Abstract
Squamous cell carcinoma of the temporal bone is an uncommon tumour normally preceded by a history of inflammation within the external auditory canal or middle ear cavity. Although the human papillomavirus (HPV) has been implicated in many head and neck malignancies, its role in the pathogenesis of carcinoma affecting the temporal bone is yet to be determined.

Polymerase chain reaction (PCR) was used to detect high-risk HPV subtypes. RNA in situ hybridization, DNA sequence analysis and p16 immunohistochemistry were employed to confirm positive samples. All specimens underwent separate investigations for the presence of latent and active Epstein-Barr (EBV) virus infection or mutations within exons 5–9 of the TP53 gene.

Genomic DNA was successfully extracted from 14/20 patient tissue samples, as determined by control PCR reactions. Three of these were shown to contain high-risk HPV DNA (21.4%). HPV16 subtype was present in all positive cases. No significant difference in disease-specific survival was detected for the papillomavirus-positive group. One patient was found to have detectable EBV on PCR, but this was not confirmed on subsequent analysis with immunohistochemistry or in situ hybridization. All TP53 mutations were restricted to the non-viral group.

This is the first report to implicate high-risk HPV16 in squamous cell carcinoma of the temporal bone by multiple analytic techniques. This study suggests the need for further investigations, and if confirmed, it may set the foundations for stratification of treatment according to a viral aetiology.

Introduction
Squamous cell carcinoma (SCC) within the temporal bone region is a rare and destructive type of malignancy with a poor prognosis. The reported annual incidence is 1–6 cases per million, which accounts for 0.3% of all tumours of the head and neck1. Regional lymph node metastases can occur in 10%–20% of the patients, but distant spread is rare2,3.

The site of origin of the invading tumour may not be obvious at presentation as the tumour progresses by direct incursion into the temporal bone and adjacent structures (parotid, temporomandibular joint, dura, brain). Multiple factors2,3,4 influence the development of SCC within epithelium adjacent to the temporal bone. Exposure to ultraviolet light has been implicated in the development of SCC, which originates in the pinna or external auditory canal (EAC)1. Chronic suppurative otitis media (CSOM) and radiotherapy have been widely reported to have an association2. SCC arising in the nasopharynx may also invade the temporal bone by extension via the eustachian tube. One theory, which may connect the various sites and their predisposing factors, is the establishment of a chronic inflammatory process, leading to metaplastic or neoplastic change4.

Within the head and neck region, the association of HPV with SCC affecting the oropharynx, hypopharynx and nasopharynx has been extensively investigated. A recent meta-analysis of the literature demonstrated that the proportion of oropharyngeal SCC caused by HPV has increased to 72.2%5. New clinical trials are currently in progress to evaluate management based on this viral aetiology6.

Human papillomavirus (HPV) is a major carcinogen; it was associated with 4.8% of total worldwide cancers in 20087. HPV has been implicated in the development of SCC in a variety of body sites, such as the cervix and anogenital region, as well as in carcinomas of the head and neck. Essentially, all (99.7%) cervical cancers are causally associated with HPV4. HPV subtypes can be divided into a high- or low-risk, depending on their frequency of association with malignant lesions. The high-risk group contain HPV types 16, 18, 31 and 45, which are responsible for majority of the oncogenic activity in cervical cancers (~75%–80%)9. The low-risk group are not oncogenic but may be associated with genital warts, HPV types 6 and 11 being responsible for >90% of such cases9.

Epstein-Barr virus (EBV) is also associated with various forms of cancer, particularly Hodgkin’s lymphoma, nasopharyngeal carcinoma (NPC) and Burkitt’s lymphoma. The global burden of EBV-associated cancer is markedly lower than HPV (prevalence <0.01%)9.

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The potential contribution of viral infection to SCCs of the whole intratemporal region has not been formally investigated. This is surprising given the high morbidity and mortality associated with this disease and the established link to chronic inflammation. Case reports and small studies have indicated that HPV may play a role. One previous study has described an association between high-risk HPV and SCC affecting the middle ear cavity, but so far, no study has described the whole intratemporal region. Papillomas of the EAC may harbour low-risk HPVs but malignant transformation is very rare. Inverted or Schneiderian papillomas of the sino-nasal region, which can invade locally, may also be associated with HPV. If a viral aetiology of temporal bone SCC can be established, this could have important diagnostic, therapeutic and prognostic implications.

**Methods and materials**

**Patients and specimens**

This study received formal approval by the National Research Ethics Service Committee of East of England (12/EE/44). Clinical samples for the period 1983–2008 were obtained from the Department of Histopathology, Addenbrooke’s Hospital, Cambridge, UK. Cases with a diagnosis of SCC affecting the external ear canal or middle ear cavity were included. Biopsies were obtained at the time of diagnosis or at the time of operation. Patient data including age, sex, smoking status, clinical presentation and disease stage were correlated to patient outcome.

**Extraction of DNA**

All samples underwent a decalcification process with trichloroacetic acid (TCA) and were stored at –70°C. DNA was eluted in purified H₂O (autoclaved + nuclease free) and stored at –20°C until further evaluation. Purity and concentration of DNA was ascertained by spectrophotometry. All samples had a 260/280 nm absorbance ratio in the range of 1.7–2.1, and were diluted with H₂O to a concentration of 1–27 ng/μl prior to PCR.

**PCR**

**HPV PCR analysis**

The PGMY09/11 L1 consensus primer set was utilized for each sample (approx. 8 ng extracted DNA), as described previously. PCR thermal cycling programme was as follows: denaturing step at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. This was followed by a final extension for 10 min at 72°C. After amplification by PGMY09/11 primers, all samples (2 μl PCR product) underwent nested PCR with the GP5+/GP6+ primer pair, as described previously. PCR thermal cycling programme was as follows: denaturing step at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 40°C for 2 min and 72°C for 1.5 min. This was followed by a final extension for 10 min at 72°C.

**HPV DNA sequence analysis**

DNA was subsequently purified from positive samples following gel electrophoresis using QIAQuick® gel extraction columns (QIAGEN Ltd, UK) and directly sequenced (Source BioScience, UK). The sequences were then aligned with known HPV types (Basic Local Alignment Search Tool, National Center for Biotechnology Information, Bethesda, USA).

**EBV PCR analysis**

PCR assay using the EP/EM₁ primer set was performed as described previously (approx. 10 ng extracted DNA). PCR cycling conditions were as follows: denaturing step at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. This was followed by a final extension for 10 min at 72°C.

**RNA in situ hybridization**

This technique utilized the Roche RNA labelling and detection kit (Roche Diagnostics, Indianapolis, IN, USA) to perform the catalyzed signal amplification method for biotinylated probes. Anti-sense digoxigenin-labelled probes for HPV16 E4, E6, E7 and L1 messenger RNA (mRNA) were utilized to discern viral activity within the cell. Sections were reviewed independently by two authors. A positive signal was noted if a dark purple colour was evident within tumour cells. Negative controls were carried out using sense probes, and positive controls were ascertained from HPV16 positive vulval intraepithelial neoplasia (VIN) samples.

**Prehybridization**

Briefly, 5 μm FFPE sections were deparaffinized and rehydrated in a graded ethanol series. The slides were then fixed in 4% parafomaldehyde solution for 5 min. After a brief wash in phosphate buffered saline (PBS), the sections were fixed in proteinase K solution (0.5 mg/ml) at 37°C for exactly 10 min. Sections were further washed in PBS and then refixed in 4% paraformaldehyde solution. A final wash in PBS was employed before and after a soak in acetic anhydride/triethanolamine solution for 10 min. The sections were dehydrated via alcohol and air-dried. Hybridization mix (1 μl of digoxigenin-labelled HPV16 E4, E6, E7 or L1 antisense/sense probe + 10 μl of RNA hybridization mix) was applied to each section and stored at 37°C overnight.

**Post-hybridization**

Slides were washed in 2× saline-sodium citrate buffer (SSC: 0.3 M NaCl, 0.03 M sodium citrate, pH 7) at 55°C for 10 min. Thereafter, the slides were subjected to RNaseA digestion
cytoplasmic stain was indicated by a granulated dark brown colour.

**TP53 sequencing**
A 1.84 kB TP53 gene segment, incorporating exons 5–9, was amplified from purified tumour DNA by PCR and sequenced directly. This region was chosen because somatic mutations outside this DNA-binding region are rare in human malignancies.

**Statistics**
Characteristics of each study population were summarized using descriptive statistics. Tumour samples were considered positive for HPV if detected by PCR and DNA sequence analysis with corroborative evidence on p16 IHC or RNA in situ hybridization. Tumour samples were also considered to have an association with EBV if detected by PCR or LMP-1 IHC.

Rates of overall- and disease-specific survival were estimated by means of the Kaplan–Meier method and were compared between these groups with the use of the log-rank test. The chi-squared test was used to analyse all other proportionate data between subgroups. Differences with values of $P < 0.05$ were considered significant.

All calculations were performed using SPSS® Version 17 (Illinois, USA).

**Results**

**Clinical data**
A total of 20 cases of temporal bone SCC involving the EAC or middle ear cavity were identified from the hospital records and entered into the study. The majority of cases (70%) displayed moderately differentiated SCC. The age at presentation varied from 37 to 79 years, with a median of 62.5 years [standard deviation (SD) ±11.3 years]. Eleven (55%) patients were men and nine were women. The mean follow-up period was for 34 months (range 1–184). Three-year disease-specific survival was 40% [standard error (SE) ±11%] with no significant difference noted when stratified by HPV status, age, sex, smoking or disease stage. The clinical and laboratory data are summarized in Table 1 and Figure 1(a, b).

**Detection of HPV by PCR**
The majority of DNA samples (14/20) showed amplification of the β-globin 268 bp product and were therefore deemed suitable for PCR evaluation of HPV. DNA extracted from an HPV11 positive genital wart sample was used as a positive control for both β-globin and HPV PCR. One sample (case 4; Table 1) showed amplification of a 450 bp product with PGMY primers. Nested PCR GP5+/GP6+ primer set showed a positive band (150 bp) in 3 patients (cases 2, 4 and 6; Table 1). DNA sequencing of positive samples revealed the HPV16 subtype in all cases. Point mutation analysis revealed the presence of minor heterogeneity.

**Detection of EBV by PCR**
One patient sample (case 19; Table 1) displayed evidence of EBV positivity when analysed by the EP5/EM3 primer set. A control sample using genomic EBV DNA (EBNA-2) revealed an 182 bp amplicon.

**RNA in situ hybridization**
In order to determine transcriptional activity within the tumour tissue, samples noted to have β-globin amplification with PCR were subjected to further analysis by RNA in situ hybridization. Two samples (each HPV+ on PCR/DNA sequence analysis) showed evidence of epithelial expression of HPV16 E6 or E7 and L1 mRNA. The control sense probe was negative. The positively stained cells were distributed throughout the entire layer of superficial neoplastic epithelium (Figure 2a–d). EBV-encoded RNA (EBER) in situ hybridization was utilized for the positive EBV sample, but it did not detect any evidence of active infection (Figure 3a–d).

**IHC**
All three HPV DNA-positive tumours underwent p16 IHC, two of which
displayed evidence of specific nuclear and cytoplasmic staining (Figure 1b). HPV DNA-negative controls did not display evidence of p16 activity. LMP-1 immunohistochemical stain for detection of EBV was negative for all samples (Table 1). A functional mutation was discovered in 3/10 HPV negative and 0/3 HPV positive samples (Table 1); however, this difference was not statistically significant ($P = 0.28$). All suspected mutations underwent forward and reverse primer sequencing.

### TP53 mutation analysis

Sufficient DNA was available for direct sequencing of exons 5–9 of the TP53 gene in majority (13/14) of the samples. The positive control for TP53 mutation consisted of DNA extracted from the human immortalized cell line K562 (chronic myeloid leukaemia). A negative (wild type) control was derived from the CaSki cell line (cervical carcinoma). A functional mutation was discovered in 3/10 HPV negative and 0/3 HPV positive samples (Table 1); however, this difference was not statistically significant ($P = 0.28$). All suspected mutations underwent forward and reverse primer sequencing.

### Discussion

This is the first study to suggest a causative role for HPV in a subset of temporal bone SCC cases, as determined by both molecular and immunohistochemical techniques. It follows a trend recently encountered in other areas of the head and neck. Further, 3 of 14 patients were found to have detectable HPV16 DNA. No significant difference in disease-specific survival was detected for the HPV-positive group. All patients were selected randomly and demographic details were similar to other reported case cohorts. Although the 21.5% HPV16 detection rate in this study represents a minority, the clinical significance may be apparent if duplicated by other centres and correlated to longer-term outcomes. The study expands on similar research conducted by Jin et al. in 1997, which was restricted to the middle ear cavity but nonetheless demonstrated high-risk HPV16 in 11/14 samples. This higher HPV detection rate may reflect ethnic composition, inter-observer bias or a methodological variation. However, potential limitations may be the absence of clinical

### Table 1 Summary of clinical and laboratory analyses

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M, male; F, female; SCC, squamous cell carcinoma; Mth, month; Diag, diagnosis; Histol, histology; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated; N/A, not applicable; F/U, follow-up duration; *= Non-malignant cause of death; WT, wild type; M, mutated.
A significant proportion of oropharyngeal cancers (40%–75%) are now thought to have HPV16 DNA integrated within their genomic DNA, with minor contributions made by other oncogenic HPV subtypes (18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68)\(^2\). The rise in HPV+ oropharyngeal carcinoma has been well documented\(^{6,22}\), and it is noteworthy that all similar cases in the temporal bone region were identified in the last decade—this may reflect the quality of samples obtained or possibly the growing importance of HPV within the head and neck region.

In this cohort, the link with EBV is less evident, with only one patient demonstrating a possible association on PCR analysis. This may represent either the true level of background infection, inhibitory pathways in vivo/vitro or inadequate detection of a low viral load. The premise for investigating this virus stemmed from the close proximity of the nasopharyngeal mucosa to the temporal region. EBV is associated with the overwhelming majority of NPC as evidenced by expression of LMP1 or EBER in tumour cells\(^23\).

At present, the definitive treatment for SCC of the temporal bone is with major surgery and free flap reconstruction with limited scope for primary medical therapy\(^1,2\). Previous studies will not have accounted for a potential viral aetiology, which may confound clinical outcomes\(^22\). Although this malignancy is comparatively rare, the morbidity associated with current treatment would provide a strong argument for change\(^2,5,22\). In common with other published datasets\(^1,3,10\), majority of the cases in this study presented with advanced stage IV disease. A practical application of this research may therefore involve targeted screening to ensure earlier diagnosis.

It is tempting to conclude from the survival analysis (Figure 1a) that HPV-associated patients showed a trend towards improved survival, but small numbers prevent this from proving statistically significant. If replicated on a larger scale, this may support studies which observe that repression of E6 and E7 (in HPV-positive SCC) leads to activation of the p53 and pRb pathways, decreased cellular proliferation and cellular growth arrest\(^24\). This situation is quite different from conventional disease (HPV-negative SCC), where an irreversible p53 mutation may be present\(^21\). It is of interest to note that all such mutations found in this study were restricted to the latter cohort; however, no clear divide was evident.

HPV is primarily transmitted through direct skin-to-skin contact during follow-up and DNA sequence analysis to exclude contamination of samples.
vaginal, oral or anal sex\textsuperscript{25}. Research conducted recently in the United States indicates that, at any single time point, 42% females may have an anogenital HPV infection, in comparison with <7\% in the oral cavity region\textsuperscript{26}. Genital–finger transmission may be possible but has yet to be conclusively proven\textsuperscript{27}. One observational study recently investigated the presence of HPV DNA on hand samples in patients with a prior diagnosis of penile HPV. Seventy percent of specimens were positive, the majority of which were of the same HPV type\textsuperscript{28}.

Non-genital routes of HPV infection have yet to be fully elucidated, but it is generally accepted that haematogenous spread is unlikely\textsuperscript{27}. It is also contentious as to whether HPV may undergo fomite transmission from person to person (e.g. towels, sheets, medical instruments)\textsuperscript{29}. Spread from mother to child (vertical or perinatal transmission) of HPV6 or HPV11 may rarely lead to juvenile onset recurrent respiratory papillomatosis\textsuperscript{24}.

Therefore, which method could potentially predispose to HPV infection within the temporal bone? The two obvious routes of ingress for the virus would include direct spread from the oral cavity through the eustachian tube or topical spread through the external ear canal. The presence of a tympanic membrane or mastoid cavity perforation will potentially influence either route. Two of the three HPV-positive patients in this study had a pre-existing tympanic membrane defect; this was also a common finding in HPV-negative disease and prevented accurate localization of the primary to the external or middle ear cavity. Figure 1b suggests a correlation with viral positivity and extended length of otological symptoms that may support a link with chronic inflammation.

The exact prevalence of HPV16 within the head and neck region is at present undetermined. The best proxy marker comes from a systematic review by Gillison et al., which demonstrated a 1\% [95\% confidence interval (CI), 0.7–1.3] prevalence rate for oral HPV16 in the general adult population\textsuperscript{26}. A comparison with the SCC temporal bone group may suggest a significant difference (1\% versus 21\%; \( P < 0.01 \)); however, this needs to be interpreted with caution due to obvious baseline differences. The rate of oral cavity HPV persistence may also be higher than currently observed at the anogenital site, facilitating the potential for malignant change\textsuperscript{25}.

The discovery of HPV DNA from this cohort of samples is insufficient evidence to lead to the conclusion that HPV is involved in the causation of the disease. HPV is common on all skin or mucosal surfaces and the detection of HPV DNA may be due to contamination. We therefore chose to investigate HPV transcriptional activity within the tumour site by RNA in situ hybridization (Figure 2a–d). The detection of HPV16 E6 or E7 mRNA may indicate transcriptional carcinogenic activity and is considered the current standard for confirmation of HPV-associated disease\textsuperscript{16,26}. Since HPV DNA in head and neck cancer is frequently found to be integrated into the host cell genome, it is of interest to detect HPV16 L1 mRNA in one of the patients. This may reflect capsid expression and active replication of HPV16—features more in keeping with an episomal status of the HPV genome.

The limitations of this study are disparate methods of sample collection, variable storage length and a decalcification technique utilizing TCA. Our observations suggest that TCA has an adverse effect on the quality/amount of extracted DNA, and as such, this

\textbf{Figure 2:} HPV detection in SCC of the temporal bone. (a) HPV16 E7 mRNA sense probe (control) shows no uptake in epithelium. (b) HPV16 E7 mRNA antisense probe shows increased uptake throughout all layers of the epithelium. (c) HPV16 L1 mRNA sense probe (control). (d) HPV16 L1 mRNA antisense probe also shows uptake throughout all layers of the epithelium.
may reduce the sensitivity for high-risk HPV subtypes. This study is restricted to the United Kingdom and may not reflect other countries with different age/ethnicity profiles. Finally, the sample size was limited due to the rare nature of this disease and therefore may not be applicable to the wider population.

This study outlines early clinical data that relate high-risk HPV16 to SCC temporal bone and as such may have a direct impact on patient care. A theoretical cause for malignant transformation may be transmission of the virus through the eustachian tube or external ear canal in combination with longstanding inflammation. The data collected suggests the need for further investigation, preferably in a prospective setting. This will have the benefit of providing fresh biopsy material, which will improve quantitative analysis.

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Abbreviations

Epstein-Barr virus (EBV); human papillomavirus (HPV); immunohistochemistry (IHC); squamous cell carcinoma (SCC).

References


Figure 3: EBER in situ hybridization (linear scale bar 200 µM; inset picture magnified × 2.5). (a) Nasopharyngeal carcinoma control reveals strong uptake of probe in comparison with (b) RNA negative probe. (c) EBV PCR-positive patient revealed no uptake of probe when compared with (d) RNA negative probe.*

*An RNA negative control probe is a single oligonucleotide, designed from zebrafish DNA. Basic Local Alignment Search Tool analysis is utilized to confirm no homology with any human sequence.

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