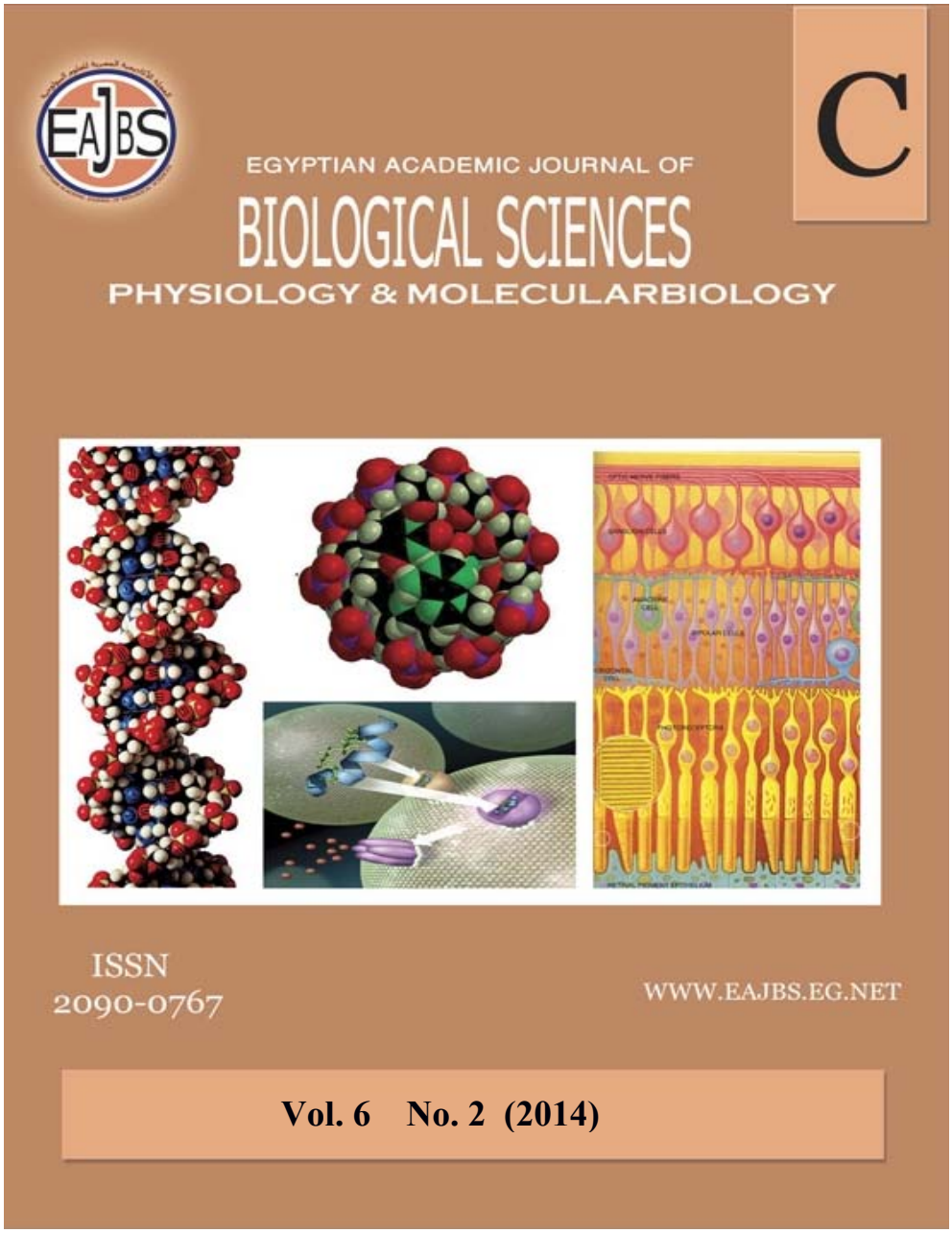

Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences ,Department of Entomology ,Faculty of Sciences Ain Shams University .

Physiology & molecular biology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers that elucidate important biological, chemical, or physical mechanisms of broad physiological significance.

www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 6(2): 31 – 38 (2014)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbs.eg.net

Association between HR-HPV infection and P53 gene mutations among Sudanese Oral Cancer Patients

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ARTICLE INFO

Article History
Received: 2014/4/10
Accepted: 2014/5/21

Keywords:
Oral lesions
Benign and malignant tumors
HPV
p53 mutation and PCR.

ABSTRACT

Objectives: The aim of this study was to determine the association between HR-HPV and p53 gene mutation among Sudanese oral cancer patients.

Methodology: In this retrospective study 200 patients with oral lesions were screened by molecular methods (PCR) for the presence of HR-HPV subtypes and Immunohistochemistery for presence of p53 gene mutation. 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).

Results: Out of the 200 patients, 12/200 (6%) were found with HR-HPV infection. Of the 12 positive patients, 10/12 (83.3%) were among cases and the remaining 2/12 (16.7%) were among control group, HPV16 was the most prevalent type. None of the sample of patients with benign tumor with positive HPV showed p53 gene mutation. From three samples obtained from patients with oral cancer who were positive HPV showed (30%) had mutations in the p53 gene. The chi- square test was shown to have significant differences between the oral cancer with HPV infection and the presence of p53 mutation

Conclusion: HPV is a risk factor for oral cancer, and not always that the incidence of cancer is caused by mutations in gene.

INTRODUCTION

Oral cancer is the most common type of cancer worldwide and is particularly in developing countries (Nelson and Rhodus, 2005; Warnakulasuriya, 2009; Marchioni, 2007). The incidence of this type of cancer remains high in the Sudan, especially among men due to the habit of Toombak use (Ahmed and Mahgoob, 2007). The high risk of human papillomaviruses (HPVs) is one of important factors in the genesis of oral carcinoma (Scully, 2002).

Many studies were shown that an integrated part of the genome corresponding to the E6 and E7. Therefore, E6 and E7 sequences are directly involved in the cellular cycle by inhibiting the normal function of p53 and pRb. Protein 53 kDa (p53) may provoke arrest cell division and guarantee for the repair of DNA. If the damage cannot be repaired, p53 may induce apoptosis and prevent the spread of DNA damage in the next generation of cells. E7 protein interacts with pRb protein that is an important ingredient for the control of cellular cycle. This interaction causes the release of the E2F transcription factor that is now free to act and may stimulate cellular division via c-myc proten. This means that certain types of HPV may cause malignant lesions even without other co-factors actions (Stankovic, *et al*. 2002; Choi and Myers, 2008). The study was undertaken to determine the association of high risk HPV infections and p53 gene mutation in oral lesions, among Sudanese patient using standard polymerase chain reaction method and Immunohistochemistery.

MATERIAL AND METHODS

Study population

One hundred and twelve males and 88 females with a median age of 43 years (range from 14 to 85 years) collected from the department of Histopathology of Sudan University of Science and Technology and Khartoum Hospital of Sudan.

Histological diagnoses of neoplastic and pre-neoplastic oral lesions were determined following the criteria proposed in the WHO (El Naggar, 2005). The study was approved by the local Ethics Committee of the Sudan University of Science and Technology and 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-20ºC 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.

Table1: 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	322
18	CAC TTC ACT GCA AGA CAT AGA	457
31	GAA ATTGCATGA ACT AAGCTCG	263
33	ACT ATA CAC AAC ATT GAA CTA	398

Immunohistochemistery:

Paraffin embedded blocks of oral cancer tissues as well as benign oral tumors were retrieved from histopathology laboratories and cut into (3 µm thick) sections using rotary microtome. The sections were mounted on poly-L-lysine-coated slides and dried in hot air oven at 60°C for 1 hour. The sections were dewaxed in xylene 5 minutes, three times, and

rehydrated through descending grades of ethyl alcohol beginning with 100% ethyl alcohol, then 90% ethanol, 70% ethanol and finally to distilled water, 4 minutes for each change, then the sections were washed 3 times with PBS, three minutes for each. The sections were boiled in the Target Retrieval Solution of Dako (*Real Envision Detection Kit, China*) in a water bath at 95°c for 30 min, then left to cool at room temperature and washed three times with PBS. 0.3% hydrogen peroxide in methanol were added to each section for 15 min to block endogenous peroxidase activity, and then washed three times with PBS. The following antibodies (Abs) were used: primary mouse monoclonal mutent p53 antibody. (Gene tech company limited, Shanghai, China) at a working dilution of 1/100, at 37°C for 30 min; After two washes in PBS, sections were incubated with Chem Mate TM En Vision of + / HRP (*Gene tech company limited, Shanghai, China*), a secondary antibody at room temperature for 30 min, then washed three times in PBS. The immunoreactivity was detected using diaminobenzidine (DAB) (Gene Tech Company limited, Shanghai, China) in a dilution 1/100 as the final chromogen for 10 min, and then washed in DW for 3 min. Finally, sections were counterstained with Mayer's Hematoxylin for 3 min, and washed in running tap water 5min, then dehydrated through a sequence of increasing concentrations of alcoholic solutions and cleared in xylene then mounted with DPX. Mutated P53 was observed only as a nuclear staining of epithelial cells, and the nuclei with clear brown color (Pu, *et al*. 2009).

RESULTS

We analyzed 200 samples of tissue, (100 oral cancer and 100 benign tumors) for the presence of HPV DNA with PCR. and p53gene mutation by Immunohisto-chemistery. HPV genomic materials using E6 and E7 primers were detected in 12/200 (6%) of oral lesions. Out of the 12 HPV; 10/12(83.3%) HPV were found in malignant lesions, whereas, 2/12(16.6%) HPV were found in benign lesions. Of these, 8/12 (66%) HPV-16, 2/12 (16%) HPV-18, 1/12 (8%) HPV-31, and 1/12(8%) HPV-33. Consequently, the risk associated with HPV infection was found to be statistically significant (P<0.001) as show in Table (2).

Table 2: Prevalence of HPV detected by PCR in samples from patients with oral Cancer and with benign tuomor.

Lesion type	Positive (%)	Negative (%)	Total
Malignant	10 (10%)	90 (90%)	100
Benign	2 (2%)	98 (98%)	100

P. value: <0.001

p53 mutations were screened by Immunohistochemistery were observed in 34 of 200 tumors, 28/200 (14%) with oral cancer, and 7/200 (3%) with benign tumor. It was observed that no samples from benign tumor patients with HPV positive for p53 gene mutation. Another samples group obtained from patients with oral cancer observed that 3 of 10 samples (30 %) were found mutation in the p53 gene. The state of the p53 gene did not show any correlation with HPV infection show in Table (3). The frequencies of patients with oral cancer were also increasing with the age (Fig1). The distribution of HPV was categorized on the basis of age, gender, site and types of tumor by HPV genotyping were presented in

Table 3: Occurrence of mutation in the p53 gene in a sample of patients with HPV infection.

Lesion type	*P53 mutation*	*total*
Malignant	*3/10(50%)*	*10*
Benign	*0l2 (0.0%)*	*2*

P. value: >0.05

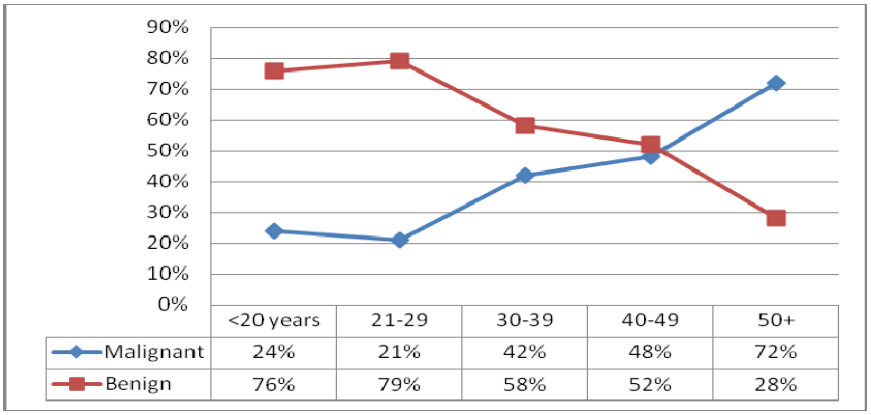

Fig. 1: Description of the study population by age.

Table 4 The description of the tumour site is described in Figure 2. The majority of oral tumours originated from the buccul mucosa and salivery gland. The majority of patients were from Khartoum followed by the western regions, as shown in Figure 3. Two of the HPV-16, one of the HPV-18, and one of the HPV-33

Table.4: Explain distribution of HPV by age, gender and site of lesion.

Variable	***Category***	**HPV genotyping**				**Total**
		16	**18**	**31**	**33**	
Tumor	*Malignant*	6	2	1	1	10
	Benign	2	0	0	0	2
	Total	8	2	1	1	12
Age	*< 20 years*	0	0	0	0	0
	21-29	0	0	0	0	0
	30-39	1	0	0	0	1
	40-49	3	2	0	1	6
	50+	4	0	1	0	5
Site of lesion	*Salivary gland*	0	0	0	0	0
	Buccal mucosa	2	0	0	0	2
	Tongue	4	1	0	0	5
	Oropharynx	1	0	0	1	2
	Jaw	1	1	1	0	3
	Gingiva	0	0	0	0	0

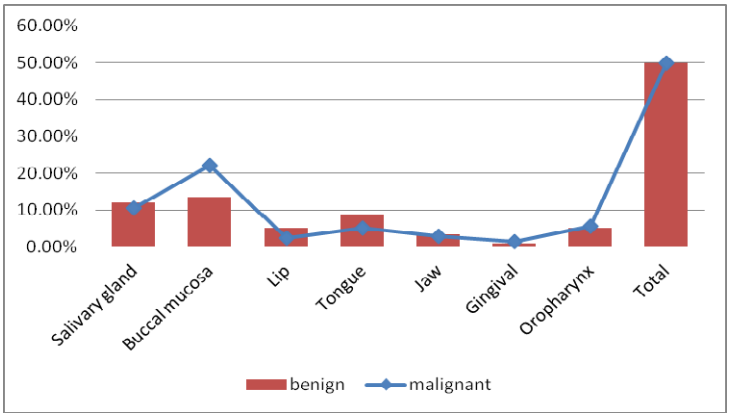

Fig. 2: Description of study subjects by the site of oral tumor.

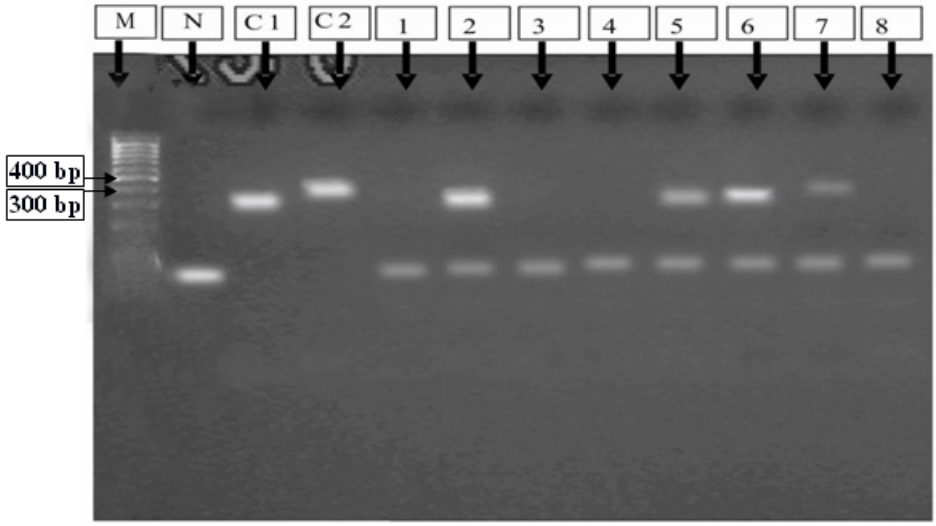

Fig. 3: PCR amplification of high risk Oral lesions samples. The products were electophoresed on 2% agarose gel and stained with ethidium bromide. Lane M:1000bp ladder, (Arrows shows 300 and 400 band), Lane N negative control, Lane C1 positive control for HPV16, Lane C2 positive control for HPV18, lane 2,5,6,7. positive tumor samples, lane 2,5,6 HPV 16 positive tumor samples. Lane 3,4,8 negative samples. Lane 7 HPV 16. positive tumor samples.

DISCUSSION

HPV infection is emerging as an important risk factor for oral cancer. Previous studies have shown that patients with HPV positive tumours actually benefit from a better overall disease-specific survival than patients with HPV-negative tumors (Deng, *et al*. 2012). In this study, we used E6 and E7 as the two key viral oncoproteins that induce and propagate cellular transformation (Wieking, *et al.* 2012). The current study has indicated a significant association between HPV infection and oral cancer in Sudan, and to the best of our knowledge this is the first report in this context from Sudan. In a study investigating the prevalence of HPV, in 55 OSCCs from eight different countries from different ethnic groups, continents, and with different socioeconomic backgrounds, the highest prevalence of HPV was seen in Sudan (65%) (Jalouli, *et al.* 2012). However, there are some studies investigating the relationship between oral cancer and HPV infection. Of these studies, a study found that HPV was in only two Sudanese cases, both of which harboured types 6 and 11: these two cases demonstrated mild epithelial dysplasia (Ibrahim, *et al.* 1998). Another study evaluated the possible role of high risk HPV 16 and 18 in oral squamous cell carcinomas (OSCC), 40 SCCs, and 15 benign lesions, HPVDNA was detected in 15% of cases (6 of 40 cases) and none of

controls (n = 15), P < 0.0001 (Ahmed and Eltoom, 2010).

The p53 gene and its product have been studied extensively ever since it became clear that more than 50% of human cancers contain mutations in this gene (Levine, 1990; Hollstein, *et al.* 1991). In regard to the association between p53 gene mutation and HPV in oral cancer, we resulted no significant association between mutation of p53 and HPV in oral cancer. Similar results were published Wrede, *et al.* (1991), that result are not significant the expression p53 mutation in HPV-positive and cervical carcinoma. another results were published by Koh, *et al.* (1998) when screened 42 cases oral squamous cell carcinomas (SCCs) were analysed for p53 mutations and human papillomavirus (HPV) infection, (38%) of the cases showed positive P53 and negative with HPV.

In the present study, most of the positive samples were identified in Tongue and buccal mucosa sites, and most types identified were HPV16 and HPV18, particularly in the Tongue tissues. HPV infections are commonly identified in the tumor tissues of patients with OSCCs, in which HPV16 and 18 are the most prevalent HPV genotypes (Wei, et al. 2012). Although, the study from Sudan (Ahmed and Eltoom, 2010), showed that HPV18 is more prevalent in the OSCCs than HPV16, but many studies from other countries have revealed the domination of HPV16 in HNSCCs in general and OSCCs in particular (Mineta, *et al* .1998; Oka, *et al*. 1999; . Klussmann, *et al.* 2001; Yamakawa-Kakuta, *et al.* 2009) Most HPV positive cases in the present study were aged 31–40, and men accounted for over 74%. Oral cancer in Sudan is lower among females (Ahmed and Mahgoob, 2009). This is because toombak use (synergistic factor to HPV) is uncommon among females, as it is considered as a social stigma in the Sudan. However, HPV-associated oropharyngeal cancers generally are diagnosed at slightly younger ages in men than in women (CDC, 2012).

There are clear limitations in our material when investigating the association of HPV and p53 gene mutation. 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 and not always that the incidence of cancer is caused by mutations in genes. 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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ARABIC SUMMARY

العلاقة بين فيروس الورم الحليمي البشري العالى الخطورة والطفرة فى الجين بى 53 فى المرضى السودانيين بآفات الفم

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5- قسم تقنية المختبرات الطبية – كلية العلوم الطبية والتطبيقية – حائل - السعودية

الهدف: تحديد العلاقة بين فيروس الورم الحليمي البشري العالى الخطورة والطفرة فى الجين بى 53 فى المرضى السودانيين بآفات الفم

طرق القياس: تم فحص 200 عينة من مرضى بافات الفم (100 مريض بسرطان الفم و 100 مريض بأورام حميدة (مجموعة ضابطة)). بواسطة تفاعل البلمره للكشف عن الفيروس الحليمي البشري و الكيمياء المناعية للأنسجة للكشف عن الطفرة فى الجين بى 53.

النتائج: تم العثور على 12 من 200 مريض بفيروس الورم الحليمى العالى الخطورة.وكان سرطان الفم**10**/12 و الأورام الحميدة 12/2. كان النوع 16 هو الاغلب فى هذه الحالات. لاتوجد طفرة فى الجين بى 53 للمرضى الذين يحملون فيروس المرض الحليمى البشري وهم مصابين باورام الفم الحميدة. وتوجد طفرة فى الجين بى 53 لثلاث عينات تم الحصول عليها من مرضى سرطان الفم ويحملون فيروس الورم الحليمي (30%) وقد لوحظ من خلال هذه الدراسة ليس هنالك علاقة بين فيروس الورم الحليمي البشري العالى الخطورة والطفرة فى الجين بى53

الخلاصة: خلصت الدراسة الى ان فيروس الورم الحليمي البشري هو أحد العوامل المسببة لسرطان الفم، وليس دائما حدوث طفرة فى الجين هو المسبب الرئيسى لجيمع حالات السرطان.