

## RESEARCH ARTICLE

# Detection of High-Risk Human Papillomavirus Genotypes 16 and 18 in Head and Neck Squamous Cell Carcinomas in Jordan

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### Abstract

**Background:** Recently, associations of the human papillomavirus (HPV) with head and neck cancer have become well established. Of particular concern, the severity and pathological outcomes of squamous cell carcinomas are remarkably affected by the genotypes of HPV present in such lesions. This study was conducted to investigate the occurrence of HPV genotypes, particularly high risk 16 and 18, among oral and laryngeal squamous cell carcinomas in Jordan. **Methods:** During the period of May 2015 to March 2016, we evaluated a total of 108 paraffin-embedded tissue samples, histologically confirmed as SCC, of both oral and laryngeal tumors for the presence of HPV DNA. DNA was extracted using a Zymogen commercial kit. HPV genotypes were detected by nested PCR using consensus primers followed by primer-specific PCR for HPV-16 and HPV-18 genotypes. The genotypes were confirmed by DNA sequencing methods. **Results:** Sixteen samples were positive for HPV DNA (14.8%) with higher rates in oral tumors compared to their laryngeal counterparts (20% and 6% respectively). The HPV-16 genotype predominated, being detected in 81.3% of the cases as a single infection and in 18.7% in combination with HPV-18. A significant association between the anatomical location and the HPV-16 genotype was observed ( $p < 0.05$ ). In contrast, no significant associations could be established with tumor grade and gender or age. **Conclusions:** A relatively high rate of high-risk HPV genotypes, especially HPV 16, is evident in head and neck cancers SCCs in Jordan. Genotyping of HPV might be of considerable value for evaluation of progression.

**Keywords:** Human papillomavirus (HPV)- squamous cell carcinoma (SCC)- genotyping

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### Introduction

Head and neck squamous cell carcinomas (HNSCC) has been globally considered one of the most increasing lesions with an estimated 620,000 new cases diagnosed annually (Jemal et al., 2011; De Martel et al., 2012). In general, the vast majority of these lesions occur in the oral cavity of the patients with high mortality rates (Jemal et al., 2011). A part from the genetic predisposition of the patients to HNSCC, several epidemiological risk factors for SCC have been identified including tobacco use and heavy alcohol consumption; however, the presence of human papillomavirus (HPV) infection has been shown to be an integral risk factor which usually complicates the situation (Bettendorf et al., 2004; Hammarstedt et al., 2006; Nasman et al., 2009). In several western countries, HPV was found in approximately 45% to 95% of oropharyngeal SCCs despite the decreasing frequency of tobacco-related cancers (Kupferman et al., 2006; Ernster et al., 2007; Chenevert et al., 2012). Interestingly, different HPV genotypes have been detected in oropharyngeal and laryngeal SCCs. HPV 16, 18, 31, 33, and 35 are considered

high risk genotypes that are associated with different pathological conditions not restricted to HNSCC (Kreimer et al., 2005). Two major oncoproteins, E6 and E7, are produced by the high-risk HPV genotypes facilitating viral replication and malignant transformation through degradation of P53 and retinoblastoma protein, which results in upregulation of the p16 protein (Lindel et al., 2001). Recent studies revealed that HPV-16 is the most common genotype identified in HPV-positive cancers especially in cervical cancers. Nevertheless, depending on geographical variation, age groups of patients and life styles, the prevalence of HPV genotypes in various communities is variable. In addition, the rates of HPV genotypes in several head and neck tumors are affected by the different detection methods which have varying sensitivities (Schmitt et al., 2011; Al Bdour et al., 2013).

To date, the presence of HPV in HNSCC is still not fully investigated in Jordan. This study is conducted to detect the rates of high-risk HPV genotypes particularly HPV 16 and HPV 18 in defined cases of HNSCC using a simple PCR-based detection and DNA sequencing methods which potentially could have important

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implications in cancer prevention and control.

## Materials and Methods

### Patients and Samples

A total of 108 Formalin-fixed, paraffin-embedded (FFPE) HNSCC tumor samples, collected during May 2015- March 2016, were kindly provided from Alpha medical laboratories, Amman, Jordan. Each tumor sample was processed to obtain 5 µm thick paraffin sections and routinely stained with H&E for histological diagnosis performed by pathology specialist of the same laboratory. The assessment of the differentiation grade was made according to Wiernik et al (1991). Epidemiological data including age and sex of patients was collected from the laboratory history records.

### DNA extraction

Approximately 8–10 sections of 5 µm each were placed in tubes containing xylene in order to remove the paraffin and subsequently washed three times with absolute, 80%, and 70% ethanol to remove xylene. The deparaffinized tissue samples were then treated with Proteinase K and allowed to complete digestion by incubated overnight. DNA purification was performed using Zymo-spin columns (Zymo Research, USA) following the manufacturer instructions. The samples were stored at 4°C. Concentration, purity and integrity of the isolated DNA were determined by measuring the OD260/OD280 ratio using UV spectrophotometry (Biorad, Germany) and by 1% agarose gel electrophoresis stained with ethidium bromide.

### PCR detection of HPV genotypes

HPV DNA was detected in the tumor samples by conventional PCR described by Hwang (1999). Consensus primers (Table 1) specific to the E6-E7 genes of the HPV virus were used. PCR reaction mixtures of 50-µl were prepared by adding 2 µl DNA to 0.4 µM each primer and 25 µl of 2× PCR Master Mix (New England Biolab, UK). Initial denaturation was performed at 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 90 s, with final extension at 72°C for 7 min. PCR products were assessed by 1% agarose gel electrophoresis with ethidium bromide staining. Presence of a 250-bp fragment was considered a positive result for HPV. Viral typing was performed for positive specimens using new sets of HPV-16 and HPV- 18 specific primers. The reaction mixtures and cycling conditions were similar to those described above. The amplified products were predicted to yield a 238 bp and 268 bp bands for HPV-16 and HPV-18 respectively. All samples were tested in triplicate for reproducibility.

### Confirmation of HPV genotypes by DNA sequencing

For DNA sequencing, 1 µL of the PCR products generated from HPV consensus PCR reaction was used for direct DNA sequencing. 1 µM of the sequencing primer (genotype specific primers) were mixed with 1 µL of the BigDye® Terminator (v 1.1/Sequencing Standard Kit), 3.5 µL 5× buffer, and 13.5 µL water in a total volume of 20

µL for 20 enzymatic primer extension/termination reaction cycles according to the instructions of the manufacturer (Applied Biosystems). After dye-terminator cleanup with Dye Ex 2.0- Spin columns (Qiagen, Germany), the reaction mixture was loaded in an automated ABI 310 Genetic Analyzer for sequence analysis. Sequence alignments were performed against various standard HPV genotype sequences stored in the GenBank database by on-line BLAST analysis. All of the samples were tested in duplicates. Controls for sample adequacy were included in the sequencing kit and were used for each run. Internal control for PCR polymerase inhibitors was used by amplification of human B globin gene (Lee et al., 2007).

### Statistical analysis

Data was analyzed using SPSS v. 20 (SPSS, Chicago IL, USA). Chi square test was used and p-values of <0.05 were considered statistically significant.

## Results

A total of 108 HNSCC tumor samples presented with primary lesions of the oral (60.2 %) and laryngeal (39.8 %) cavities were included in this study. The mean age of patients was 58.2 years (range 46 - 78); 71.3% of the patients were males however, no information regarding tobacco use and alcohol consumption was available. Regarding the HNSCC stage; 50 % of the tumors were well-differentiated, 37% moderately differentiated, and 13% were poorly differentiated SCCs. HPV DNA was detected in 16/108 (14.8%) of the HNSCC cases tested

Table 1. Primers Used in This Study

Primer type	PCR Product size
Consensus primers	
<i>HPV CF</i> 5'-TGTCAAAAACCGTTGTGTCC-3'	250 bp
<i>HPV CR</i> 5'-GAGCTGTCGGTTAATTGCTC-3'	
Genotype 16 specific primers	
<i>HPV 16F</i> 5'-TGTCAAAAGCCACTGTGTCC-3'	238 bp
<i>HPV 16R</i> 5'-GAGCTGTCAATTAATTGCTC-3'	
Genotype 18 specific primers	
<i>HPV 18F</i> 5'-TGCCAGAAACCGTTGAATCC-3'	268 bp
<i>HPV 18R</i> 5'-TCTGAGTCGCTTAATTGCTC-3'	
Genotype specific sequencing primers	
<i>16-Seq</i> 5'-GCTGCCATATCTACTTCAGA-3'	
<i>18-Seq</i> 5'-GCTTCTACACAGTCTCCTGT-3'	NA
Beta globin primers (Internal Control)	
<i>B-GL F</i> 5'-ACACAACCTGTGTTCACTAGC-3'	
<i>B-GL R</i> 5'-CAACTTCATCCACGTTCCACC-3'	

NA, not applicable

Table 2. Distribution of HPV-Positivity by Tumor Grade, Sex, and Anatomical Location of Tumors

Characteristics	Total		Positive HPV		HPV-16		HPV-18	
	N	%	N	%	N	%	N	%
Anatomical location								
Oral	65	60.2	14	21.5	14	100	2	14.3
Laryngeal	43	39.8	2	4.7	2	100	1	50.0
P-value			< 0.05		NS		NS	
Gender								
Male	77	71.3	10	13.0	10	100	2	20.0
Female	31	28.7	6	19.4	6	100	1	16.7
P-value			NS		NS		NS	
Tumor Grade								
Well differentiation	54	50.0	9	16.7	9	100	2	22.0
Moderate differentiation	40	37.0	5	12.5	5	100	0	0.0
Poor differentiation	14	13.0	2	14.3	2	100	1	20.0
P-value			NS		NS		NS	

NS, not significant where p value was greater than 0.05.

in this study. Of the positive cases for HPV, HPV-16 genotype was detected as a single infection in 13 (81.3%) and in combination with HPV-18 genotype in 3 (18.7%) of tumor specimens. In regards to anatomical location of the tumors, the oral cavity reported the highest rate of HPV infection with 21.5% compared to the laryngeal cases which reported 4.6% ( $p < 0.05$ ). HPV-16 genotype was also the most frequent genotype in both types of tumor lesions. HPV-18 was detected in 2 samples of the oral lesions (14.3%) and in one laryngeal tumor sample (50%).

HPV-16 genotype was found in 16.7%, 12.8% and 11.1% of well-differentiated, moderately differentiated and poorly differentiated cases of SCC. No association was found between tumor grade and positivity for HPV-16 ( $p > 0.05$ ). HPV-18 genotype was not detected in moderately differentiated tumors while it was detected in 22% and 50% of well differentiated and poorly differentiated tumors respectively (Table 2). The risk of testing positive for HPV-16 increases in women with rates of 19.4% compared to 13% among males, however, no significant difference could be deduced in relation to gender.

## Discussion

Recent epidemiologic evidence postulates different oncogenesis of HNSCC based on HPV infection in addition to the increased risk due to alcohol consumption and tobacco use (De Petrini et al., 2006).

The present study was mainly conducted for HPV identification in HNSCC samples, using archived formalin fixed parafrin embedded specimens which, although the DNA might not be conserved similarly to fresh frozen specimens, are the most commonly used specimens for routine histopathology in cancer patients. HPV infection was reported in 14.8% of HNSCC cases included in this study which is in agreement with results from other regions (Badaracco et al., 1999). Findings obtained from PCR-based regional studies in Asia and

Africa showed similar findings (McKaig et al., 1998; Li et al., 2011). In contrast, higher prevalence was found in North America and Europe (Van den Brule et al., 1989). A higher prevalence was reported when advanced nested and real-time PCR techniques, which are much more sensitive than the conventional PCR protocol described here, were applied in the investigation (Sotlar et al., 2004; Guily et al., 2011). In addition, DNA hybridization methods and microarray based studies provided higher rates for HPV infection in HNSCC (Didelot-Rousseau et al., 2006).

Despite the high incidence of high-risk HPV genotypes, especially genotypes 16 and 18, reported in several regional studies at all the anatomical locations, the role of HPVs in the etiology of head and neck cancers is still controversial. In this study, HPV genotype 16 was predominant in positive cases of HPV infections. HPV genotype 16 was observed either as a single infection in most of the cases or with HPV genotype 18 in few cases (18.7%). This is in agreement with results from Major et al., (2005) in which, different HPV DNA profiles were observed in cancer cases with regards to the prevalence, physical state, genotypes, and copy number of HPV DNA.

Interestingly, higher rate of HPV genotype 16 infection was observed in OSCC compared to laryngeal tumors ( $p < 0.05$ ). Based on these results, it could be suggested that infection with HPV genotype 16 is a major risk factor in OSCC. however, due to the limited information regarding some risk factors such as tobacco and alcohol use or sexual behavior, it was difficult to establish such a statistical association with HPV genotype-16.

It has been reported that frequency of HPV DNA in laryngeal sites varies between 3% and 85% (Venuti et al., 2000; Vietía et al., 2014). Although the laryngeal epithelium is commonly colonized by HPV as confirmed by IHC staining demonstrating the expression of HPV structural proteins, the relationship of HPV with squamous carcinoma in the larynx is not fully well established (Syrjänen et al., 2005). In a study conducted in 2005, approximately one quarter of tested laryngeal

tumours and 19% of the controls the reported incidence of HPV infection in laryngeal SCC (Torrente et al., 2011). In France, 5% of larynx cancers in different public and private hospitals in France was reported however, higher prevalence of HPV among laryngeal tumors was observed in studies included higher numbers of patients (Kreimer et al., 2005). This is in agreement with our study which report low prevalence of HPV in laryngeal tumors 4.6%.

Some studies showed higher prevalence of other HPV genotypes like HPV-6 DNA in HNSCC cases although in low copy numbers (Vietia et al., 2005). Compared to a study conducted by Ringstrom et al. (2002) which reported higher rates of HPV-positive tumors among younger patients, our study could not establish an association between HPV and age of patients. Furthermore, no significant association between patient's sex and HPV-associated tumors was observed in this study. Previous international reports showed that men were more affected (Miller et al., 2001; Shiboski et al., 2005) while others have suggested OSCC is more frequent in women probably because women's exposure to known oral carcinogens such as tobacco and alcohol has increased (Gillison et al., 2000). Some researchers have found no association between HPV and gender (Torrente et al., 2011).

A higher percentage of HPV 16-positive cases was observed in SCC cases presented well differentiation however, no association between HPV genotype 16 and histopathological tumor grade could be established in this study which is in consistence with other studies (Miller et al., 2001; Shiboski et al., 2005). In contrast, well-differentiated OSCCs were more strongly associated with HPV genotype 16 as observed in other studies. In addition, some studies showed a high frequency of poorly differentiated OSCC with HPV-16 genotype (Miller et al., 1996; Elango et al., 2011). Increasing the number of HPV-positive cases of poorly differentiated OSCC would be required to properly investigate the role of HPV-16 genotype in such tumor lesions.

To the best of our knowledge, this is the first study to investigate the epidemiology of HPV genotypes colonization and risk factors for HNSCC in Jordan using advanced molecular techniques. Future work is needed to elucidate the role of HPV-16 genotype in the oncogenesis of OSCC with emphasis on tumor related signaling pathways like hedgehog pathway.

In conclusion, HPV 16 accounted for the majority oncogenic HPV genotypes and is a major risk factor for OSCC cases in Jordan. Need for HPV genotyping would help in controlling HPV transmission and occurrence in patients with HNSCC.

#### List of abbreviations

HNSCC, squamous cell carcinoma  
HPV, Human Papillomavirus  
FFPE, Formalin-fixed, paraffin-embedded

#### Competing Interests and funding information

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#### Author contributions

LA, IS and EA conducted the molecular biology work. IA is the histopathologist who was responsible for collecting samples, evaluation of tumor grade and patient's information. LA, EA and TA interpreted the results. TA and LA guided the project, coordinated laboratory work, and analyzed the data. All authors read and approved the final manuscript.

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