IMMUNOHISTOCHEMICAL SCREENING FOR HUMAN PAPILLOMA VIRUS AMONG SUDANESE PATIENTS WITH NASOPHARYNGEAL CARCINOMA

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ABSTRACT

Background: Nasopharyngeal carcinoma (NPC) is a malignant tumor which arises in surface epithelium of the posterior wall of the nasopharynx. Epstein-Barr virus (EBV) is a well known causative agent for this tumour; recent reports have implicated oncogenic Human Papillomavirus (HPV) in a subgroup of these tumours. The aim of this study was to screen for the presence of HPV in (NPC) in Sudan. Methods and Materials; Paraffin-embedded tumor specimens from 30 patients with NPC were screened for HPV. HPV detection was performed by immunohistochemistry targeting type 6, 11, 16, 18, 31, 33, 42, 51, 52, 56 and 58. Results: Of the 30 cases included in this study, there were 6 (20%) keratinising squamous cell carcinomas (KSCCs), 20 (66.7%) non-keratinising carcinomas, differentiated type (NK-D) and 4(13.3%) non-keratinising carcinomas undifferentiated type (NK-U). HPV was detected in two cases out of thirty (6.6%), this positivity was observed only in non-keratinising carcinomas, differentiated type (NK-D). Conclusion: Our data indicated infection with HPV is rare in NPC, though larger studies are needed to verify these findings.

KEYWORDS: Nasopharyngeal carcinoma, Human papillomavirus.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a tumour that arises in the epithelium surface of the posterior nasopharynx, and shows a peculiar geographic and ethnic distribution. The highest incidence rates of NPC are found among the southern Chinese population and in isolated northern populations such as Eskimos and Greenlanders (30 to 80 cases per 100,000 per year [Parkin et al., 1997]. Intermediate incidence (8to 12 cases per 100,000 per year) was reported in the Mediterranean basin, especially among the Arabic populations of North Africa (7-10% of all cancers among men), where NPC is also the commonest tumour of the ear, nose and throat region [Benider et al., 1995; Chaouki and El Gueddari. 1991]

The World Health Organisation (WHO) has classified NPC according to histological criteria into keratinizing squamous cell carcinoma (KSCC, formerly WHO type I), non-keratinising carcinoma differentiated type (NK-D, formerly WHO type II), non-keratinising carcinoma undifferentiated (NK-U, formerly WHO type III) and basaloid squamous cell carcinoma (BSCC) [Barnes. 2005]. The etiology of NPC is multifactorial, including environmental factors, genetics, and infectious agents, particularly the Epstein- Barr virus (EBV). [Chang and Adami. 2006; Wei and Sham. 2005]. Epstein-Barr virus

(EBV), a ubiquitous human herpesvirus, is recognized as a primary etiologic agent in non-keratinizing NPC (WHO type II/III). In contrast, keratinizing carcinomas (WHO type I) lack a consistent association with EBV suggesting differences in the pathogenesis of NPC [Niedobite et al., 1991; Nicholls et al., 1997]. Additional established risk factors for NPC in endemic areas include consumption of salt-preserved fish, tobacco exposure, and certain human leukocyte antigen class I genotypes [Chang and Adami. 2006]. In non-endemic regions, tobacco exposure has been associated with type I NPC but not type II or III NPC [Vaughan et al., 1996]. High-risk human papillomavirus (HPV), particularly oncogenic HPV subtype 16, has recently been established as the primary etiologic agent in a subset of head and neck squamous cell carcinomas, the majority of which localize to the oropharynx and exhibit improved prognosis compared to patients with HPV-negative tumors [Gillison et al., 200; Ang et al., 2010). Given the similarities between the epithelium and lymphoid tissue of the oropharynx and nasopharynx, there has been recent interest in the potential role of HPV in NPC carcinogenesis. Reports, however, have been inconsistent likely due to limited patient numbers and ethnic and geographic differences in study populations. Several studies have detected HPV in NPC with some

demonstrating a dichotomy between EBV and HPV infection [Lo et al., 2010; Robinson et al., 2013] and others reporting cases of EBV and HPV co-infection predominantly in patients from endemic regions [Laantri et al., 2011; Tyan et al., 1993]

MATERIAL AND METHODS
The analyses were performed on formalin fixed paraffin embedded tissue from30 patients with histopathologically confirmed NPC collected from different histopathology laboratories in Khartoum State, Sudan. The geographical and histoclinical data were achieved from the patients files , The malignant epithelial tumors were classified according to WHO into type 1 – keratinizing squamous cell carcinomas,(K.S.S.C), type 2 – non-keratinising carcinoma differentiated type (NK-D) and type 3 – non-keratinising carcinoma undifferentiated (NK-U). Samples were used to investigate the positive rate of HPV; Immunostaining was performed according dextral polymer method, using monoclonal antibodies (HPV-Ab-3 -Thermo Fisher) against HPV types (6, 11, 16, 18, 31, 33, 42, 51, 52, 56 and 58.). The data was analyzed, using the statistical programs software Statistical Package for the Social Sciences (SPSS) version (11.5), Chi square test and different statistical measures were calculated.

The Immunochistochemical procedure was done as follows
One section (3μm) from formalin-fixed, paraffin-embedded tumors was cut and mounted onto salinized slides (Thermo). Following deparaffinization in xylene, slides was rehydrated through a graded series of alcohol and was placed in distilled water. Samples were steamed for antigen retrieval for HPV using high PH (9) by water bath at 95c. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and methanol for 10 min, and then Slides was incubated with 100 μl of primary antibodies for 20 min at room temperature in a moisture chamber. After washing with PBS for 3 min, binding of antibodies was be detected by incubating for 20 minutes with dextran labeled polymer ((Thermo -ultra vision). Finally, the sections washed in three changes of PBS, followed by adding 3, 3 diaminobenzidine tetra hydrochloride (DAB) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex for up to 5 min. Slides was counterstained with haematoxylin.. Each slide was evaluated with investigator then the results were confirmed by consultant histopathologist.

Statistical analysis
The results were analyzed statistically by the chi2 test. The level of significance was set at 95% (a = 0.05) for all tests.

RESULTS
The total number of the obtained tissue blocks was 30 blocks with all their records studied and analyzed. Age

of patients ranged between 15 and 80 years with median age of 47.5 years Table 1. Patients were 23 (76.7%) males and 7 (23.3%) females; the male to female ratio was 3.3: 1 as illustrated in table 2

HISTOLOGICAL SUBTYPE
Of the 30 cases included in this study, there were 6 (20%) keratinising squamous cell carcinomas (KSCCs), 20 (66.7%) non-keratinising carcinomas, differentiated type (NK-D), 4(13.3%) non-keratinising carcinomas, undifferentiated type (NK-U) as shown in (Figure 1). There is no statistically significant correlation between NPC subtype and sex group as in table 4.

HPV status
HPV was detected in two cases out of thirty (6.6%), this positivity was observed only in non-keratinising carcinomas, differentiated type (NK-D)

Table 1: Distribution of the study population by age

Age Group	No of Patients	Frequency
10-20	2	6.7%
21-30	2	6.7%
31-40	3	10%
41-50	14	46.7%
51-60	4	13.3%
61-70	4	13.3%
71-80	1	3.3%

Table 2: Distribution of the study population by sex

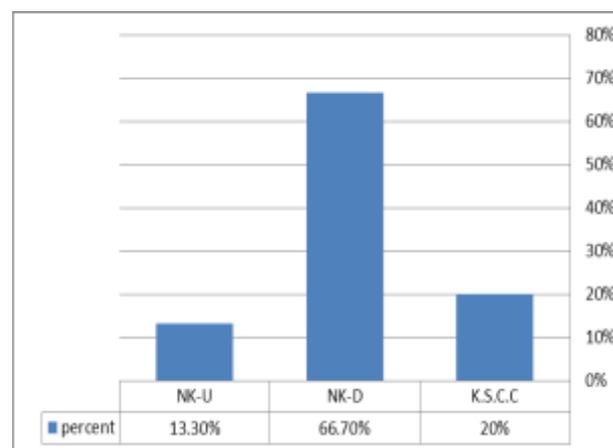| Gender	Frequency	Percent
Male	23	76.7%
Female	7	23.3%
Total	30	100

Figure 1: the frequency of NPCs histological subtypes

Table 4: the correlation between NPC subtype and sex

Histological subtype	Gender		Total	P value
	Male	Female		
K.S.C.C	6	0	6	
NK-D	14	6	20	0.345
NK-U	3	1	4	
Total	23	7	30	

DISCUSSION

It is widely accepted that EBV is etiologically associated with NPC; but it is proven that other co-factors might be involved in the carcinogenesis process. HPVs are considered to be one of those factors since they possess the ability to transform epithelial cells and a significant number of NPC biopsies harbour HPV DNA [De Villiers et al., 2005; Kreimer et al., 2005]. However, while the biological behaviour of HPV-related oropharyngeal carcinoma is now becoming increasingly established, little is known about the clinical significance of this virus in NPC. This study therefore aimed to screen the presence of HPV in NPC.

Our data may not reflect the situation in the general population, especially as we worked on a size-reduced series (30 cases), but it will have a value   in keeping with those previously reported studies, median age and sex distribution of NPC were not different from what was reported in the literature; most of patients were men over 40 years (Vokes et al., 1993). Males were more affected by nasopharyngeal cancer than females ((67.7% versus 23.3% respectively) with a sex ratio of 3.3:1. These findings were in agreement with a previous Sudanese published study by Ameera, et al, their results showed that; "in the gender frequency of patients with NPC, (79.1% males versus 20.9% female). [Ameera et al., 2014]

We report here that only two cases were immunostain positive for HPV 6.6% (2/30) from Sudanese NPC biopsies, which reported in WHO type 2 – non-keratinising carcinoma differentiated type (NK-D). However, the number of HPV (+) NPC in our series was too small to draw any definitive Conclusions. The reported incidence of HPV in NPC ranges from 9–52.9% [Rassekh et al., 1998; Mirzamani et al., 2006; Lo et al., 2010; Singhi et al., 2012]. Although the lack of consensus on the relationship between HPV and NPC may be due to the small numbers of patients in the studies published to date, geographic variations, and racial differences between study populations and non-standardized viral detection methods. In Tyan et al's study of 30 Asian patients with WHO-II/III NPC, 46% of these tumors were positive for high-risk HPV, and consistently these tumors were also positive for EBV.[Tyan et al., 1993] In Mirzamani et al's study of 20 Iranian patients with WHO-II/III NPC whose tumor samples were studied using in situ hybridization, 95% were EBV positive and 10% were also positive for high-risk HPV.[Mirzamani et al.,2006] Hørding et al looked at 38 Danish and Inuit patients and found that 3 (20%) of 15 WHO-I NPC were high-risk HPV positive and EBV negative while all 23 WHO-II/III NPC were HPV negative and EBV positive.[Hørding et al., 1994). Giannoudis et al's study of 63 Greek patients showed that 9 (33%) of 27 WHO-I NPC but only 3 (8%) of 36 WHO-II/III NPC were high-risk HPV positive, and no HPV positive tumors were also positive for EBV.[Giannoudis et al., 1995). Rassekh et al found that high-risk HPV was present only in association with EBV and was present in 3 (30%) of 10 WHO-I NPC and 4 (57%) of 7 WHO-III NPC.[Rassekh et al., 1998] A study of a mixed Caucasian and Asian population by Punwaney et al. showed that 2 (50%) of 4 WHO-I NPC and 5 (19%) of 26 WHO-II/III NPC were high-risk HPV positive; co-presence of EBV was found in two Caucasians with WHO-II/III.[Punwaney et al., 1999] Finally, in a recent publication, Maxwell et al reported one Asian patient with WHO-II/III NPC who was high-risk HPV negative and EBV positive and four Caucasian patients with WHO-II/III NPC who were high-risk HPV positive and EBV negative[Maxwell et al., 2009]. Compare with this summary of the literature, In our study the positive rate of HPV is less than reported in the above mentioned studies , this may be due to the small sample size or the less sensitive applied immunostain technique compare with PCR or ISH techniques.

CONCLUSIONS

Our study found that HPV are rare in Sudanese patients with NPC Further studies with larger number of specimens are recommended.

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