Detection of Human Papilloma Virus Types 16 and 18 among Sudanese Patients with Oral Squamous Cell Carcinoma

Hussain Gadelkarim Ahmed[*,1] and Faris Merghany Eltoom[2]

[1]*University of Khartoum, 102, Faculty of Medical Laboratory Science, Khartoum, Sudan*

[2]*Department of Histopathology and Cytology, Faculty of Medical laboratory Science, Khartoum, Sudan*

Abstract: To evaluate the possible role of high risk Human Papilloma viruses (HPV) 16 and 18 in oral squamous cell carcinomas (OSCC), 40 SCCs and 15 benign lesions were analyzed for the presence of HPV DNA. The investigation followed a case-control design. Cases consisted of patients diagnosed with OSCCs. Controls were patients diagnosed with benign oral lesions. Information concerning all study subjects were retrieved from hospital records.

Genomic DNA was isolated from the formalin-fixed paraffin embedded tissue (FFPET) specimens and tested for detection of HPV DNA by polymerase chain reaction (PCR).

Pearson Chi-square test for statistical significance (P value), with the 95% confidence level and confidence intervals were used. HPVDNA was detected in 15% of cases (six out of 40 cases), and none of controls (n=15), P <0.0001. Among the six positive cases four were HPV type 18 and the remaining two were type 16.

These results provide evidence supporting causal association between HPV infection and oral SCC in Sudan.

Keywords: HPV, OSCCs, Sudan.

INTRODUCTION

Oral cancer incidence and mortality rates vary widely across the world, and the highest rates are registered in developing countries and more common among males than among females [1]. Incidence rates of oral cancer have been rising in most regions of the world [2].

Many studies have identified tobacco and alcohol consumption, and their combined effect, as the primarily important determinants for the development of oral cancers [3,4].

Other factors have been related to the oral cancer, such as diet low in fruit and vegetable [5], poor oral hygiene [6], and indoor air pollution [7].

The role of Human Papilloma Virus (HPV) in the etiology of cervical is firmly established [8,9]. HPV infection has also been postulated as a potential risk factor for OSCC. Several studies have detected HPV DNA in a considerable proportion of oral cancers, with wide variations from 0% to 100% prevalence in oral tissues, perhaps reflecting the inherent variations in the different populations, [10-12] as well as the detection methods used [13,14].

Oral cancer is tremendously increasing in Sudan, particularly among men [15]. Several studies have linked the etiology of oral cancer in the Sudan to the habit of Toombak use (Tobacco Specific Nitrose amine (TSN)) rich tobacco [16-19]. However, no recent study has investigated the association between HPV and OSCC, although, some prior studies have reported a lack [20] or low association between HPV and OSCC among Sudanese patients [21].

Therefore, the objective of this study was to examine the association between HPV types 16 and 18 and OSCC assessed by detection of HPV DNA in OSCC tissues, as well as, in benign oral lesions.

MATERIALS AND METHODS

A total of 55 patients, 41 males and 14 females (male/female ratio, 2.9:1), aged between 31 and 70 years with mean age of 49 years, 40 were diagnosed as having OSCCs (ascertained as cases) and the remaining 15 with benign oral lesions (ascertained as controls), were investigated for the presence of HPV-16 and -18. The diagnosis was based on clinical examination and histological features of the biopsy. OSCCs were graded as well, moderate and poorly differentiated using Smith and Pindborg criteria [22].

The sample included full coverage of patients with oral lesions referred to our hospital within one-year time.

Ethical consent was obtained from ethical committee of the Faculty Research Board and Hospital.

DNA EXTRACTION

Genomic DNA was isolated from the formalin-fixed paraffin embedded tissue (FFPET) specimens for which adjacent sections were examined by microscopy for assessment of the presence of adequate tumor tissue and the proportion of stromal tissue. All OSCC samples used for

*Address correspondence to this author at the University of Khartoum, 102, Faculty of Medical Laboratory Science, Khartoum, Sudan;
Tel: +249912698998; Fax: +24983775183;
E-mail: hussaingad1972@yahoo.com

DNA extraction showed >60% tumor tissue in each case. Cellular DNA was extracted from each paraffin embedded tissue block using 30 to 50 micron sections. DNA was extracted by DNA extraction kit purchased from *Sacace biotechnologies-Casera–Italy*. The standard protocol of kit was performed with the following modifications [23]. First, the step of xylene/ethanol extraction of the wax was eliminated. Second, the lysis buffer of each kit was added directly to the tissue section in a microcentrifuge tube. Third, the tissue section immersed in lysis buffer was heated to 98°C for 15 minutes before the addition of proteinase K. Briefly, for the HighPure DNA preparation kit, 200 µL of lysis buffer was added to a tissue sample in a 1.5-mL microcentrifuge tube. The microcentrifuge tube was then placed in a heating block at 98°C for 15 minutes and was briefly cooled at room temperature for 5 minutes. Proteinase K solution (40 µL of 20 mg/mL) was added to the heat-treated tissue section. The tissue sample was incubated at 68°C for 45 minutes. Binding buffer (200 µL) was added to the sample, and the sample was incubated for 10 minutes at 72°C. After incubation, 100 µL isopropanol was added to the sample. The sample mixture was transferred to a HighPure Tube Assembly. The genomic DNA was retained on the column and washed twice with 500 µL of wash buffer before eluting with 200 µL of 10 mmol/L Tris-HCl (pH 8.0). For the DNA extraction kit, 180 µL of buffer ATL was added to a tissue sample in a 1.5-mL microcentrifuge tube. The microcentrifuge tube was then placed in a heating block set at 98°C for 15 minutes and briefly cooled at room temperature for 5 minutes. Proteinase K solution (20 µL) was added to the heat-treated tissue section. The tissue sample was incubated at 68°C for 45 minutes. Buffer AL (200 µL) was added to the sample, followed by incubation for 10 minutes at 72°C. After incubation, 200 µL ethanol was added to the sample, and the mixture was transferred to Spin Column. The genomic DNA was retained on the column and washed twice with 500 µL of wash buffer before eluting with 200 µL of 10 mmol/L Tris-HCl (pH 8.0) at room temperature.

POLYMERASE CHAIN REACTION (PCR)

Total cellular DNA (100ng/µL) was amplified by PCR. HPV16 and 18 type specific primers were used for conventional mutiplex PCR. These primers were designed to detect L1 open reading frame of HPV16 and 18. One microlitre (100 ng/µL) of DNA was mixed with 50µL PCR mix (125 mM dNTPs and 0.5 units of Red hot Taq polymerase, 50mM KCl, 10mM Tris HCl pH 8.3, 2mM MgCl2 and 200 mg/ml bovine serum albumin (BSA). The PCR was initiated by hot start at 95°C for 5 minutes, then 30 cycles (denaturation 95°C/40sec; annealing 50°C/60sec and extension 72°C/ 90sec. Then last one step for extension at 72°C for 10 minutes). Ten microlitres of the PCR product was mixed with 2 loading solutions in 2% Agarose gel electrophoresis and run for 60 minutes, then stained by ethidium bromide and photographed by gel documentation system (Gel mega, digital camera and software in a computer). According to the manufacturer HPV16/18 kit (*Sacace technologies- Casera – Italy)* manual, the PCR product length for HPV16 and 18 were 325bp and 425bp respectively.

RESULTS

A total of 40 cases (patients with OSCCs) and 15 controls (patients with benign lesions) were included in the study. The cases included; carcinomas of the oral cavity proper, carcinomas of the lip vermilion. Among cases, the most common cancer site was tongue, with 12 cases (30%), followed by lower lip, palate, lower jaw and floor of the mouth constituting 9 (22.5%), 8 (20%), 6 (15%) and 5 (12.5%) respectively. Among controls (patients with benign oral lesions) the most common conditions are, hemangiomas, inflammation, hyperplasia and leukoplakia representing 6 (40%), 5(33%), 2 (13.5%) and 2 (13.5%), in this order. Table **1** summarizes the distribution of subjects according to demographic characteristics. Most cancer patients were aged 45–65, and men accounted for over 74% of participating subjects, with male female ration of 2.9: 1, as indicated in Fig. (**1**). Only, four cancer patients were identified as tobacco users. HPV was detected among 6 (15%) of the cases, four were HPV type 16 and two were HPV type 18 as shown in Table **2**.

Table 1. Distribution of Study Subjects According to Demographic Characteristics

Variables	Categories	OSCC		Benign Lesions	
		N	%	N	%
Age	<35	3	7.5	6	40
	36-45	10	25	7	46.8
	46-55	11	27.5	1	6.6
	56-65	10	25	1	6.6
	66+	6	15	0	0
	Total	40	100	15	100
Gender	Male	28	70	13	86.7
	Female	12	30	2	13.3
Resident	Khartoum	4	10	4	26.8
	North	18	45	6	40
	East	10	25	1	6.6
	South	6	15	3	20
	West	1	2.5	0	0
	Center	1	2.5	1	6.6
Pathology	Well differentiated	20	50		
	Moderated	13	32.5		
	Poor	7	17.5		

According to the clinical examination and histological features, well, moderate and poor differentiated grades of OSCCs were identified in 50%, 32.5% and 17.5% of the cases, respectively. The great majority of cases were coming from North Sudan (high Toombak consumption), followed by East, South and Khartoum state constituting, 45%, 25, 15 and 10% respectively. Notably, North State population representing approximately, 0.0825% of the total Sudan population, hence, Khartoum State representing about 30%

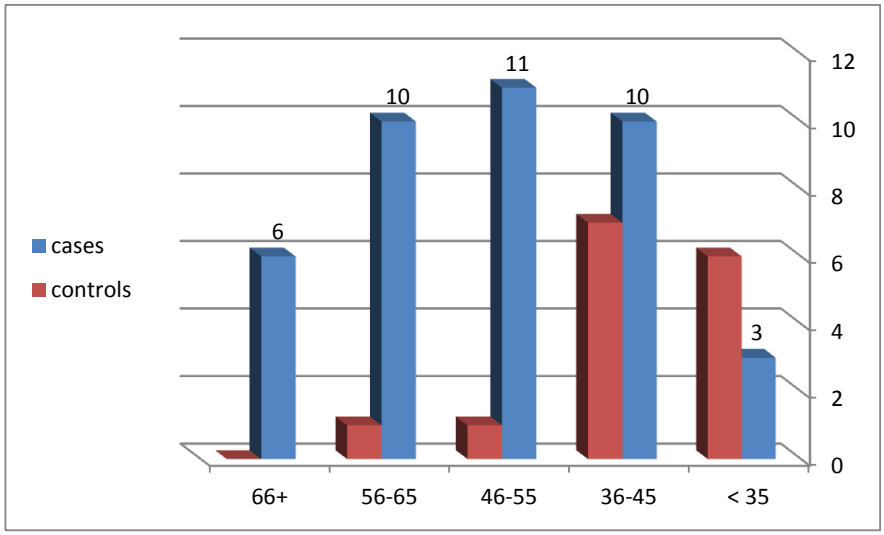

Fig. (1). Description of the cases and controls by age.

Table 2. Distribution of Cases and Controls According to HPV DNA Detection in Oral Tissues

Variables	Categories	HPV Type 16		HPV Type 18	
		N(+ve)	%	N(+ve)	%
Age	<35	0	0	0	0
	36-45	1	50	0	0
	46-55	0	0	2	50
	56-65	1	50	2	50
	66+	0	0	0	0
	Total	2	100	4	100
Gender	Male	2	100	2	50
	Female	0	0	2	50
Resident	Khartoum	0	0	0	0
	North	1	50	3	75
	East	0	0	0	0
	South	1	50	0	0
	West	0	0	1	25
	Center	0	0	0	0
Pathology	Well differentiated	1	50	3	75
	Moderated	1	50	1	25
	Poor	0	0	0	0
Site	Tongue	1	50	2	50
	Flour of the mouth	1	50	1	25
	Lower lip	0	0	1	25

of the total Sudan population, as shown in Fig. (**2**). Reference to the cases with poor, moderate and well differentiated OSCCs, 57%, 30.8%, and 50%, respectively, were from northern State; 43%, and the remaining 43%, 15.3% and 25%, respectively, were from the eastern State; 0%, 53% and 5% were from South States; 0%, 7% and 15% were from Khartoum State;0%, 7% and 0% were from central states; 0%, 0%, and 5%, respectively, were from western States. Approximately, 66.7% of the females presented with poorly or moderately differentiated OSCCs, hence, about 42.5% of the males presented with the same conditions.

DISCUSSION

The epidemiological association of HPV with OSCC, was well established [24,25]. The prevalence of oral carcinomas reported to be associated with HPV has varied widely. This is due to differences both in the population studied and in the sensitivity of the assay used for HPV detection [26,27]. The current study has provided some evidence of an association between HPV and the development of OSCC among Sudanese patients. After controlling for the confounding effect of socio-demographic factors (Table **3**), Toombak dipping, tobacco use, and alcohol drinking we found a strong association between high-risk HPV types 16, 18 and risk of oral cancers, using detection of DNA to HPV 16 and 18 P <0.0001). The first study from the Sudan in this context was done by Ibrahim *et al.* in 1998 [20] when they studied formalin-fixed, paraffin-embedded oral carcinomas from Sudanese snuff dippers (n=14) and oral carcinomas from Sudanese (n=14), by in situ hybridization (ISH) using the cocktail HPV OmniProbe and the ViraType probe and polymerase chain reaction (PCR) using the general HPV primers GP5+/GP6+. All oral carcinomas were negative for HPV DNA with the PCR. Another study in the same year examined the association of oral mucosal lesions from Sudanese (9 hyperplasias/40 dysplasias) with HPV infection. HPV was found in only 2 Sudanese cases, both of which harbored both type 6 and type 11: both these cases demonstrated mild epithelial dysplasia. However, their study concluded that HPV genome is found infrequently in oral lesions from Sudanese Toombak-dippers, suggesting that these viruses may not play a prominent role in the early stages of carcinogenesis in these subjects [21]. These are the only published data from Sudan in relationship between HPV and development of OSCC. Although, many studies have related the occurrence of

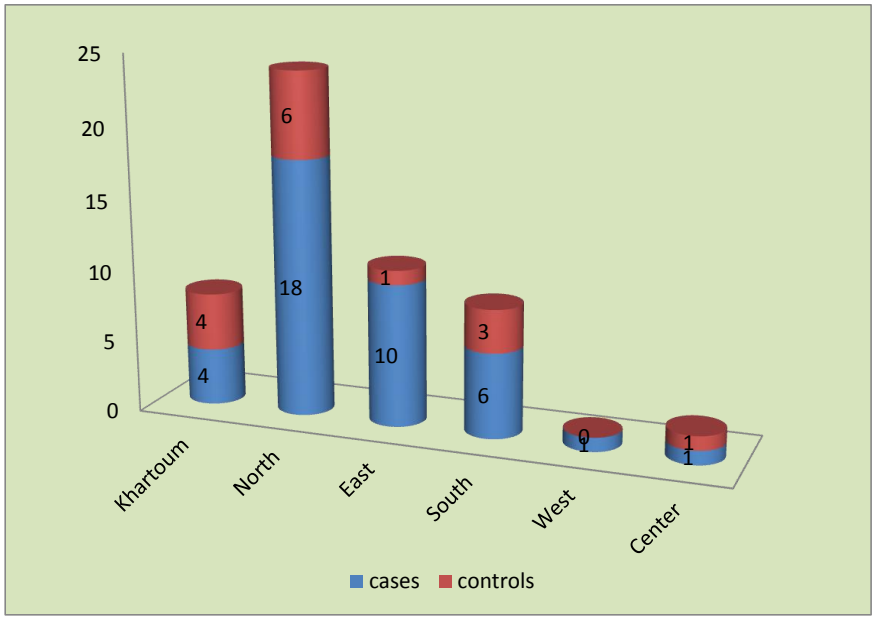

Fig. (2). Description of the study subjects by residency.

OSCCs to the use of Toombak [15-21], this study provides support for the contributing role of HPV infection in etiology of oral cancer in Sudan.

Table 3. Distribution of Study Subjects According to Socio-Demographic Factors Confounders

Variables	Categories	OSCC		Benign Lesions	
		N	%	N	%
Socio-demographic confounders	Toombak use	2	5	0	0
	Smoking	0	0	0	0
	Alcohol use	0	0	0	0

In the current study, the most common cancer site was tongue (30%), followed by lower lip (22.5%). In a recent study, HPV was detected in 36% (13/36) of oral tongue cancer patients, compared with 4% (1/25) of the control. In the HPV-positive group of oral tongue cancers, HPV-16 was the most common type and its prevalence rate was 85% (11/13). Of the HPV-16 infected oral tongue cancers, the integration rate of HPV-16 was 55% (6/11). The HPV-16 positive group showed shallower stromal invasion than the HPV-16 negative group (p=0.045). HPV-16 may be one of the causative factors in early squamous cell oral tongue carcinoma and be associated with its depth of invasion [27]. Although, high levels of lip cancer have been reported [28], most cases of the lower lip carcinomas among Sudanese patients were attributed to the use of Toombak, as the majority of the habitual use to place the dip at the vestibule of the lower lip. Although most of the participants have claimed not to use tobacco, social stigma to announce their smoking status, especially in the context of oral cancer, may have precluded accurate information gathering. A study from the Sudan found that among 62 patients with oral carcinoma 81% had used Toombak, and that the carcinoma arose primarily at or near those sites where the quid was kept [29]. Another study showed that 54.2% of Sudanese patients with OSCCs (Toombak users) were found with lesions at lower lip (at the site of saffa)[15].

Most cancer patients in the present study were aged 45–65, and men accounted for over 74%. Ahmed and Mahgoob [15] found that 86.6% of oral cancers among Sudanese patients occurring in males with mean age of 48 years. There were 2 female OSCC patients positive for HPV 18 (non-tambook users) in support of HPV causality for OSCC. This is because Toombak use is uncommon among females, as it is considered as a social stigma in the Sudan.

Although, northern states are inhibited by the lowest population, the great majority of cases were coming from there. Though, some studies have reported this before, but no one knows the exact causes. Idris *et al.* [30] found a relatively high frequency of oral malignant neoplasms, particularly squamous carcinomas, in men of North Sudan and of the Gaalein tribe, which lives in northern Sudan. Eastern side has shown relatively high occurrence of OSCCs with the absence of HPV infection, but no study from there has explored the possible causes.

As it is shown in the results, many patients came with advance stages of the disease, particularly those from remote states. Patients with squamous-cell carcinomas from the South and West of Sudan even die before being diagnosed or treated, while patients with slowly growing odontogenic and salivary neoplasms may survive. Therefore, neoplasms of odontogenic origin constitute the main pattern of tumors admitted to Radio Isotope Centre Khartoum (RICK) from South and West Sudan. Nonetheless, this pattern of oral neoplasms is probably endogenous in these regions, since similar types of oral neoplasms are observed in neighboring African countries [30,31].

HPVs are known to cause cancers of the cervix. Molecular biology has provided some evidence as to the specific mechanisms involved in the HPV-related carcinogenesis. There are many similarities between oral and cervical

oncogenesis and many of the HPV induced changes in the cervix may also be applicable to the oral mucosa. However, unlike in the cervix, HPV integration into host DNA is not common in oral cancer [32].

The current study provides support for the contributing role of HPV 18 and 16 infection in etiology of oral cancer in Sudan. One of the limitations of the present study is the relatively small number of patients with OSCC, which limited the precision of risk estimates.

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