Lack of evidence of human papillomavirus-induced squamous cell carcinomas of the oral cavity in southern Germany

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S U M M A R Y

Objectives: The aim of the present study was to identify HPV-attributable SCC of the oral cavity (OSCC) in a cohort of patients from southern Germany.

Materials and methods: A sensitive PCR-enzyme immunoassay (EIA) was followed by a more specific HPV-DNA by PCR–EIA could be detected in 25.1% (69/275) of the tumors, but ISH was negative in about 25% of head and neck squamous cell carcinomas.

Viral DNA can be detected in some tumors by a sensitive PCR, but absence of ISH signals indicates that the HPV-attributable fraction is smaller than estimated from PCR positivity. p16INK4a expression in a focal pattern (p = 0.09) was observed.

Conclusion: Viral DNA can be detected in some tumors by a sensitive PCR, but absence of ISH signals indicates that the HPV-attributable fraction is smaller than estimated from PCR positivity. p16INK4a/Ki-67 co-expression is detectable in a fraction of OSCC irrespective of the HPV status.

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Introduction

High-risk human papillomaviruses (HR-HPVs) are necessary for the development of virtually all cervical cancers, a substantial proportion of other anogenital cancers, and viral DNA can be detected in about 25% of head and neck squamous cell carcinomas. HPV-induced malignant transformation requires the overexpression of the viral oncogenes E6 and E7 and the sole detection of viral DNA cannot be regarded as evidence for a causal involvement of HPV in tumor development. Data on HPV-association with oropharyngeal SCC primarily involving the tonsil and base of tongue are increasingly compelling by demonstrating that viral oncogenes and associated biomarkers are overexpressed and that patients with these likely true HPV-driven tumors have a better prognosis, highlighting the potential diagnostic relevance of identifying HPV-induced tumors in the head and neck region.

The evidence for a causal involvement of HPV in SCC development at other sites of the head and neck region, including the oral cavity, is less clear. Each year, nearly 300,000 new cases of oral cancer are identified worldwide. Tobacco and alcohol are strong risk factors and genetic alterations, such as affecting CDKN2A are...
important carcinogenic drivers.\textsuperscript{10} HPV DNA has been found in a percentage of oral SCC (OSCC) with an average of 20.2\% (95\% confidence interval 16.0–25.2\%) in 60 studies on 4195 patients in a recent meta-analysis.\textsuperscript{11} However, data on the percentage of OSCC that is causally linked to HPV oncogene expression are scarce and currently suggest that the fraction of HPV-attributable cancers might be much smaller than estimated from HPV DNA PCR positivity,\textsuperscript{12} although there is no clear evidence yet on which is the gold standard test to identify HPV-driven OSCC. Detection of transcriptionally active viral oncoproteins by HPV E6/E7 mRNA assays has been used and yielded positive results ranging from 0\% in 50 patients from Latin America and Central Europe,\textsuperscript{13} 5.9\% in 409 patients from the USA\textsuperscript{12} to 14.7\% in 68 patients from Japan.\textsuperscript{14} Similarly, in situ hybridization (ISH) of viral genomes is considered more specific for relevant HPV than PCR, most likely because the latter may easily pick up contaminations of HPV genomes that are not associated with the cancer, however potentially with a lower sensitivity of ISH than DNA and RNA amplification techniques.\textsuperscript{15,12} Furthermore detection of p16\textsuperscript{INK4a} overexpression is discussed as a marker for HPV-driven head and neck cancers due to the indirect transcriptional activation of p16\textsuperscript{INK4a} expression by the HPV E7 oncoprotein.\textsuperscript{16,17} However, while for tonsillar cancers p16\textsuperscript{INK4a} overexpression has been described strongly associated with HPV and is discussed as a simple diagnostic tool to identify HPV-associated tonsillar SCC,\textsuperscript{18} data for OSCC are less clear and one recent study reports that over 50\% of p16\textsuperscript{INK4a}-positive OSCC are negative for HPV E6/7 expression.\textsuperscript{12}

The aim of the present study was to (a) determine the likely HPV-attributable fraction in a large German cohort of SCC located in the oral cavity by combining a sensitive PCR followed by a more specific HPV ISH assay and (b) to apply an immunohistochemical dual-staining for p16\textsuperscript{INK4a} and the proliferation marker Ki-67 in order to assess whether co-expression of p16\textsuperscript{INK4a}/Ki-67 is a better surrogate marker for HPV in OSCC than p16\textsuperscript{INK4a} alone, based on the hypothesis that combined p16\textsuperscript{INK4a} and Ki-67 expression might specifically discriminate oncogene-induced p16\textsuperscript{INK4a} expression from cell-cycle arrest inducing senescence-associated p16\textsuperscript{INK4a} expression\textsuperscript{18} and (c) to evaluate the prognostic impact of the HPV and p16\textsuperscript{INK4a}/Ki-67 status on patients' outcome.

Materials and methods

Patients and tumor material

Included in the study were patients with squamous cell cancers located in one of the following localizations: floor of mouth, anterior tongue, mandibular alveolar process, buccal mucosa, maxillary alveolar process or lip mucosa and treated between 1988 and 2005 at the Departments of Oral and Maxillofacial Surgery of the University Hospital of Tübingen or Heidelberg, Germany. Formalin-fixed, paraffin-embedded (FFPE) pre-therapeutic tumor tissue from the patients was retrieved from the pathology archives and the tissue bank of the National Center for Tumor Diseases (NCT), Heidelberg, Germany following the institutional ethical approvals and patients were excluded from the study if no material was available. The histopathologic diagnosis of SCC was verified by a pathologist in addition to the previous evaluation for routine diagnostics. Clinical data were retrieved from the patients' hospital files.

Detection and genotyping of human papillomavirus DNA by PCR

DNA was extracted from FFPE tissue sections using the DNeasy Blood & Tissue Kit by Qiagen, Hilden, Germany. HPV detection was performed by amplification of a viral consensus L1 sequence using biotinylated GP5+6+ primers as described previously\textsuperscript{19} followed by an enzyme immunoassay (EIA) by binding to streptavidin-coated microtiter plates (Roche, Mannheim, Germany). Hybridization to a mix of HPV type 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, 66 and 69-specific digoxigenin-labeled probes followed by detection with alkaline phosphatase-labeled anti-digoxigenin Fab fragments (Roche, Mannheim, Germany), Alkaline Phosphatase Yellow substrate (Sigma) conversion and measurement of the optical density (OD) at 405 nm. Samples with OD threefold exceeding background OD were considered positive. Amplification of beta globin was performed to ensure sufficient DNA integrity.

Detection of HPV by in situ hybridization

In situ hybridization for HPV on FFPE sections was performed for all HPV DNA positive tumors. The GenPoint (Dako, Glostrup, Denmark) HPV probe set hybridizing DNA from oncogenic types 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, and 68 and the GenPoint™ Detection System (Dako) for visualization was used according to the manufacturer instructions. Cervical cancer sections were used as positive control and sections were processed in parallel without probe incubation as negative control.

Immunohistochemical staining for p16\textsuperscript{INK4a}/Ki-67

FFPE sections from all tumors were analyzed for p16\textsuperscript{INK4a} and Ki-67 expression using the CINtec\textsuperscript{®} PLUS dual-staining kit (mtm Laboratories AG, Heidelberg, now Roche Diagnostics), according to the manufacturer's protocol applying some minor modifications for use in histology as described previously.\textsuperscript{18} The staining is based on two monoclonal antibodies, one directed to human p16\textsuperscript{INK4a} protein (clone E6H4\textsuperscript{™}) and the other to human Ki-67 protein (clone 274-11 AC3). p16\textsuperscript{INK4a} is visualized by a horseradish peroxidase-mediated conversion of 3,3'diaminobenzidine (DAB) chromogen leading to a brown cytoplasmic staining and Ki-67 by alkaline phosphatase-mediated conversion of Fast Red chromogen leading to a red nuclear staining. Slides were evaluated according to p16\textsuperscript{INK4a} expression patterns (diffuse = continuous clinical expression beginning in the basal and parabasal tumor cell layers and to a variable extent continuously reaching the remaining tumor areas, focal = expression in cell clusters or single cells, may be extensive, but does not include clonal expression beginning in basal and parabasal cell layers, negative = no expression of p16\textsuperscript{INK4a} in the tumor cells) as well as the presence of cells co-expressing p16\textsuperscript{INK4a} and Ki-67.

Statistics

Absolute numbers and frequency distributions are provided for categorical variables and mean with minimum, maximum and standard deviation are provided for age (years). Independent samples T test was used to assess differences in patient's age between different groups of categorical variables and Fisher's exact test or Chi-square test was used to assess differences between categorical variables. Unknown clinical or histopathologic data are reported as “unknown” and excluded from calculations. Kaplan–Meier analysis was used to estimate survival times.

Results

Patients and tumors characteristics

Oral SCC from a total of 275 patients were included. Mean age of the patients at tumor diagnosis was 59.9 years (min 16, max 93, standard deviation 11.3 years), most patients were male (206/275, 74.9\%) and the most frequent tumor localization was the floor of the mouth (93.8\%).
of mouth (107/275, 38.9%). The majority of the patients underwent only surgical tumor resection (196/275, 71.3%), followed by 12.7% (35/275) of the patients receiving surgery and adjuvant radiotherapy. Detailed description of the cohort is shown in Table 1.

Prevalence of human papillomavirus

Archival tissue from all 275 patients was analyzed for DNA of oncogenic mucosal HPV types by PCR (HPV-EIA). A total of 69 (25.1%) tested positive and was further characterized by ISH for mucosal high risk HPV. While cervical cancers used as positive controls for HPV ISH regularly displayed nuclear spots in tumor cells (Fig. 1A and B), this was not observed in any of the analyzed HPV-EIA-positive OSCC (Fig. 1C and D).

HPV DNA detection by PCR was not significantly associated with patient's gender, age, tumor localization, tumor grade or stage or the year of tumor diagnosis (Table 1).

Expression patterns of p16INK4a, proliferation (Ki-67 expression) of p16INK4a-expressing cells and correlation with HPV status

Expression of the cell cycle regulator p16INK4a was analyzed immunohistochemically in conjunction with the proliferation marker Ki-67 applying a dual-staining protocol. The majority of all tumors (211/275, 76.7%) did not express p16INK4a. A diffuse p16INK4a expression of the entire tumor area was observed in 6.2% (17/275) of all SCC (example in Fig. 2C and D). Seventeen percent (47/275) of the tumors displayed focal p16INK4a expression of cell clusters of varying extent, but not reaching the entire tumor area in a diffuse, continuous pattern (example in Fig. 2A and B). All 17 tumors with diffuse p16INK4a expression pattern contained cells co-expressing p16INK4a and Ki-67, while this was not observed in any of the 47 tumors with focal p16 expression patterns, where p16 expression appears restricted to the central tumor region and Ki-67 expression to the invasion front (Fig. 2). In 11 samples without p16INK4a expression in the tumor, p16INK4a was detectable in adjacent non-invasive cells.

Although diffuse p16INK4a expression correlated with HPV-EIA positivity (p < 0.001) and 11 of the 17 diffusely p16INK4a-positive tumors were HPV-EIA-positive, 6 of diffusely p16INK4a-positive tumors were HPV-negative and the majority of HPV-EIA-positive tumors did not diffusely express p16INK4a (Table 2). Focal p16 expression was observed to equal extents in HPV-EIA-positive and negative tumors (Table 2).

Prognostic significance of HPV and p16INK4a status

In order to assess whether tumor HPV DNA presence detected by EIA or tumor HPV DNA presence in diffusely p16INK4a-positive tumors only was associated with overall (OS) and disease-free survival (DFS), Kaplan–Meier plots were computed (Fig. 3). The mean follow-up time of the patients was 37 months (max 183.6, min 0.2). Neither the sole HPV status (OS p = 0.88, DFS p = 0.86) nor the combined HPV/diffuse p16INK4a expression status (OS p = 0.80, DFS p = 0.35) was significantly associated with overall and disease-free survival. When evaluating the impact of the p16INK4a expression status only, there was a trend towards better overall survival of patients whose tumor expressed p16INK4a in a

### Table 1

Characteristics of the 275 patients in relation to HPV and p16INK4a expression status.

<table>
<thead>
<tr>
<th>HPV-PCR</th>
<th>p16INK4a expression</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Diffuse</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>143</td>
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<tr>
<td>&gt;60 years</td>
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<tr>
<td>Male</td>
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<tr>
<td>Tumor localization</td>
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<td>Floor of mouth</td>
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<tr>
<td>Tongue</td>
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<tr>
<td>Mandibulalveolar</td>
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<td>Buccal mucosa</td>
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<td>Maxillar alveole</td>
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<td>Lip mucosa</td>
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<tr>
<td>Tumor grade</td>
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</tr>
<tr>
<td>G2</td>
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</tr>
<tr>
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<tr>
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</table>
focal pattern (=p16\textsuperscript{INK4a}-positive/Ki-67-negative cells) compared to no (\(p = 0.09\)) or diffuse p16\textsuperscript{INK4a} expression (\(p = 0.25\)).

Table 2  
Numbers of HPV-EIA-negative and positive tumors in relation to p16 expression status.

<table>
<thead>
<tr>
<th>p16 Immunohistochemistry</th>
<th>HPV-EIA Negative</th>
<th>Focal</th>
<th>Diffuse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>164</td>
<td>36</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>47</td>
<td>11</td>
<td>11</td>
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Discussion

In the present study a large cohort of patients with SCC located in the oral cavity from two German university hospitals was investigated for HPV in the tumor tissue by means of different approaches. Although viral DNA was detectable in 25% of the tumors by PCR using a HPV-EIA and thereby meeting frequencies reported from other studies\(^8\), no HPV was detectable using an ISH protocol commonly yielding clear signals in cervical cancers (10/10 positive in\(^8\)), its precursors (20/20 high grade dysplasia positive in\(^8\)), HPV16-associated oropharyngeal cancers (136/146...
positive in\textsuperscript{15}) and also in all cervical cancers included in each staining run in our study (example in Fig. 1A and B). Thus the results provide further evidence that the percentage of HPV-induced OSCC is most likely frequently overestimated when viral DNA is detected by PCR. Although it has to be recognized that ISH lacks maximum sensitivity and there have been tumors described in the literature where viral oncogene expression might suggest HPV-association but ISH was negative (4/24 OSCC in\textsuperscript{12}), ISH has the advantage that it is a highly specific test to visualize viral genomes directly in the tumor and can be used on older archival tumor material where RNA integrity is limited.\textsuperscript{21}

For tonsillar SCC immunohistochemical detection of p16\textsuperscript{INK4a} has shown promising performance in diagnosing HPV-associated cancers\textsuperscript{15}, however the more accurately the anatomic localizations were stratified in recent studies, the more the relevance of p16\textsuperscript{INK4a} overexpression for SCC in non-oropharyngeal sites, such as the oral cavity, appears less clear.\textsuperscript{12,22} Our results confirm that although the majority of OSCC are p16\textsuperscript{INK4a}-negative, p16\textsuperscript{INK4a} may be found overexpressed also in HPV-negative tumors (6/17 diffusely p16\textsuperscript{INK4a}-positive tumors) in a pattern mimicking the diffuse expression usually seen in HPV-associated tumors. Whether the 11 HPV PCR–EIA-positive diffusely p16\textsuperscript{INK4a}-positive tumors overexpress viral oncogenes could not be determined. However, absence of ISH signals in all of these tumors argues against a contribution of HPV in the majority of them. One might hypothesize that p16\textsuperscript{INK4a} expression in tumors which are not induced by HPV might be the consequence of a senescence-like tumor reaction and that p16\textsuperscript{INK4a} expressing cells in these tumors are therefore cell cycle arrested (Ki-67-negative). However, our results disprove this hypothesis as p16\textsuperscript{INK4a}-expressing cells that retained proliferation capacity (co-expressing p16\textsuperscript{INK4a} and Ki-67) were found in both, HPV-positive and negative tumors. While lacking cell cycle arrest in p16\textsuperscript{INK4a}-expressing cells in cervical cancers has been explained by HPV oncogenes that are known to disrupt the downstream players of p16\textsuperscript{INK4a} (retinoblastoma protein),\textsuperscript{18} it may be speculated that other oncogenic stimuli that disrupt this pathway may result in proliferating p16\textsuperscript{INK4a}-expressing cells. Interestingly, patients with focally p16\textsuperscript{INK4a}-expressing OSCC tended to have a better overall survival than patients with tumors lacking p16\textsuperscript{INK4a} expression (P = 0.09) or diffusely overexpressing p16\textsuperscript{INK4a} (p = 0.25) in the present study. As focally p16\textsuperscript{INK4a}-expressing tumors never showed p16\textsuperscript{INK4a}-positive cells co-expressing Ki-67, which indicates cell cycle arrest, it is conceivable that these tumors have successfully entered a senescence state, potentially resulting in slower tumor progression.
growth and better outcome. It will be important in future prospective studies to confirm this finding.

For oropharyngeal cancers involving the tonsil and base of tongue the prognostic relevance of the HPV status is compelling.6–8 With respect to the prognostic relevance of HPV in OSCC there is no such clear evidence in the literature, with few studies reporting a better outcome for HPV-positive patients.23–25 Few reporting a worse outcome26–28 and others finding no effect.29,30 The HPV PCR–EIA status and also the combined HPV/p16INKnAa status did not correlate with disease-free and overall survival in our study. Considering that the result of viral E6/E7 expression in single tumors located in the oral cavity described in the literature1,2,4 indicates HPV-driven cancers, it will be important to evaluate whether here the HPV status is of prognostic relevance – with the limitation of such studies that E6/7-positive cancers are considered as the gold standard for HPV-driven OSCC, which is unfortunately difficult to prove by additional evidence. Furthermore different E6/E7 assays are likely to perform heterogeneous in terms of their analytical characteristics and considering the estimated small fraction of HPV-induced oral cavity cancers large cohorts will have to be analyzed to evaluate the prognostic relevance.

In summary, we here add further evidence that HPV-attributable cancers in the oral cavity are rarely found although viral DNA by PCR has been detected in ours and other studies in a larger percentage in cancerous and also normal epithelium of the oral cavity.31 Apparently the induction of HPV-driven transformation occurs particularly at certain anatomic sites, the uterine cervix, the anal canal and tonsils, all of which are characterized by transition of squamous cell epithelium into glandular or reticular epithelium (tonsillar crypt). While on the one hand these zones might facilitate access of the virus to basal cells and thereby increasing the risk for infection in general, there are also results showing a distinct population of cells with unique morphology and gene-expression profile at the cervical transformation zone,32 which might play a role in controlling viral oncogene expression in these cells.

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