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

Miriam Reuschenbach [a,b,*], Katinka Kansy [c], Kira Garbe [a,b], Svetlana Vinokurova [a,b], Christa Flechtenmacher [d,e], Csaba Toth [d,e], Elena-Sophie Prigge [a,b], Oliver C. Thiele [c], Siegmar Reinert [f], Jürgen Hoffmann [c], Magnus von Knebel Doeberitz [a,b], Kolja Freier [c]

[a] Department of Applied Tumor Biology, Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 224, 69120 Heidelberg, Germany
[b] Clinical Cooperation Unit Applied Tumor Biology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 224, 69120 Heidelberg, Germany
[c] Department of Oral and Maxillofacial Surgery, University of Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany
[d] Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 224, 69120 Heidelberg, Germany
[e] National Center for Tumor Diseases (NCT) Tissue Bank, Im Neuenheimer Feld 221, 69120 Heidelberg, Germany
[f] Department of Oral and Maxillofacial Surgery, University of Tübingen, Osianderstr. 2, 72076 Tübingen, Germany

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SUMMARY

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 in situ hybridization (ISH) to detect high risk human papillomavirus (HPV). An immunohistochemical dual-staining for p16^{INK4a} and the proliferation marker Ki-67 was used to assess whether co-expression of p16^{INK4a}/Ki-67 is a better surrogate marker for HPV in OSCC than p16^{INK4a} alone, based on the hypothesis that combined p16^{INK4a} and Ki-67 expression might specifically discriminate oncogene-induced p16^{INK4a} expression from cell-cycle arrest-inducing senescence-associated p16^{INK4a} expression.

Results: HPV-DNA by PCR–EIA could be detected in 25.1% (69/275) of the tumors, but ISH was negative in all of them. Diffuse p16^{INK4a} overexpression was detected in 11 HPV PCR-positive tumors, but also in 6 HPV PCR-negative tumors. p16^{INK4a}-expressing cells in diffusely positive tumors co-expressed Ki-67, irrespective of the HPV status. Neither the sole HPV status nor combined HPV/p16^{INK4a} status nor the sole p16^{INK4a} status was significantly associated with disease free or overall survival, however a trend towards better overall survival of patients whose tumor expressed p16^{INK4a} in a focal pattern (=p16^{INK4a}-positive/Ki-67-negative cells) compared to no p16^{INK4a} expression ($p = 0.09$) 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. p16^{INK4a}/Ki-67 co-expression is detectable in a fraction of OSCC irrespective of the HPV status.

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.[1,2] HPV-induced malignant transformation requires the overexpression of the viral oncogenes E6 and E7[3] 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[4,5] 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.[6–8]

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.[9] Tobacco and alcohol are strong risk factors and genetic alterations, such as affecting *CDKN2A* are

Abbreviations: DFS, disease-free survival; EIA, enzyme immunoassay; HPV, human papillomavirus; HR-HPV, high-risk human papillomavirus; ISH, in situ hybridization; OS, overall survival; OSCC, squamous cell cancer of the oral cavity; PCR, polymerase chain reaction; SCC, squamous cell cancer.

* Corresponding author at: Department of Applied Tumor Biology, Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 224, 69120 Heidelberg, Germany. Tel.: +49 6221 56 35591; fax: +49 6221 56 5981.

E-mail address: miriam.reuschenbach@med.uni-heidelberg.de (M. Reuschenbach).

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important carcinogenic drivers.[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.[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,[12] although there is no clear evidence yet on which is the gold standard test to identify HPV-driven O SCC. Detection of transcriptionally active viral oncogenes 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,[13] 5.9% in 409 patients from the USA[12] to 14.7% in 68 patients from Japan.[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.[15,12] Furthermore detection of $p16^{INK4a}$ overexpression is discussed as a marker for HPV-driven head and neck cancers due to the indirect transcriptional activation of $p16^{INK4a}$ expression by the HPV E7 oncoprotein.[16,17] However, while for tonsillar cancers $p16^{INK4a}$ overexpression has been described strongly associated with HPV and is discussed as a simple diagnostic tool to identify HPV-associated tonsillar SCC,[15] data for OSCC are less clear and one recent study reports that over 50% of $p16^{INK4a}$-positive OSCC are negative for HPV E6/7 expression.[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 $p16^{INK4a}$ and the proliferation marker Ki-67 in order to assess whether co-expression of $p16^{INK4a}$/Ki-67 is a better surrogate marker for HPV in OSCC than $p16^{INK4a}$ alone, based on the hypothesis that combined $p16^{INK4a}$ and Ki-67 expression might specifically discriminate oncogene-induced $p16^{INK4a}$ expression from cell-cycle arrest inducing senescence-associated $p16^{INK4a}$ expression[18] and (c) to evaluate the prognostic impact of the HPV and $p16^{INK4a}$/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[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, 35, 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 GP5+6+-EIA-positive tumors. The GenPoint (Dako, Glostrup, Denmark) HPV probe set hybridizing DNA from oncogenic types 16, 18, 31, 33, 35, 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 $p16^{INK4a}$/Ki-67

FFPE sections from all tumors were analyzed for $p16^{INK4a}$ and Ki-67 expression using the CINtec® 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.[18] The staining is based on two monoclonal antibodies, one directed to human $p16^{INK4a}$ protein (clone E6H4™) and the other to human Ki-67 protein (clone 274-11 AC3). $p16^{INK4a}$ 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 $p16^{INK4a}$ expression patterns (diffuse = continuous clonal 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 $p16^{INK4a}$ in the tumor cells) as well as the presence of cells co-expressing $p16^{INK4a}$ 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