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Original Article

Screening for high risk human papilloma virus (HR-HPV) subtypes, among Sudanese patients with oral lesions

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Abstract: HR-HPV subtypes are strongly linked to etiology of many human cancers including oral cancer. The epidemiology of infection with different HPV genotypes greatly varies in different countries. The aim of this study was to identify and genotype the HR-HPV subtypes in oral tissues obtained from Sudanese patients with oral lesions. In this retrospective study 200 patients with oral lesions were screened by molecular methods (PCR) for the presence of HR-HPV subtypes. Of the 200 patients, 100/200 were patients with oral cancer (ascertained as case group) and 100/200 were patients with non-neoplastic oral lesions (ascertained as control group). Out of the 200 patients, 12/200 (6%) were found with HR-HPV infection. Of the 12 positive patients, 8/12 (66.7%) were among cases and the remaining 4/12 (33.3%) were among control group. The distribution of different genotypes was: type HPV 16 6/12 (50%), HPV18 4/12 (34%), HPV 31 1/12 (8%) and HPV 33 1/12 (8%). In view of these findings, HPV particularly subtypes 16 and 18 play a role in the etiology of oral cancer in the Sudan.

Keywords: Oral lesions, HR- HPV, PCR, Toombak, smoking

Introduction

Oral cancer is the most common type of cancer worldwide and is particularly in developing countries [1-3]. The incidence of this type of cancer remains high in the Sudan, especially among men due to the habit of Toombak use [4]. The high risk of human papillomaviruses (HPVs) is one of important factors in the genesis of oral carcinoma. Earlier investigator showed that the relationship between HPV and oral cancer development and progression [5, 6]. Infection with the HPV showed a strong association in the development and progression of carcinomas. The cancer associated with HPV contains HPV DNA that is integrated into the host cell genome and thereby responsible for E6 and E7 viral gene expression [7]. The viral gene E6 and E7 shows high ability for the binding and lcause the alteration in the functions of tumor suppressor gene with their oncogenic potential. After all, the oncogenic potentiality of viral genes causes changes in the apoptosis and DNA repair mechanism and causes overexpression of tumour suppressor genes. A few studies made on sudanese patients of detection of HPV 16 and 18 with oral squamous cell carcinoma (OSCC). They found that association between HPV infection and OSCC in Sudan [8]. The aim of this study was to determine the differences subtype of HPV infections in oral lesions, among Sudanese patient using standard polymerase chain reaction method.

Materials and methods

Study population

The Cases were collected from the department of Histopathology of University of Science and Technology and North Khartoum Hospital of Sudan.

Table 1. Sequences of type-specific PCR primers used in this study

HPV-genotype	Sequence (5′-3′)	Amplification (bp)
16	CAC AGT TAT GCA CAG AGC TGC	457
18	CAC TTC ACT GCA AGA CAT AGA	322
31	GAA ATTGCATGA ACT AAGC TCG	263
33	ACT ATA CAC AAC ATT GAA CTA	398

Table 2. Distribution of the study population by site of lesion and gender

Site of lesion	Male	Female	Total
Salivary gland	24	22	46
Buccal mucosa	41	30	71
Submandibular	6	9	15
Tongue	19	8	27
Oropharynx	16	5	21
Jaw	5	10	15
Gingival	1	4	5
Total	112	88	200

The comprised 112 males and 88 females with a median age of 43 years (range 4 to 85 years). Histological diagnoses of neoplastic and pre-neoplastic oral lesions were determined following the criteria proposed in the WHO [9]. The study was approved by the local Ethics Committee of the Sudan University of Science and Technology and North Khartoum Hospital.

DNA extraction

Tissue sections were deparafinized with xylene and rehydrated with different concentrations of ethanol and double distilled water (DDW). Then DNA extraction was performed using DNA Extraction Kit *(Beijing Aide Lai Biotechnology Co., Ltd, China).* The entire extracted DNA was stored at -20°C until PCR.

Polymerase chain reaction (PCR)

Total cellular DNA (100ng/μL) was amplified by PCR. HPV types (16, 18, 31 and 33) with specific primers were used for conventional mutiplex PCR (**Table 1**). These primers were designed to detect E7 and E6 open reading frame of HPV. Amplification was performed according to HPV kit (*Sacace technologies-Casera - Italy)*. Approximately 0.2 μg of extracted DNA was amplified in each 50 μl PCR reaction containing 100 mM of each dNTPs, 1 U of Taq DNA polymerase, 2.5 μl of 10X PCR buffer, 20 pmol of each primer. The reaction mixture was first heated at 94°C for 4 min and amplification was done for 30 cycles using PCR program.

The amplified products were resolved by electrophoresis on the 2% agarose gel and stained with ethidium bromide and visualized on a UV Transilluminator (**Figure 3**).

Results

Total numbers of 100 histopathologically confirmed cases of neoplastic lesions and 100 cases of inflammatory lesions of oral as control were taken. Their ages ranged from 4 to 85 years with a mean age of 43 year. The frequencies of patients with oral cancer were increasing with the increase of age. Hence, those with benign oral lesions, the frequencies of ages of patients were decreasing with the increase of age, as shown in **Figure 1**. The incidence of carcinoma was predominantly seen among males (62%) patients as compared to the (38%) females, giving the ratio was 1.2:1.0 (**Figure 2**). The maximum number of controls was in < 34 years age group followed by 40-55 years. The incidence of benign tumour in both gender were relatively similar with 50 cases each whereas in malignant lesions the incidence were more common among males compared to females. The frequencies of patients with oral cancer were also increasing with the age (**Table 2**). The cases were distributed on the basis of site of lesions. A high frequency of oral lesions was seen among patients in salivary glands lesions

Table 3. Distribution of age, gender and site and types of oral lesion by HPV genotyping

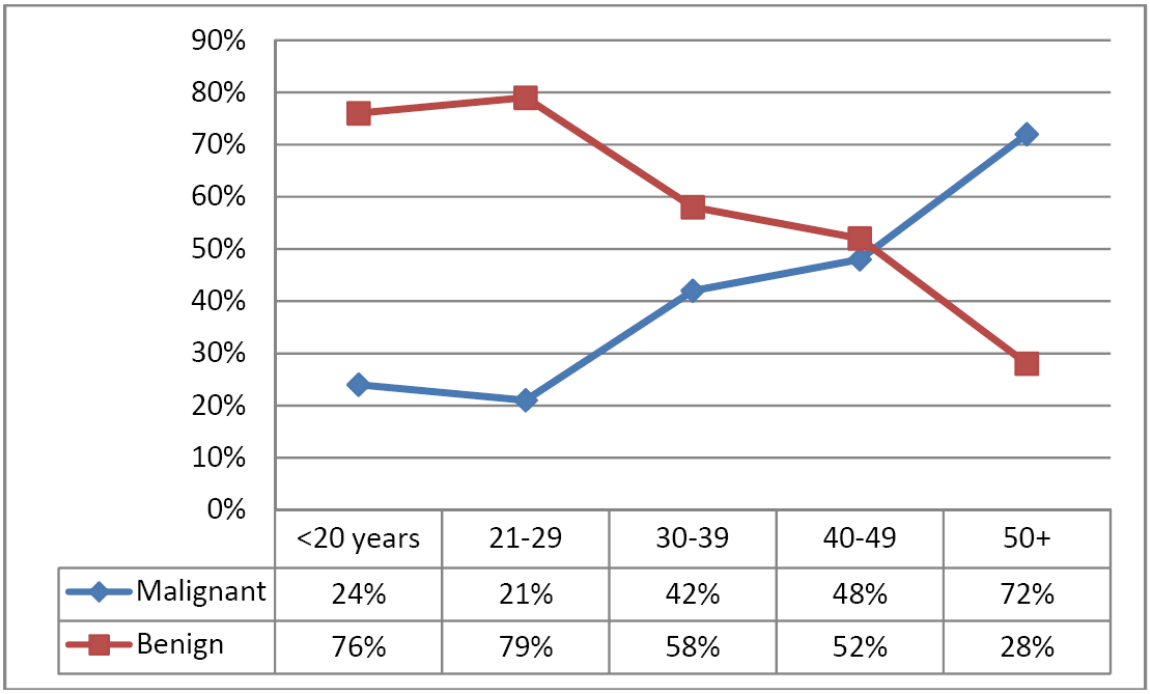
Variable	Category	HPV genotyping				Total
		16.00		31.00	33.00	
Age	< 20 years	0	1	0	0	1
	21-29	0	0	0	0	0
	30-39	1	2	0	0	3
	40-49	3	1	0	1	5
	50+	2	0	1	0	3
Gender	Male	4	2	0	1	7
	Female	2	2	1	0	5
Site of lesion	Salivary gland	0	1	0	0	1
	Buccal mucosa	2	0	0	0	2
	Tongue	2	1	0	0	3
	Oropharynx	1	0	0	1	2
	Jaw	1	1	1	0	3
	Gingiva	0	1	0	0	1
Types of lesion	Squamous cell carcinoma	5	1	1	1	8
	Pleomorphic adenoma	0	1	0	0	1
	Inflammation	1	1	0	0	2
	Reactive hyperplasia	0	1	0	0	1

with 46 (23%) whereas in submandibular with15 (7.5%) when we analyzed the incidence of lesion in study group on the basis of gender. In our study group the incidence of lesions were high in men as compared to women (**Table 2**). The difference of incidence among gender was statically insignificant. The distribution of HPV was categorized on the basis of age, gender, site and types of lesion by HPV genotyping were presented in **Table 3**. HPV genomic materials using A6 and A7 primers were detected in 12/200 (6%) of oral lesions. Out of the 12 HPV; 8/12 (66.7%) HPV were found in malignant lesions, whereas, 4/12 (33.3%) HPV were found in benign lesions. Of these, 6/12 (50%) HPV-16, 4/12 (34%) HPV-18, 1/12 (8%) HPV-31, and 1/12 (8%) HPV-33 (**Figure 3**). Consequently, the risk associated with HPV infection was found to be statistically significant ($P<0.001$).

Discussion

Oral cancer consistently ranks as one of the ten most frequently diagnosed cancers worldwide [10]. The major etiologic factors in the genesis of carcinoma of oral constitute tobacco chewing/smoking, alcohol consumption HPV [11-14]. The exact role for HPV in the pathogenesis of OSCC therefore remains controversial, mainly because the detection rates of HPV DNA varies with 0% to 100%. The study made on Sudanese patients and we found that this type of cancer is the common among all type of cancer. The reason behind this is not well known but might be due to the use of Toombak [15-17]. In the present study, incidence of tumors was high in males as compared to females. The variation of incidence of oral cancer in gender may be due to the variation in the environmental, dietary exposures, innate sexual characteristics, this finding is accordance with earlier investigators and they showed that high incidences of tumors was in men than women [4, 18, 19]. In our study the peak incidence of oral cancer was observed in the age group of 49-60 years. The reasons for this remain unclear, but it might be due to the cumulative effects of long time exposures to carcinogens, the failure of DNA repair mechanisms and aging [20]. Earlier study also showed that the incidence of oral cancer was high in older age people [4, 18, 21].

Tumorigenesis and tumor progression of oral cancer are thought to result from changes in the function of tumour suppressor and apoptotic genes [10, 22]. Earlier investigators showed that HPV plays an important role in the genesis of oral carcinoma through inactivation of tumour suppressor gene. In the present



	<20 years	21-29	30-39	40-49	50+
Malignant	24%	21%	42%	48%	72%
Benign	76%	79%	58%	52%	28%

Figure 1. Description of the study population by age.

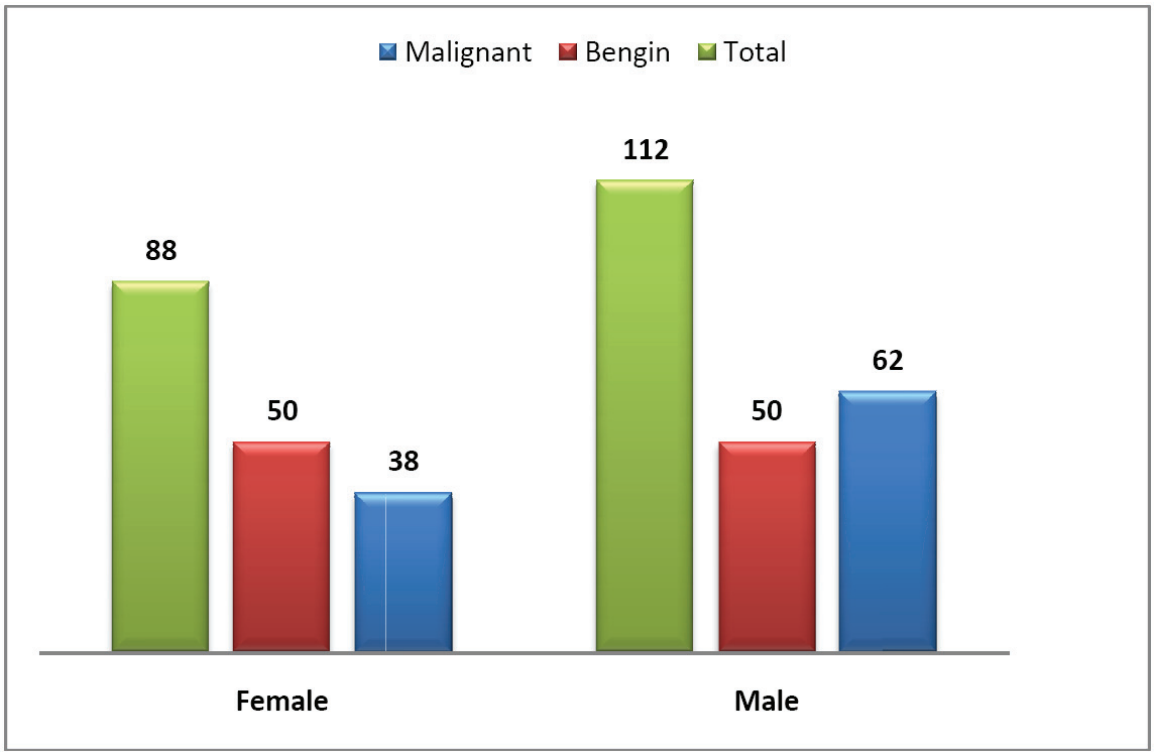


Figure 2. Distribution of the study population by oral lesions and gender.

study, HPV was detected in 12/200 (6%) of oral lesions Out of 12 HPV; 8/12 (66.7%) were found in malignant lesions, whereas, 4/12 (33.3%) were found in benign lesions. This differences

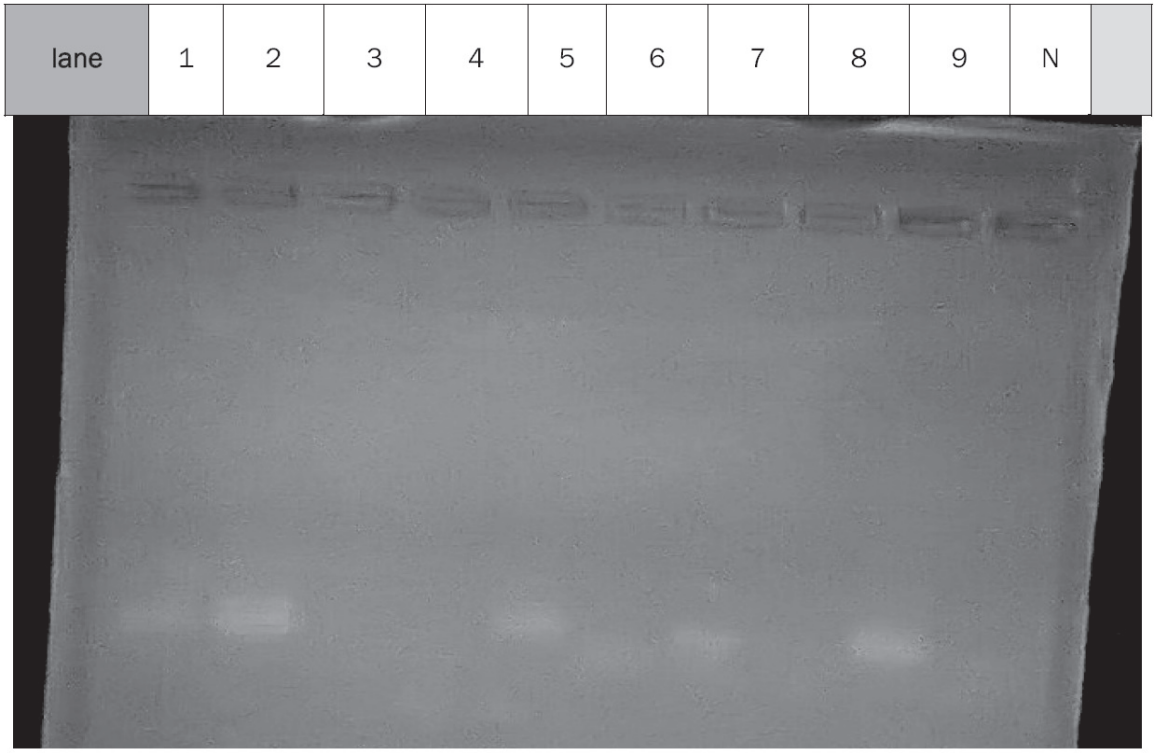


Figure 3. PCR amplification of high risk Oral lesions samples. The products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Lane (N): Negative control, lane 1-, 2, 5, 6, 7.9. positive tumor samples, lane 1, 2, 5 HPV 18 positive tumor samples. Lane 3, 4, 8 negative samples. Lane 6 HPV 33. positive tumor samples. Lane 7, 9 HPV 16 positive tumor samples.

of positivity were found to be statistically significant (P<0.001). Our also findings showed that HPV prevalence increased with lesion severity with 10% in normal oral mucosa, 22% in benign leukoplakia, 26% in intraepithelial neoplasia, 29% in verrucous carcinoma, and 46% in oral squamous cell carcinoma. Another study showed that HPV 16 was the most prevalent type and was found in 89.7% and 95.5% ora-pharyngeal and oral cavity carcinoma cases respectively [23], which was similar to our findings. This little variation of our findings with other investigations, it might be due to the different type of aetiological factors. HPV is now the major cause of oropharyngeal cancer in developed countries with 45-90% of cases as well as developing countries [11, 24, 25].

Our observed showed that the strong association between HPV infections and the pathogenesis of OSCC, this is similar with other findings [8, 26, 27]. Earlier the incidence rate of OSCC was high in the patients of Sudan [28]. The incident rate were also high in the another part of the world like Egypt 6.4%, Ethiopia 11.6%, Tchad 6.7%, Europe is 5.5%, United States is 4.5%, in India 19.9% [28]. However, the exact mechanism behind this variation is not well defined but it might be due to geographic variation and various etiological risk factors.

In this study, the genotyping pattern of HPV subtypes was high in malignant lesions as compared to benign tumors. The incidence of HPV 16, 18 was detected in 6 (50%), 4 (34%) cases respectively. The remaining types of HPV 31 and HPV 33 were found in only one case (8%) for each type. Our findings with 50% incidence of HPV 16 are consistent with earlier report in oral lesions [29]. In this study we found that the incidence rate was increasing according to the grade of the tumour that was statically significant. Another report also showed that HPV 16, 18 and 33 was high in high grade tumor of the head and neck [30]. We are reporting first time about all four type genotype HPV 16, 18, 31 and 33 and its association with oral cancer in Sudanese patients. Earlier report in Sudanese

subjects showed that about HPV 16 and 31 and its vital role in the genesis of this type of cancer [31]. Although, the study from Sudan showed that HPV18 is more prevalent in the OSCCs [8, 18], as our findings and HPV18 was the next most common HPV type (8%) [11, 32]. These findings were in agreement with another study that reported in the premalignant lesion with 55% HPV-16 [25].

In the present study, most of the positive cases were identified in tongue, jaw and oropharynx sites, and most common types were HPV 16 and HPV 18, particularly in the oral lesions. These findings support other studies conducted in different part of the world with HPV in many sites of oral region particularly tongue and oropharynx. However, HPV infection has been found to be strongly associated with oropharyngeal cancer [33-35].

There are clear limitations in our material when investigating the prevalence of HPV. The patients with oral lesions were selected among patients with clinical symptoms and not processed at the same time as normal oral samples and tumor samples, and also we do not have knowledge about the patient's socioeconomic status, nutritional status, previous health history nor family relations. A major limitation of our study is the lack of information regarding alcohol intake and smoking habits.

In summary, these data reinforce the clinical importance of HPV-associated OSCC in the Sudan population. The high prevalence of HPV 16 genotypes in population suggests towards vaccination for HPV genotypes as an important parameter for reducing cancer risk due to HPV infection.

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