.04 and *p*-value = 0.002, respectively) (**Table 2**).

Table 1: Description of the study population.

Age group			Study Subject		Total
			Normal	OSCC	
20-40	Sex	Female	6	3	9
		Male	9	5	14
	Total		15	8	23
41-60	Sex	Female	10	14	24
		Male	6	14	20
	Total		16	28	44
≥ 61	Sex	Female	7	6	13
		Male	11	9	20
	Total		18	15	33
Total	Sex	Female	23	23	46
		Male	26	28	54
	Total		49	51	100

The study population was composed of 49 healthy humans and 51 Oral Squamous cell carcinoma patients (OSCC). The normal subjects involved 23 females and 26 males while the OSCC patients were 23 females and 28 males. The Age of the study population was divided to three groups; (20-40), (41-60) and (≥ 61).

Table 2: Expression of HPV-16 and HPV-18 in normal and cancerous oral squamous tissues using the IHC and PCR.

	Positive		*p*-value *	Negative		*p*-value
	Normal	Cancerous		Normal	Cancerous	
HPV-16 (IHC)	1 (2.04%)	5 (9.8%)	0.1	48 (97.95%)	46 (90.2%)	0.1
HPV-16 (PCR)	2 (4.08%)	3 (5.9%)	0.68	47 (95.9%)	48 (94.1%)	0.68
***p*-value	0.56	0.47		0.56	0.47	
HPV-18 (IHC)	4 (8.2%)	9 (17.6%)	0.16	45 (91.8%)	42 (82.4%)	0.16
HPV-18 (PCR)	0	0	-	49 (100%)	51 (100%)	-
***p*-value	0.04	0.002		0.04	0.002	

*The significance value of the difference between the normal and cancerous tissues. **The significance difference between the IHC and PCR. There was insignificant difference between the expression of HPV-16 and HPV-18 in the normal and cancerous tissues. The HPV-18 detection by IHC was significantly different the its detection by PCR i.e. the IHC was significantly sensitive for the HPV-18 detection compared to the PCR.

Table 3: The odd ratios of the IHC and PCR results of HPV-16 and HPV-18 for detecting the OSCC.

		HPV-16 IHC	HPV-16 PCR	HPV-18 IHC	HPV-18 PCR
Numerator	Cancer positive	9.8	5.9	17.6	0
	Normal positive	2.04	4.08	8.2	0
	Ratio	4.8	1.45	2.15	-
Denumerator	Cancer negative	90.2	94.1	82.4	100
	Normal negative	97.95	95.9	91.8	100
	Ratio	0.92	0.98	0.89	1
Odd ratio (OR)		5.22	1.48	2.42	-

The odds of having the OSCC were 5.22 and 1.48 higher given being positive for HPV-16 using the IHC and PCR, respectively compared to being negative for HPV-16 while the odd of having OSCC was 2.42 higher being positive for HPV-18 using the IHC compared to being negative.

Table 4: Sensitivity and specificity of the HPV-16 and HPV-18 detected by IHC and PCR in the OSCC tissues.

	HP-16 IHC	HPV-16 PCR	HPV-18 IHC	HPV-18 PCR
Positive cancerous tissues	5	3	9	0
Total positive tissues (normal and cancerous)	6	5	13	0

Sensitivity %	83.3	60	69.2	-
Negative normal tissues	48	47	45	49
Total negative tissues (normal and cancerous)	94	95	87	100
Specificity%	51.1	49.5	51.7	49

The highest sensitivity was for HPV-16 and HPV-18 (IHC) since they were able to detect 83.3% and 69.2% of the OSCC tissues, respectively. If an oral squamous tissues was negative for HPV-16 and HPV-18 by IHC or PCR, it was approximately 50% normal tissues (specificity).

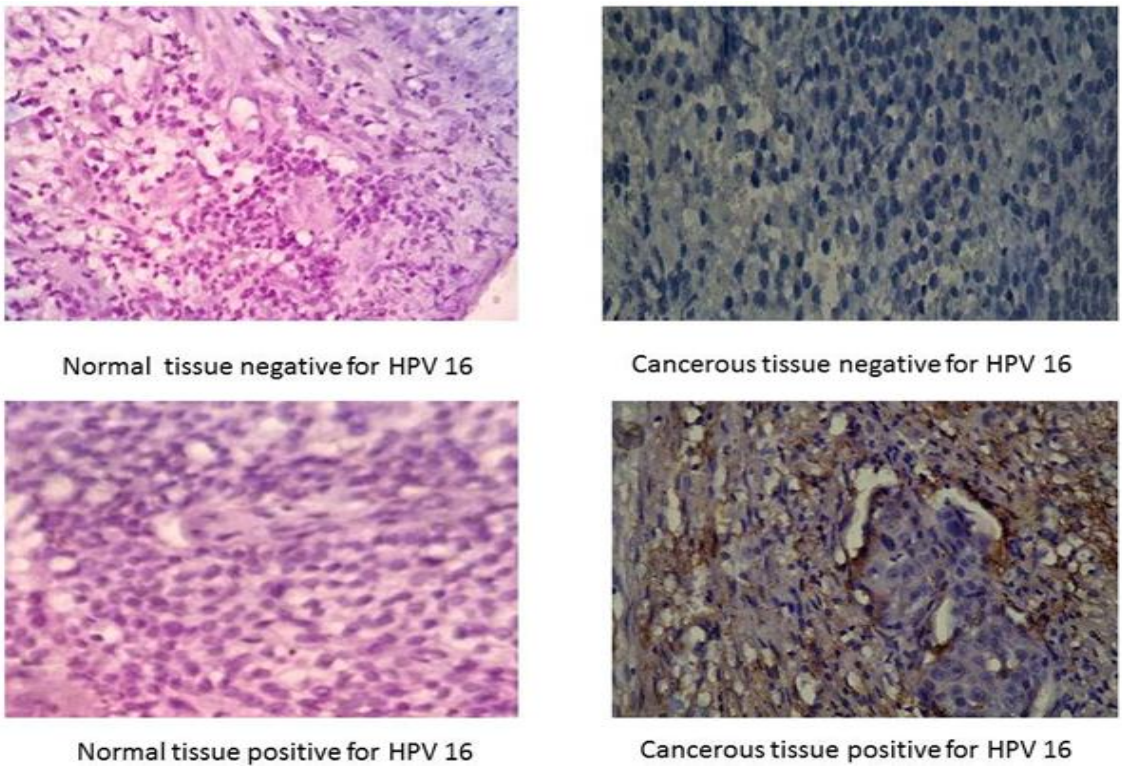

Fig 1: Representative IHC staining results of HPV 16 in the normal and cancerous oral tissues. The HPV 16 positive normal and cancer tissues were one and five, repectively.

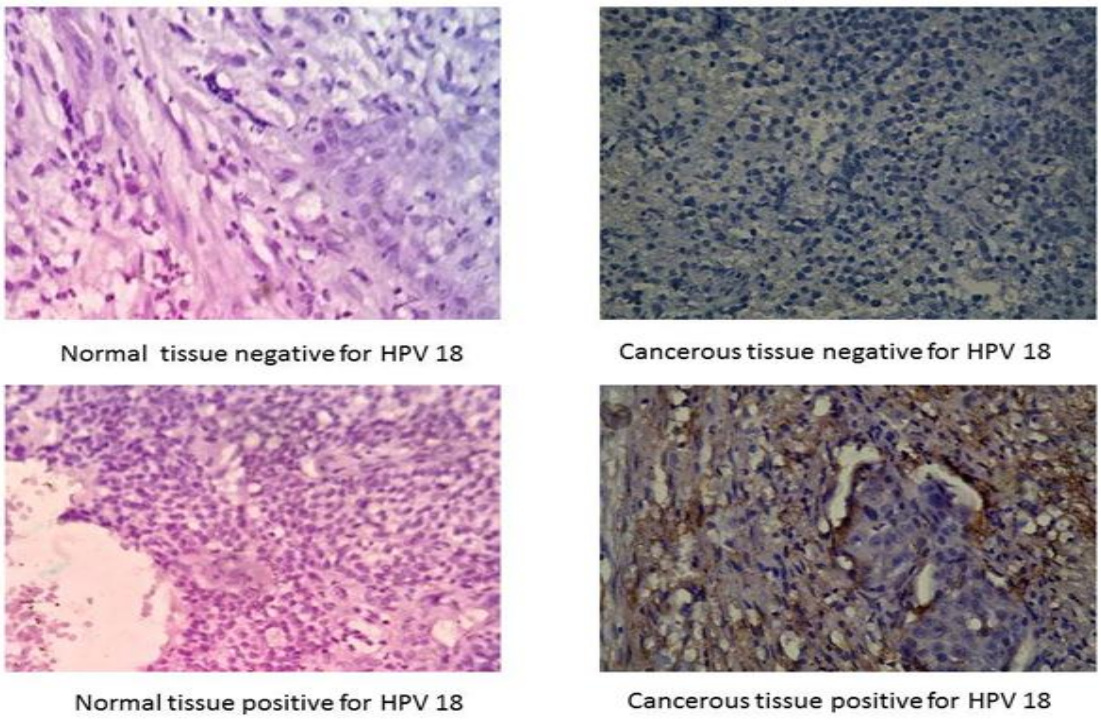

Fig. 2: The IHC staining results of HPV 18. Four normal and nine cancer tissues were positive for HPV 18.

DISCUSSION

Our study reported insignificant differences between the percentages of HPV-16 and HPV-18 positive cases in the normal and cancerous oral squamous tissues. The odd of having OSCC if positive for HPV-16 was 5.22 higher than being negative for HPV-16. Detection of HPV-18 by IHC was significantly sensitive compared to its detection by PCR.

Some of the previous studies showed that the gene (PCR) and protein (IHC) expression of HPV-16 and HPV-18 in cancerous oral mucosa smears was significantly more than its expression in normal oral smears.[4-6]

Other studies stated that the expression of HPV-16 and HPV-18 in cancerous oral mucosa was insignificantly different compared to their expression in normal oral mucosa.[7-9] Other studies carried out researches on oral cancers without involving normal oral samples; three studies conducted on Chinese and Indian populations found that HPV was not expressed in tissue samples from oral squamous cell carcinoma patients. However, the Chinese studied detected the HPV-16 and HPV-18 in the tissues and serum of OSCC patients and oral potentially malignant disorders (OPMD) patients.[10-12] Patil in 2014 studied 30 OSCC samples and concluded that HPV infection was associated with OSCC.[13]

This study reported that oral smears positive for HPV-16 and HPV-18 were associated with increased risk for OSCC (odd ratios were 5.22 times and 2.42 times, respectively). Chen et al, (2002) reported 11.21 and 6.57 odd ratios for HPV-16 and HPV-18 infection and OSCC, respectively.[14] A study conducted on Chinese population proved that HPV infection was strongly associated with increased risk for OSCC with odd ratio of 7.21 for HPV-16/HPV-18 and 7.59 for HPV-18 alone.[15] Human papilloma viruses were non-significantly associated with oropharyngeal cancer in a case control study conducted in Thailand (adjusted odd ratio was 5.83).[16]

The sensitivity of the HPV-16 and HPV-18 using the IHC of this study were 83.3% and 69.2%, respectively while the specificity of the two HPV proteins were about 50% using the IHC and the PCR. Unlike the findings of this study, Sritippho et al, (2016) mentioned that the sensitivity and specificity of HPV-16/18 with OSCC were 40% and 79.3%, respectively.[17] The findings of the study of Fonmarty et al., (2015) were comparable to our findings since they reported that HPV-16 test had 94% sensitivity and 82% specificity for oropharyngeal squamous cell carcinoma.[18] Another research investigated HPV in oral smears from oropharyngeal squamous cell cancer (OPSCC) patients and controls (had benign or malignant thyroid nodules) and found that the specificity and sensitivity of HPV were 90.5% and 79.1%, respectively.[19]

This study proved that there was an insignificant difference between the IHC and PCR in the detection of HPV-16 while the IHC was significantly sensitive compared to the PCR of HPV-18. Contrarily, Awan et al. (2017) concluded that the PCR is more sensitive than IHC in the detection of HPV in OSCC.[20] Lee et al. (2016) found that the immunohistochemistry sensitivity for the detection of HPV was comparable to that of PCR.[21] Prigge et al. (2017) concluded that IHC for HPV detection in Oropharyngeal Squamous Cell Carcinoma (OPSCC) had high sensitivity and moderate specificity compared to PCR and when they combined the IHC and DNA PCR the specificity was significantly optimized while the sensitivity was not affected.[22]

As this research is a descriptive case control study, it suffers from the small number of samples and its findings cannot be generalized. A survey study with statistically acceptable number of samples is highly recommended.

The detection of HPV-16 and HPV-18 by IHC and PCR in the normal and cancerous oral smears was insignificantly different. The sensitivity and specificity of HPV-16 using the IHC were 83.3% and 51.1%, respectively while their values when detected by PCR were 60% and 49.5%, respectively. The detection of HPV-18 by IHC was with 69.2% sensitivity and 51.7% specificity. There was insignificant variation between the detection of HPV-16 by IHC and PCR while the detection of HPV-18 by IHC was significantly sensitive compared to the PCR.

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