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Association of Human Papillomavirus Type 6 and 11 with Head and Neck Squamous Cell Carcinomas in Sudanese Patients

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Abstract: The aim of this study was to know the relation between HPV type 6 and 11 and the head and neck squamous cell carcinomas in Sudanese patients and to study the histopathology of those neoplasms. About 59 HNSCCs samples, including 28 previously HPV type 16 negative extracted DNA samples and 31 formalin fixed paraffin-embedded tissues were used for conventional polymerase chain reaction to detect HPV type 6 and 11. Statistics Package of Social Sciences (SPSS) was used to analyze the data. In those biopsies, there were 34 male squamous cell carcinomas (58%) and 25 female squamous cell carcinomas (42%). DNA of HPV type 6 and 11 was each detected in about 25% of the examined samples. The DNA samples that were HPV type 16 negative revealed 14 positive samples for HPV type 6 and total absence of HPV type 11. The age category 41-50 years was found to be the most affected age category with HNSCC in both genders. It constituted 27% of HPV type 6 positive samples and 13% of HPV type 11 positive samples. The most frequent site for HNSCC is the nasopharynx that constituted about 64% (38 sample) of the total examined samples, 53% of the HPV type 6 and 27% HPV type 11 positive samples were from that site. Most of positive samples for HPV type 6 were poorly differentiated. Most of positive samples for HPV type 11 were well differentiated. Most of positive samples for HPV type 6 were non-keratinized (60%) whereas 60% of HPV type 11 were keratinized. The relation between HPV type 6 and 11 and the gender age site of SCC, differentiation and keratinisation was found to be insignificant. Further studies are needed investigate the relation between HPV genotypes (HPV type 6, 11 and 16) to probably induce multiple infection required for the development of malignant lesions.

Key words: HPV type 6, HPV type 11, squamous cell carcinomas, male, female, infection, Statistics Package of Social Sciences (SPSS)

INTRODUCTION

Human Papillomavirus (HPV) is an epitheliotropic virus (Montaldo *et al*., 2010) that infects skin or mucosal cells (Anonymous, 2011). The potentially oncogenic HPV is divided into high and low risk types. They were classified according to their affection in epithelial cells and the ability to perform cellular transformation. The high-risk HPV, such as 16, 18, 31, 33, 35, 52, 58, 59, 68, 73 and 82 are responsible for malignancies while the low risk sub types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81) are rarely found in carcinoma (Chaudhary *et al*., 2009).

Many studies had connected the infection with HPV to the aetiology of head and neck cancer in different parts of the world. The term head and neck cancer has been widely adopted in the recent literature and includes lesions at several anatomical sites: Lip, oral cavity, nose and paranasal sinuses, nasopharynx, oropharynx, hypopharynx and larynx (Chaudhary *et al*., 2009).

Head and Neck Squamous Cell Carcinoma (HNSCC) is the 6th most common cancer with an annual incidence of approximately 400,000 worldwide (Chung and Gillison, 2009). It is recorded to be rising in most regions of the world. (Chaudhary *et al*., 2009). Head and neck cancer accounts for 15% of all cancer cases in men worldwide (Parkin *et al*., 1993) and approximately 270.000 additional cases of them occur in women per year (Franceschi *et al*.,1990). In developing countries, upper respiratory and digestive tracts cancers are the most frequent cancer in males and the third most frequent in females (Parkin *et al*., 1993).

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Although, the principal risk factors for head and neck cancer remain tobacco and alcohol use, Human Papillomavirus (HPV) has recently been found to be etiologically associated with 20-25% of HNSCC, mostly in the oropharynx (Chung and Gillison, 2009).

HPV type 6 and 11 are associated with head and neck cancers (Kaya *et al*., 2001; Lee *et al*., 2008). In the Sudan, HPV type 18 and 16 were detected in oral cancer (Jadelkareem and Mergeny, 2010) and upper respiratory and digestive tract cancer (Husain *et al*., 2012). However, no research was done to study the presence of the low risk HPV types in the cancers of Sudanese patients in these sites. Therefore, this study is an attempt to find out (if any) the relation between HPV low risk types (1 and 11) in Sudanese patients with HNSCCs with special reference to HPV type 16 negative HNSCCs.

MATERIALS AND METHODS

In a descriptive cross sectional study, 59 samples were used to study the presence of HPV type 6 and 11 in HNSCCS. These samples included 28 previously extracted DNA samples were known to be HPV type 16 negative and 31 formalin fixed paraffin-embedded tissues of patients previously diagnosed to have HNSCCs. Those 31 samples constituted the samples of all patients presented during year 2010 to Dr. Elmubarak Laboratory, Radiation and Isotopes Centre of Khartoum (RICK) and the National Health Laboratory, Department of Histopathology (Khartoum, Sudan). Tiny samples which were <15 mg were excluded. The 31 paraffin-embedded formalin fixed samples were processed and stained with haematoxylin and eosin stain according to the methods of Bancroft (2008).

A DNA extraction kit (Real Genomics. Genomic DNA extraction kit, Mini) was used to extract the DNA from the tissue samples. Small sections (up to 25 mg) were sliced from the paraffin-embedded tissue and processed as described by the manufacturer.

The methods adopted for the preparation of the PCR mix and the PCR protocol used for the detection of the HPV type 6 and 11 in the ready extracted type 16 negative samples and the newly extracted DNA from tissue samples of head and neck neoplasms were those described by Sacacce company.

First reaction mix was prepared in one new tube (5 μL of PCR-mix-1, 10 of 2.5×buffer-blue and 0.5 of hot start polymerase). After preparing the required quantity of tubes for samples and controls, 15 μL of reaction mix was poured in each tube before adding 10 μL of the appropriate template DNA. About 10 μL of DNA-buffer was added to the tube of negative Control (C-) and 10 μL of positive control (C+) was added to the tube of positive control. PCR-mix-1 tubes were closed and transferred to the thermal cycler. The protocol used for the amplification was that described by Sacacce HPV 6 and 11 PCR kit (Table 1).

Table 1: PCR program used for amplification of HPV type 6 and 11

Steps	Temperature (°C)	Time (min)	Cycle
1	95	Pause	
2	95	15	1
3	95	1	42
	65	1	
	72	1	
4	72	1	1
5	10	Storage	

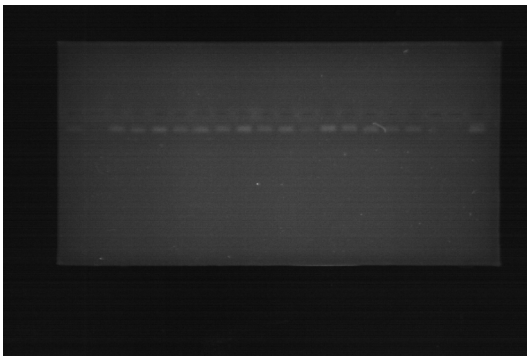

Fig. 1: HPV type 6 positive DNA samples detected under UV light

Agarose gel electrophoresis was used to monitor the DNA. About 10 μL from the coloured template DNA was poured in wells made in the gel using combs. The gel was over laid with a 1×TBE loading buffer. A power source (servo electrophoresis, Gmblt, max power 150 VA heidelberg) was adjusted to give 6 volts/cm of gel for 30-45 min for running the gel and was connected to the electrophoresis-tank. The gel was seen in a UV light emitting machine. Fluorescent bands were said to be positive for presence of the DNA of the tested HPV type (Fig. 1). Statistics Package of Social Sciences (SPSS) was used to analyze the data in the cross tab and Chi squire.

RESULTS

About 59 tissue, biopsies Head and Neck Squamous Cell Carcinomas (HNSCCs) were included in this study. In these biopsies, there were 34 male squamous cell carcinomas (58%) and 25 female squamous cell carcinomas (42 %).

DNA of HPV type 6 and HPV type 11 was each detected in 15 (about 25%) samples out of the 59 examined samples. The examined HNSCC DNA samples which were HPV type 16 negative revealed 14 positive samples for

HPV type 6 and total absence of HPV type 11.

Table 2: HPV type 6 and 11 in the different age categories of Sudanese patients with HNSCC

	Age (years)							
HPV type	11-20	21-30	31-40	41-50	51-60	61-70	71-80	Total
6 +ve	2	2	2	4	2	3	0	15
11 +ve	0	2	2	2	1	6	2	15
Total	2	4	4	6	3	9	2	30

Patient age ranges from 10-80 years were categorized in 7 groups with an interval of 10 years. The number of positive samples for presence of HPV type 6 and 11 is shown in Table 2. The age category 41-50 years was found to be the most affected age category with HNSCC in each gender and in the 2 genders collectively. It constituted about 42% (25 sample) of the total examined samples, 27% of HPV type 6 positive samples and 13% of HPV type 11 positive samples.

The number of HPVC positive cases increases with age till 41-50 age group and then drops in the following age ranges to reach zero in the age group 71-80 fluctuation of the number of type 11 positive cases in the different age groups was detected, it shows a linear phase followed by a drop in age 51-60 and peaks at age 60-70.

In the collected samples of HNSCCs, the following sites were detected: Buccal mucosa, gingival mucosa, tongue, mucosa of lower lip, nose, sinonasal area, pharynx, nasopharynx, oropharynx, larynx, pyriform fossa, post crecoid, oesophagus, palate and tonsillar tissues. The most frequent site for HNSCC is the nasopharynx that constituted about 64% (38 sample) of the total examined samples, 53% of the HPV type 6 and 27%, 11 positive samples were from that site. The nasopharyngeal SCCs tended to increase with age but it was not detected in the age group 70-80 years. The other HPV type 6 positive samples were 2 pharyngeal tissue, 2 nasal polyps, 1 oropharyngeal, 1 post-crecoid tissue and 1 tonsillar tissue. HPV type 11 positive samples were 4 lower lip, 1 buccal tissue, 1 Laryngeal tissue, 1 tongue tissue, 1 sinus mass, 1 sinonasal mass and 2 samples were label only as HSCCs.

Most of the SCC types named according to the site were found to be more frequent in males, this is reflected also in the total number of male and female samples that were collected randomly.

Most of positive samples for HPV type 6 were poorly differentiated, they constituted about 47%. All the moderately differentiated sections were HPV type 6 negative. Most of positive samples for HPV type 11 were well differentiated, they constituted about 53% of the total well differentiated tissues.

The numbers of keratinized and non-keratinized squamous cell carcinomas were almost the same as they constituted 49 and 51%, respectively. They differ within the site and HPV type. Most of positive samples for HPV type 6 were non-keratinized (60%) whereas 60% of HPV type 11were keratinized. The relation between HPV type 6 and 11 and the gender age site of SCC, differentiation and keratinisation was found to be insignificant.

DISCUSSION

This study was set up to know the Association of Human Papillomavirus type 6 and 11 in 28 HPV type 16 negative HNSCCs DNA samples and 31 HNSCCs formalin fixed paraffin-embedded tissue samples collected from Sudanese patients. In this study, the technical classification used in the local hospitals and laboratories for these neoplasms was adopted. This technical classification describes many parts for a main site in the classification of HNSCC in the literature.

Over the past 20 years, there has been an increasing interest in HPV types with regards to their potential role in the pathogenesis of Oral Squamous Cell Carcinoma (OSCC) in the whole world (Syrjanen *et al*., 1983; Trottier and Franco, 2006). This interest has been rose in the Sudan also in the last few years (Jadelkareem and Mergeny, 2010; Husain *et al*., 2012). As the results indicates the relation between HPV type 6 and 11 and HNSCCs is insignificant, similar results for HPV type 16 in upper respiratory and digestive tract SCCs in Sudanese patients were obtained before by Husain *et al*. (2012). Contradictory results were reported by Miller and White (1996) on HPV, they considered it as an independent risk factor for OSCC compared to other known factors, such as alcohol and smoking. This difference may be due to differences in HPV types detected and the detection methods used.

The results together with the previous study of HPV type 16 suggest the probable role of the other risk factors, such as tumbak dipping, tobacco smoking and alcohol drinking in the development of HNSCCs in Sudanese patients. Some of these risk factors were said to increase the risk by 80-fold in the most heavily exposed individuals and the condition is further aggravated by nutritional deficiencies that are all not uncommon in the Sudan (Franceschi *et al*., 1990; Munoz and Castellsague, 1994).

The results substantiate that of Chang *et al*. (1991) who reported presence of HPV 6 in squamous cell carcinoma and those of Chen *et al*. (1994), Kaya *et al*. (2001), Szentirmay *et al*. (2002), Herrero *et al*. (2003) and Gungor *et al*. (2007), who reported presence of HPV type 6 and 11 in epithelial malignant transformation, although they have been classified as

types of low risk.

In this study, the percentage of HPV type 6 and 11 can be considered as high percentage bearing in mind the small number of the samples. HPV type 6 was detected in about 25% of the examined samples which is much near to the results of Herrero *et al.* (2003) who detected it in 29.3% of their examined samples. Other researchers, such as Szentirmay *et al.* (2002) reported comparatively low percentage, they detected it together with type 11 in 1.33% of the specimens. Chen *et al.* (1994), reported higher percentage of HPV type 6 than the percentage detected in the study whereas the results of the same researchers are contradictory to ours in that they do not detect HPV type 11.

As suggested before, contradictory results reported on HPV infection are mainly due to differences in detection methods and the epidemiological characteristics of the observed populations the latter, include the difference in the risk factors which is more clear in the Sudan due to the suspected use of tumbak dipping, tobacco smoking and alcohol drinking (Nielsen *et al.*, 2008; Parkin *et al.*, 2008).

The ratio of affected males and females with HNSCCs HPV type 6 positive was 1.8:1 and 1.5:1 for HPV type 11. For both types 6 and 11 more positive samples were detected in males (60-64%). Similar results were obtained by Marais *et al.* (2006) who observed a different frequency between the two genders with a major presence of HPV-DNA in males compared to females, the researchers recorded also description of similar results in previous studies. Boffeta *et al.* (1992) and Farshadpour *et al.* (2007) recorded HPV as specific risk factor for the development of oral squamous cell carcinoma in association with other important risks, these risk factors include tobacco smoking, tumbak and alcohol which are commonly used by Sudanese males and rarely used by Sudanese females. These risk factors may aggravate the condition, acts as co-factors or work as predisposing factors in Sudanese men. It may also be due to poor oral hygiene which is more common in men (Rosenquist, 2005).

An increased number of HNSCCs with age was observed. It peaks at age 41-50 years and decreases again. This increment is accompanied with an increased positivity of HPV type 6 that peaks at 41-50 years. This can be explained by the fact that malignant process is multifactorial and some of these factors aggravate the condition with time as the immune defences that weakened with age and enter as an influencing factor (Lesourd, 2006).

Most of positive samples for HPV type 6 were poorly differentiated, they constitute about 47% whereas the majority of HPV type 11 (53%) were well differentiated. The degree of differentiation reflects the degree of malignancy and this suggests that HNCCs that harbour HPV type 11 are of better prognosis compared to type 6.

CONCLUSION

Keratinisation within the HPV types showed a marked difference as it appears to dominate in HPV type 11 positive samples whereas in HPV type 6 the non-keratinized are the more frequent. The association between HPV genotypes (HPV type 6, 11 and 16) to probably induce multiple infection required for the development of malignant lesions should be thoroughly investigated.

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