SHORT REPORTS

Human papilloma virus in squamous carcinoma of the head and neck: a study of cases in south east Scotland

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Several studies have found human papillomavirus virus (HPV) in tissue from head and neck squamous cell carcinomas (HNSCCs), although the number of positive cases varies greatly from study to study. The extent and molecular epidemiology of HPV in HNSCC were assessed within cases drawn from southeast Scotland by performing broad-spectrum, real-time HPV polymerase chain reaction (PCR) on DNA extracted from 100 cases of HNSCC in formalin-fixed, paraffin wax-embedded material. All HPV-positive specimens were genotyped and sampled by laser capture microdissection. Pure samples of tumour, and, where possible, dysplastic and normal epithelium were then submitted for further HPV PCR and genotyping to investigate the sensitivity of the technique in small tissue samples. 10 of 100 cases tested positive for HPV, with 8 of these being derived from Waldeyer's ring. HPV DNA was found in adjacent epithelium in two of four cases where this was available. These findings confirm that HPV is likely to be involved in a subset of HNSCC in this population and that successful amplification of HPV nucleic acid is possible even using small amounts of paraffin wax-embedded tissue.

Involvement of persistent human papilloma virus (HPV) infection in the development of squamous carcinoma of the cervix is now established. Accumulated data have also shown the presence of HPV DNA in head and neck squamous cell carcinomas (HNSCCs), suggesting a similar aetiological role for HPV in these tumours. Specifically, several studies have implicated the oropharynx and, in particular, Waldeyer's tonsillar ring as a predilection site for HPV-associated carcinogenesis,^{1–3} and have suggested that these tumours represent a distinct clinical subgroup of squamous carcinomas with an improved prognosis.⁴ This evidence has led to suggestions that HPV DNA detection might be used in the clinical management of HNSCC⁵ provided that the detection technology was sufficiently robust for preserved pathological specimens.

MATERIALS AND METHODS

Case selection

In all, 101 archival cases of HNSCC were selected, composed of 25 women and 76 men aged 21–87 years (mean age 61.45 years). The cases included 64 laryngeal carcinomas (mean (SD) age 62.63 (10.73) years) and 36 carcinomas from around Waldeyer's ring (tonsil, base of tongue, posterior pharynx and uvula; mean (SD) age 61.06 (11.19) years). A total of 24 controls of benign tonsillar tissue were also identified, comprising 14 females and 10 males aged 14–87 (mean 35.1) years.

DNA extraction and PCR protocol

Thick sections were cut from each paraffin wax block and DNA was extracted using lysis buffer containing proteinase K at 50°C for 4 h. Real-time PCR incorporating HPV-specific broad-spectrum primers was performed. Full details of the methods

are as described by Cubie *et al.*⁶ A 150 nucleotide product was amplified using the GP5+6+ primers⁷ on a LightCycler. A positive HPV product was identified via Tm of generated amplicon visualised by incorporation of Sybr Green dye during amplification. Any positive sample identified by the real-time protocol was subjected to the Roche line blot assay for genotyping, which involved amplification using the PGMY primer set⁸ designed to generate a 450 nucleotide amplicon. Control primers for the amplification of the β globin gene were also included in the PCR to assess the integrity of the cellular extract. Amplicons were then hybridised to a nylon strip containing 27 immobilised type-specific HPV probes and 2 levels of β globin control probes according to the technique described by Gravitt *et al.*⁹

Laser capture microdissection

Each tumour that tested positive for HPV DNA was microdissected using laser capture microscopy. Sections of thickness, 7 μ m were cut, dried and counterstained with Mayer's haematoxylin. Tumour epithelial cells were microdissected on to caps (CellPix II, Arcturus), DNA was extracted as above and PCR was repeated on these samples. For each case, two separate samples composed purely of tumour cells were taken, one containing approximately 100 cells and one with approximately 300 cells. Tissue was also taken from adjacent normal or dysplastic epithelium where applicable.

RESULTS

None of the 24 controls tested positive for HPV DNA by real-time PCR whereas 10 of 101 (10%) cases did. Two cases from the laryngeal group yielded weak HPV DNA PCR-positive results, which could not be repeated. These two cases did not test HPV positive after application of the PGMY primer set, and therefore could not be genotyped. Eight cases derived from Waldeyer's ring contained HPV DNA by real-time PCR. All eight were strong, repeatable positives and genotyping was possible in every case.

The mean age in the 10 positive cases was 50.6 years (95% CI 44.11 to 57.09). The mean age in the 91 HPV-negative cases (excluding controls) was 63.3 years (95% CI 61.17 to 65.5). This age difference is significant (unpaired Student's *t* test, *t* = −3.69, *p* < 0.001).

HPV genotyping showed that 7 of 8 strong-positive cases contained HPV type 16. The eighth strong-positive case contained HPV type 11. No mixed infections were detected (all 8 positives tested positive for β globin; table 1).

HPV DNA was not detected in microdissected tissue from the two weakly positive carcinomas from the larynx. Of the eight cases from Waldeyer's ring, five tested positive for HPV DNA when microdissected (table 2). HPV DNA was detected in one of three microdissected samples of adjacent dysplastic epithelium. The adjacent epithelium in case 7 had a non-dysplastic,

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus virus; PCR, polymerase chain reaction

Table 1 Analysis of human papilloma virus-positive tumour biopsy specimens

HPV-positive case	Real-time PCR positive	PGMY/genotype positive	Site of tumour	HPV type
1	✓	✓	Tonsil	16
2	✓	✓	Tonsil	16
3	✓	✓	Tongue base	16
4	✓	✓	Tonsil	16
5	✓	✓	Tonsil	16
6	✓	✓	Posterior pharynx	16
7	✓	✓	Tongue base	16
8	✓	✓	Uvula	11
9	✓wk	×	Vocal cord	—
10	✓wk	×	Pyiform fossa	—

HPV, human papilloma virus; PCR, polymerase chain reaction; wk, weak positive result via real-time PCR.

The two weak real-time positives did not test positive after application of the PGMY primer set and thus could not be typed.

Table 2 Human papilloma virus PCR results in microdissected samples from positive cases

HPV-positive case	Normal epithelium	Dysplastic epithelium	Tumour (100 cells)	Tumour (300 cells)
1	0	NA	0	0
2	0	0	+	0
3	0	NA	0	0
4	NA	0	+	+
5	0	NA	+	0
6	0	+	0	0
7	+	+	0	+
8	NA	NA	+	+
9	NA	NA	0	0
10	NA	NA	0	0

HPV, human papilloma virus; NA, not available; PCR, polymerase chain reaction; 0, no HPV DNA detected; +, HPV DNA detected.

*In this case, the adjacent epithelium was not overtly dysplastic. Instead, an area with a warty appearance was sampled.

warty appearance and this epithelium and the background normal epithelium contained HPV DNA.

DISCUSSION

A previous study of HPV in head and neck carcinoma in a UK population concentrated on oral lesions, with HPV found in up to 46% of normal and tumour tissues.¹⁰ We have investigated the presence of HPV in tonsillar and laryngeal carcinomas, and our findings replicate the results of similar studies performed in other countries, confirming that any future HPV-directed diagnostic and treatment strategies for HNSCC are relevant to this UK-based population. We have confirmed that HPV seems to have a predilection for involvement in carcinomas arising in Waldeyer's ring, suggesting that detection of HPV in this subset of tumours may be of clinical relevance not least because HPV-positive carcinomas seem to have a better prognosis, possibly because of a greater sensitivity to radiation treatment than conventional HNSCC.^{11–12} Paradoxically, a recent study found that HPV-positive HNSCCs were associated with earlier nodal metastasis and suggested that screening high-risk populations for HPV may be valuable in preventing premalignant lesions progressing to carcinoma.¹³

Microdissection confirmed that HPV DNA was present in carcinoma cells. The detection of HPV in some of the small microdissected samples has shown the sensitivity of these techniques in formalin-fixed paraffin wax-embedded material, which is of relevance, as biopsy specimens submitted from head and neck sites may be limited in size.

The presence of HPV DNA in abnormal epithelium adjacent to the invasive carcinoma in two cases and normal epithelium in one case suggests that the detection of HPV in the epithelium of Waldeyer's ring could indicate an increased risk of neoplastic transformation with possible future diagnostic utility, as is being

developed for the cervix. Previous studies that investigated the presence of HPV DNA in normal head and neck epithelium found wide-ranging results,¹⁴ and therefore further work in this area is necessary before HPV testing can be used clinically to identify patients who are potentially at risk of head and neck malignancy.

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Copper:caeruloplasmin ratio

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Investigation of copper status can be a diagnostic challenge. The non-caeruloplasmin-bound copper (NCC) has deficiencies; accordingly, the copper:caeruloplasmin ratio has been suggested as an alternative index of copper status. A reference interval for this index was derived. In addition to making the interpretation of copper easier, the copper:caeruloplasmin ratio should also enable adjustment for relatively high caeruloplasmin concentrations without recourse to producing gender- and age-derived intervals. The copper:caeruloplasmin ratio has weaknesses similar to those identified for NCC in that immunological methods used for caeruloplasmin can cross react with apocaeuloplasmin and there is no standardised method for caeruloplasmin. Caeruloplasmin assays also have uncertainty from precision, bias and specificity and, accordingly, method-related differences may have a large effect on the copper:caeruloplasmin ratio in a manner similar to the NCC.

Copper studies are performed to identify copper deficiency or excess. Wilson's disease (primary copper excess), an autosomal recessive condition,¹ results in copper deposition in the hepatic parenchymal cells, brain, iris and kidney. Its diagnosis can be a challenge.² Secondary copper excess is rare in the developed world. Childhood primary copper deficiency has a poor prognosis and is often caused by an inherited defect in copper absorption (Menke's disease).³ Adult primary copper deficiency can cause a neurological condition similar to the extrapyramidal signs of Wilson's disease.⁴ Secondary copper deficiency is possible in patients receiving long-term enteral nutrition⁵ and with overuse of zinc supplementation.⁶ Copper studies are often requested to diagnose copper deficiency and to prevent neurological deterioration with supplementation.

The vast majority of copper in serum is bound to caeruloplasmin.⁷ Laboratories often do not take age and gender into account when reporting serum copper and caeruloplasmin reference intervals.⁸ Furthermore, caeruloplasmin has 6–8 copper atoms per molecule, with the majority being tightly bound.^{9–10} Heterogeneity in the number of copper atoms per caeruloplasmin molecule is possible, so any formula assuming

six copper atoms per molecule may be valid only in certain situations.

Caeruloplasmin largely determines the copper concentration,^{11–12} and plays a critical role in the interpretation of copper results. Some patients with Wilson's disease have copper and caeruloplasmin within their respective reference intervals,^{1–9} usually with copper being in excess of the caeruloplasmin concentration. Furthermore, about 2% of the population are heterozygous for P-type ATPase mutations,¹ and often have caeruloplasmin results around the lower reference interval. Non-caeruloplasmin-bound copper (NCC) has been advocated as a superior diagnostic tool for Wilson's disease¹¹ to try to overcome such issues. There are problems associated with the current use of the NCC; in particular, it produces physiologically impossible negative results in a significant number of patients.¹² To be effective in distinguishing patients with Wilson's disease from normal patients, the NCC may be expressed as a percentage of total copper. Furthermore, any index for the study of copper status should ideally have the potential to be used in the investigation of excess and deficiency copper states. Accordingly, new indices have been proposed, such as copper corrected for caeruloplasmin¹³ and the copper:caeruloplasmin ratio.¹⁴ The first study included only normal patients, whereas the second included patients with Wilson's disease who predominantly presented with neurological symptoms. We derived our own reference interval for this index.

Using the copper and caeruloplasmin data for 338 patients without Wilson's disease or copper deficiency,¹² we calculated the copper:caeruloplasmin ratio ($[\text{copper}] \times 0.132 / [\text{caeruloplasmin}]$) where copper is in $\mu\text{mol/l}$ and caeruloplasmin is in g/L .

Mean, median, SD and 2.5th and 97.5th centiles by both parametric and non-parametric methods for the copper:caeruloplasmin ratio were derived from measurements of copper (Varian Spectra 20) and caeruloplasmin (Tinaquant Kit; Hitachi 912; Roche, Welwyn, Hertfordshire, UK) assays. The copper:caeruloplasmin ratio ranged from 3.9 to 12.97 $\mu\text{mol/g}$, with the mean and median being 6.81 and 6.83 $\mu\text{mol/g}$, respectively. The SD was 0.87 $\mu\text{mol/g}$ and, accordingly, the parametric reference interval was 5.08–8.55 $\mu\text{mol/g}$ and the non-parametric 2.5th

Abbreviation: NCC, non-caeruloplasmin-bound copper