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Human Papillomavirus (HPV) and Oral Squamous Cell Carcinoma in a UK Population: Is there an Association?

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Abstract

Objective: Head and neck cancer (HNC) is one of the foremost causes of death throughout the world. Human papillomavirus (HPV) is an evolving important risk factor for HNC, especially for individuals that do not smoke and do not drink alcohol. However, studies from Europe have reported ambiguous prevalence of HPV in HNC, particularly in oral squamous cell carcinomas (OSCC). Therefore, the aim of this study was to establish the prevalence of HPV infection and elucidate its association with OSCC patients in a UK population.

Materials and Methods: The presence and association of HPV was investigated in a total of (n=124) HNC patients. Samples were obtained from archived biopsies taken between 1990 and 2003. HPV screening was performed by the use of polymerase chain reaction (PCR) using the GP5+/GP6+ and the SPF1/2 consensus as primers and by *in situ* hybridization (ISH). Samples of viral warts that were immunohistochemically positive for HPV and fibroepethelial polyps (FEP) were used, as positive and negative controls, respectively.

Results: Of the 124 patients with HNC, 2.4% (3/124) demonstrated the presence of HPV, 1.8% (2/113) were OSCC. Two samples were from the tongue and one from oropharyngeal (tonsils). HPV16 was the associated virus type in all positive samples. However, no significant association was observed between HPV positivity and other clinico-pathological variables including age and sex of the patients, stage, and malignancy differentiation.

Conclusion: This report is among the few to investigate the prevalence and association of HPV with HNC in a UK population. The results revealed that HPV infection is low in OSCC in this UK population during this time period. This may suggest that HPV infection may have less important role in OSCC carcinogenesis compared to other risk factors. This information can help improve targeted therapy when treating cases of OSCC in the UK.

Keywords: HPV; HNC; HNSCC; Oral cancer; OSCC; UK; Risk factor; Carcinogenesis

Introduction

Head and neck cancer (HNC) is a group of cancers that afflict the diverse structures in the head and neck region. Such tumours may originate in the oral tissues, nasal cavity, throat, larynx and the salivary glands. Globally, HNC is the ninth most common cancer and accounts for more than 90% of squamous cell carcinomas [1]. HNSCC has been associated to environmental risk factors such as tobacco and alcohol [2]. However, there is a small population (15%-20%) of HNSCC that occur in people who do not smoke and do not consume alcohol, suggesting that other factors, such as human papillomavirus (HPV) may play a role [3-5].

The association between HNSCC and HPV was first suggested by Syrjanen, et al., when he noted microscopic histopathological changes normally associated with HPV infection (koilocytes) in HNSCC lesions [6]. A couple of years later, Loning et al., confirmed this association when he isolated HPV DNA in both premalignant and malignant oral lesions using *in situ* hybridization [7]. Since then, HPV has been implicated in HNC and oropharyngeal cancer [1,8,9]. The mechanisms by which it works differ greatly from those of alcohol and tobacco users [10].

HPV is a diverse group of viruses from the papillomaviridae family. There is an estimated 100 human HPV types, although a larger number is presumed to exist, but these have not been fully sequenced [11]. HPV's role in the etiology of carcinogenesis was first identified in cervical cancer where more than 90% of these cancers can be associated to HPV infection [12]. Clues such as the detection of HPV16 and 18 DNA sequences in cell lines derived from cervical carcinoma and their involvement in altered cell growth due to cellular mutations and altered genomic integrity, sustain the role of HPV in cervical carcinoma [13,14].

HPV's etiologic role has been supported by epidemiologic and molecular evidence in a subset of oropharyngeal cancers (OPC) through pathways similar to those in cervical cancer [15]. Park et al., and others have reported that oral epithelial cells become immortal when they express E6 and E7 oncoproteins from HPV16 [16,17]. Viral

expression of E6 and E7 of HPV16 were detected more often in oropharyngeal tumours and less often in OSCC [18-20]. The HPV16 E6 and E7 proteins inactivate p53 and pRb, respectively, with an associated lack of p53 mutations [18-20], a reduction in pRb expression and overexpresion of p16 proteins [19]. This alteration in the cell cycle regulation pathways are seen most frequently in oropharyngeal tumours, specifically in tonsillar tumours [18-20].

Epidemiologic case-series studies showed a large discrepancy in HPV prevalence from 0% [21,22] to 100% [23] in HNSCC cases. However, HPV16 was consistently the most prevalent type (86.7%) [4] associated with HNSCC [24]. However, the detection of viral DNA in either biopsy or resection samples is not sufficient for the definitive implication of an etiologic role in the pathogenesis of HNSCC. HPV biological activity is needed to prove causation such as the expression of viral oncoproteins (E6 and E7), the inactivation of the p53 and pRb pathways (not through p53 and pRb mutation), the clonal association at all cancer stages (preinvasion, invasion, and metastasis), and the viral physical state and load (integrated *vs.* episomal and low load *vs.* high load) [24]. Some studies have suggested genetic subclasses of HNSCC based on HPV infection and activity within the host cell nucleus [2,25]. Further studies also suggested that HPV positive HNSCC showed a 2 to 5-fold increase in mutations making HPV a serious etiologic factor [26]. However, studies from Europe have reported ambiguous prevalence of HPV in HNC, particularly in oral squamous cell carcinomas (OSCC). Therefore, the aim of this study was to establish the prevalence of HPV infection and elucidate its association with OSCC patients in a UK population.

Materials and Methods

Patients and archival tissue samples

Ethical approval was attained from the Joint Research and Ethics Committee at the Eastman Dental Institute (EDI) and Hospital for patients who had incisional or excisional biopsies, or surgeries, for a primary HNSCC lesion. The ethical approval fully abided with the World Medical Association Declaration of Helsinki. Cases were obtained from the archived biopsies, of specimens archived between 1990 and 2003, of the histopathological records of the EDI and the Department of Pathology of University College London, UK. All samples were fixed in a 4% dilution of 10% formalin saline concentrate and were then processed and embedded in paraffin wax. Patients who underwent pre-operative radiotherapy or chemotherapy were excluded.

The following information was retrieved from the patients' notes: date of birth, gender, and site of primary tumour, date of diagnosis, differentiation, date and type of surgery, and TNM staging. Tumours were clinically classified according to the gold standard TNM system as follows: Stage I, T1N0M0; Stage II, T2N0M0; Stage III, T3N0M0, or any T with N1 M0; and Stage IV, any T with N2M0, N3M0, or any N with M1.

In addition, the mode of invasion was objectively graded according to Odell et al. [27] as follows: Grade 1: well delineated borderline; Grade 2: infiltrating cords, bands and strands; Grade 3: small groups or cords of less than 15 cells; and Grade 4: marked extensive cellular dissociation in single cells or small groups. Two independent pathologists reviewed 10% of the hematoxylin/eosin-stained slides. Cases were divided into sample and control groups. The control group consisted of positive and negative controls (13 cases and 18 cases).

DNA extraction from paraffin embedded tissue

Meticulous measures were taken to avoid cross-contamination. To extract DNA from paraffin blocks, the Qiagen Mini-kit was used following the manufacturer's instructions. Tissue sections were cut from each tissue block ~ 25 μm thick and put in a sterile microtube for further processing. The resulting kit DNA extraction solution was either used for PCR reactions or stored at -20°C.

HPV screening by PCR

PCR-quality control: To circumvent contamination and false positive results, all PCR-related work was carried out in specific areas within the PCR laboratories. Viral-free DNA and negative controls consisting of PCR reagents with no DNA, were used to assess and detect crossover contamination. Furthermore, the transfer of DNA to the PCR buffer was carried out with aerosol-resistant pipette tips. In addition, we used oral papillomas and viral warts that were immunohistochemically positive for HPV as our positive controls to avoid false negative results. A negative control group of fibroepethelial polyps (FEP) were analysed to avoid false positives. The thermal profiles used had been described in previous studies [28].

Using the GP5+/GP6+ consensus primers: The GP5+/GP6+ L1 consensus non-degenerate primer set was used as the primary method for the detection of HPV. This detected a broad range of HPV types that generated a PCR product of approximately 150 bp. The annealing temperature used in the PCR experiment was relatively low [29]. The PCR mixture was made of 5 μl 10X PCR buffer II, 4 μl of $MgCl_2$, 0.4 μl of dNTP, 0.2 μl of GP5+/GP6+ primers, 0.2 μl of Ampli Taq Gold and 1 μl of DNA in an ultimate volume of 50 μl. Conditions for PCR included: activation of Ampli Taq Gold for 7 min at 95°C, followed by 40 cycles of 45 s at 93°C, 45 s at 40°C, 90 s at 72°C, with a final allowance of 5 min at 72°C. Then the annealing temperature was changed to 46°C to improve the product yield. A separate set of positive and negative PCR controls was carried out with each experiment.

Using the SPF1/2 consensus primers: The L1 consensus primers SPF1/2 set (comprising six primers) developed by Kleter et al. [30], were the universal primers that were used to detect HPV-DNA, and they formed a 65bp product. The PCR was performed in an ultimate reaction volume of 50 μl that contained 1 μl of isolated DNA, 5 μl of 10X PCR buffer II, 5 μl MgCl2, 0.4 μl of dNTP, 0.6 μl of SPF mix primers, and 0.2 μl of Ampli Taq Gold. Conditions for PCR were as follows: activation of Ampli Taq Gold for 7 minutes at 95°C, 40 cycles of denaturation for 45 s at 93°C, annealing for 45 s at 45°C, and extension for 90 s at 72°C with a final allowance of 5 min at 72°C.

Electrophoresis of PCR products: The Mini-Protean 3 Electrophoresis was used to mix an 8% gel. The gel mixture was instantly loaded between the glass plates and well-forming combs inserted into place. After 15 min the combs were removed and the sample wells rinsed with 1 X TBE buffer. The apparatus was assembled following the manufacturer's instructions. The 2.5 μl of loading buffer was mixed with 5 μl of the PCR products before loading into wells. The gels were run at 200 V for 35-40 min. Gels were then separated from the glass plates, stained in ethidium bromide (2.5 μl/ml) for 5-10 min and observed under ultra-violet light and photographed.

HPV cloning and sequencing: The purification of the PCR putative major protein L1 product was carried out using Quiaquick PCR purification kit (Qiagen) and sequenced in one direction using the T7

primer. The TOPO TA cloning kit for sequencing (Invitrogen) was used to clone the purified PCR product into vector pCR4-TOPO. Nine positive recombinant clones were randomly chosen, and sequencing of the insert DNA was done by using the CEQ DTCS Quick start kit (Beckman-Coulter). All major capsid protein L1 sequences that had at least 98% identity were considered to belong to the same species.

HPV screening by in situ hybridization (ISH): Sialanized slides (DAKOCytomation) were used to prevent section detachment. Xylene was used to dewax the sections which were then hydrated and microwaved in Dako Target Retrieval Solution at pH 6.0, for 20 min on high power. They were left to stand for 10 min and then rinsed in tap water. Then, 1-2 drops of a biotinylated wide spectrum HPV probe (DAKOCytomation), rubber glue and coverslip were added. Sections were denatured for 6 minutes on a 90°C hot plate followed by hybridization for 60 min at 37°C. Slides were subsequently washed in 2 changes of 0.05% Tween 20 in TBS (TBS/Tween) on a shaker to remove the coverslips. They were then immersed in stringent wash solution (Dako stringent wash was diluted 1/50 in distilled water) at 37°c for 30 min and rinsed in 2 changes of TBS/Tween on a shaker. The slides were placed in an incubation tray after circling the section with a hydrophobic pen. Streptavidin-AP was applied for 20 minutes and rinsed in TBS/Tween. Slides were further incubated for 60 minutes in dark after application of BCIP/NBT. Finally, the slides were rinsed in distilled water, and counterstaining was carried out with 0.1% nuclear fast red in 5% aluminium sulphate; they were then dehydrated, mounted and examined under Olympus BUZ light microscopy.

Results

Patients and archival tissue samples

Our study population comprised 166 patients that presented with a histologically-confirmed diagnosis of HNSCC, of which 158 cases were OSCC and 8 were oropharyngeal SCC. Twenty-one of the 166 patients were excluded because the DNA was degraded and could not be analysed. An additional 21 cases were further excluded due to lack of TNM staging data. The majority of tumour specimens (104) were obtained from resections of the primary tumour and the remaining 20 tumours were obtained from biopsies.

The final cohort (n=124) included patients whose age ranged from 27-97 years (median=62, mean=60 ± 14.3 years) with a male to female ratio of 2.2:1 (85:39). The primary tumours were located from the oral cavity (n=113) including tongue (45 cases), floor of the mouth (19 cases), alveolus (35 cases), buccal mucosa (9 cases), and palate (5 cases). Five cases were from the lip and 6 from the oropharynx/tonsil (n=6). Of these, 2 cases were in the oropharynx and 4 were in the tonsils. Ninety percent (112/124) were conventional SCC, and 10 percent (12/124) were verrucous carcinoma.

Among our sample, 60 percent (74/124) presented with locally advanced stage III or IV disease. Sixty-eight percent of the patients (84/124) were diagnosed as moderately differentiated, 19% (24/124) were well differentiated, and the remainder were poorly differentiated (16/124). Seventy-two percent (89/124) had bands or cords at the invasive front, 16% (20/124) were diffuse, and the remainder (15/124) had pushing front. Of the 16 fibroepithelial polyps (negative controls) that were suitable for analysis, 4 were males and 12 were females. Age ranged from 18 to 78 with a median age of 44.5 years.

HPV screening, cloning and sequencing

Of the 124 patients with HNC, 2.4% (3/124) revealed a positive presence of HPV as indicated by a 150 bp on 8% poly acrylamide gel electrophoresis of the PCR products (GP5+/GP6+, SPF1/2 set) (Figure 1).

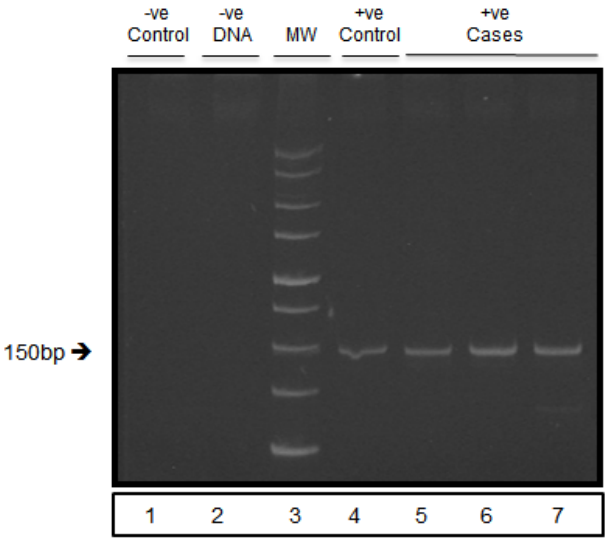

Figure 1: Detection of HPV in HNSCC specimens by PCR, with L1 consensus primers (GP5+/GP6+). 150 bp products were visualized on electrophoresed 8% poly acrylamide gel with ethedium bromide. Lane 1; negative HPV control, lane 2; HPV negative DNA control (FEP), lane 3; molecular weight marker (MW), lane 4; HPV positive DNA control (HPV positive, HIV-associated warts), lanes 5,6, and 7; positive HNSCC.

The HPV detection rate in OSCC was 1.8% (2/113). Cases in the tongue had a higher detection rate (4.4%, 2/45) versus other oral sites 0% [floor of the mouth (0/19), alveolar mucosa (0/35), buccal mucosa (0/9) and palate (0/5)]. Specific sequencing and cloning of the L1 major capsid protein PCR product distinctly revealed that HPV16 was the type present in all positive samples.

Of the 13 positive control samples, 100% revealed a positive presence of HPV as indicated by a 150 bp and/or a 65 bp fragment on 8% poly acrylamide gel electrophoresis of the PCR products. The detection rate in the negative controls was 0%. Further analysis of the PCR HPV positive cases and 13 randomly selected negative cases by *in situ* hybridization confirmed the PCR analysis results-just a few HPV-infected nuclei within the tumour were detected in each HPV positive tumour (Figure 2).

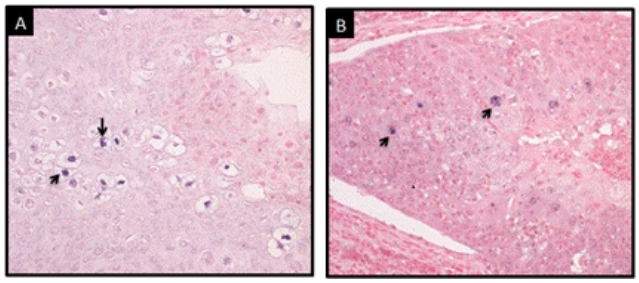

Figure 2: Detection of HPV by *in situ* hybridization with a biotinylated wide spectrum HPV probe. (A) A positive HPV control (HPV positive papilloma), positive nuclei stain blue (arrows). (B) A Positive HPV tonsillar, basaloid SCC. Positive nuclei are few and scattered within the tumour islands (arrows).

Both HPV positive OSCC samples were moderately/poorly differentiated and were from the tongue. However, the HPV-positive oropharyngeal tumour was a basaloid SCC that was moderately differentiated and from the tonsils. A summary of demographic data and clinical information of the HPV-positive cases are presented in table form.

Due to the low number of the positive sample results, an association between HPV positivity and other clinico-pathological parameters such as sex and age of the patients, as well as the stage and differentiation of the malignancy, could not be verified at this moment.

Discussion

This report is one of a few large case-series studies carried out to assess the possible prevalence and subsequent association of HNC, and specifically, OSCC with HPV infection in the UK. The low HPV detection rate in HNC (2.4%) in general, and OSCC (1.8%) specifically, is somewhat lower than other previously reported studies [5,24,31]. In this study, HPV16 infected all of the three positive tumours consistent with other studies (Table 1) [4].

Location	No. of Samples/ Type	Gender	Age	HPV Type	Histological Diagnosis	Stage	Grade	Mode of Invasion
Oral (Tongue)	1 (OSCC)	F	63	HPV16	Conventional SCC	IV	Moderately/poorly differentiated	Cords
Oral (post 1/3 of the tongue)	1 (OSCC)	M	49	HPV16	Conventional SCC	III	Moderately differentiated	Pushing
Oropharyngeal (Tonsils)	1 (OSCC)	F	65	HPV16	Basaloid SSC	IV	Moderately differentiated	Cords

Table 1: Summary of Demographic Data and Clinical Information of HPV Positive Cases.

Studies detecting HPV in HNSCC and OSCC were mostly case series that identified HPV at various rates 0% [21,22] to 100% [23] with or without control comparison groups [19,20,32,33]. This variation in HPV prevalence might be due to geographic etiological backgrounds and methodological/technical sampling approaches or errors.

More than 100 HPV types are known to infect humans, and there is no single consensus primer that detects all types. However, both consensus primers that were used (GP5+/GP6+ and SPF1/2 set) detect HPV types that are frequently detected in lesions of the oral cavity, including HPV16. Additionally, to guarantee sensitivity and specificity of the PCR assays, we used PCR that was directed towards a DNA sequence found in all cells (4 gene rearrangements in lymphoma) to eliminate non-amplifiable samples. Then, positive and negative HPV control groups were added for comparative analysis. Furthermore, with each run of PCR, a negative DNA, a negative non-DNA and a positive DNA sample were added.

Formalin fixation introduces artefacts that are related to the concentration and length of fixation. Extensive cross-linking of proteins to DNA causes fixation-induced DNA degradations. This results in DNA that is often fragmented yielding relatively short PCR fragments, which might result in negative PCR [34]. This is not the case when the amplification is less than 200 bp [21], which is the reason for using the GP5+/GP6+ and SPF1/2 set of primers in such samples. These L1 consensus primers form 150 bp and 65 bp products, respectively. This increases the sensitivity in HPV detection in such samples.

Additionally, the prevalence of HPV appeared to be inversely proportional to the study sample size-specifically in oral cancers. This might reflect a selection bias [4]. Small case-series studies (n=<100) that utilized PCR-based detection methods reported a high detection rate of 11.2% (8/71) [35] and 61.5% (16/26) [36]. Larger similar studies reported a low detection rate of HPV in OSCC (4.4%; 15/338) [24], which is comparable to the detection rate of 1.8% (2/113) in this study.

Moreover, this variation in prevalence might be due to diverse patient population analysed from different geographic locations with different rates of endemic infection. Asian studies reported a high HPV detection rate in OSCC ranging from 15% (15/100) [37] to 100% (20/20) [23]. In comparison, European studies reported a lower HPV detection rate (0%; 0/33) [21] and (4.4%; 15/338) [24]. This is similar to our low reported rate of 1.8%.

Recent literature clearly supports the theory that oropharyngeal and tonsillar cancers are more likely to be associated with HPV than other head and neck tumours. Hence, they are phenotypically considered a different entity-usually poorly differentiated, basaloid SCC, and occur in patients who don't smoke and don't consume alcohol [32,38]. Anatomically, the perimeter between the oral cavity and the oropharynx is the posterior 1/3 of the tongue. This border is often not well demarcated especially in tumours detected at later stages, which might lead to anatomical misclassification and a negative inflation in

the number of cases in the oral cavity that test positive for HPV [4,15]. Thus, we believe that our results are genuine, and they reflect a true low HPV prevalence in OSCC. This has been shown by others who utilized similar detection methods in a similar population. However, HPV might be associated with a particular subgroup of HNSCC-specifically oropharyngeal and tonsillar tumours and probably with a small fraction of OSCC. This might also depend on the population geography.

In conclusion, our study is one of the first to investigate the incidence and association of HPV with HNC, specifically OSCC, in a UK population. Our results have shown low HPV infection in HNC in general and in OSCC specifically, in the UK population over this study period. This may suggest that HPV infection may have a less important role in OSCC carcinogenesis compared to other risk factors. This information can help improve targeted therapy when treating cases of OSCC in the UK.

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