Genetic Signatures of HPV-related and Unrelated Oropharyngeal Carcinoma and Their Prognostic Implications

Jens P. Klussmann,1 Jeroen J. Mooren,5,6 Martin Lehn,1 Sandra M.H. Claessen,5 Markus Stenner,1 Christian U. Huebbers,1,3 Soenke J. Weissenborn,5 Inga Wedemeyer,2 Simon F. Preuss,1 Jens P. Klussmann, Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University of Cologne, Kerpenerstr. 62, 50924 Cologne, Germany. Phone: 49-221-478-4750; Fax: 49-221-478-7449; E-mail: peter.klussmann@uni-koeln.de.

Author’s Affiliations: Departments of 1Oto-Rhino-Laryngology, Head and Neck Surgery, and 2Pathology, 3Jean-Uhrmacher Institute, 4Institute of Virology, University of Cologne, Cologne, Germany; and Departments of 5Molecular Cell Biology and 6Otorhinolaryngology, Head and Neck Surgery, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, the Netherlands.

Received 6/5/08; revised 11/1/08; accepted 11/4/08; published OnlineFirst 02/17/2009.

Grant support: Jens P. Klussmann, Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University of Cologne, Kerpenerstr. 62, 50924 Cologne, Germany. Phone: 49-221-478-4750; Fax: 49-221-478-7449; E-mail: peter.klussmann@uni-koeln.de.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Jens P. Klussmann, Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University of Cologne, Kerpenerstr. 62, 50924 Cologne, Germany. Phone: 49-221-478-4750; Fax: 49-221-478-7449; E-mail: peter.klussmann@uni-koeln.de.

© 2009 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-08-1463

Abstract

Purpose: Patients with human papillomavirus (HPV)-containing oropharyngeal squamous cell carcinomas (OSCC) have a better prognosis than patients with HPV-negative OSCC. This may be attributed to different genetic pathways promoting cancer.

Experimental Design: We used comparative genomic hybridization to identify critical genetic changes in 60 selected OSCC, 28 of which were associated with HPV-16 as determined by HPV-specific PCR and fluorescence in situ hybridization analysis and positive p16INK4A immunostaining. The results were correlated with HPV status and clinical data from patients.

Results: Two thirds of OSCC harbored gain at 3q26.3-qter irrespective of HPV status. In HPV-negative tumors this alteration was associated with advanced tumor stage (P = 0.013). In comparison with HPV-related OSCC, the HPV-negative tumors harbored: (a) a higher number of chromosomal alterations and amplifications (P = 0.03 and 0.039, respectively); (b) significantly more losses at 3p, 5q, 9p, 15q, and 18q, and gains/amplifications at 11q13 (P = 0.002, 0.03; 0.001, 0.02, 0.004, and 0.001, respectively); and (c) less often 16q losses and Xp gains (P = 0.02 and 0.03). Survival analysis revealed a significantly better disease-free survival for HPV-related OSCC (P = 0.02), whereas chromosome amplification was an unfavorable prognostic indicator for disease-free and overall survival (P = 0.01 and 0.05, respectively). Interestingly, 16q loss, predominantly identified in HPV-related OSCC, was a strong indicator of favorable outcome (overall survival, P = 0.008; disease-free survival, P = 0.01) and none of these patients had a tumor recurrence.

Conclusions: Genetic signatures of HPV-related and HPV-unrelated OSCC are different and most likely underlie differences in tumor development and progression. In addition, distinct chromosomal alterations have prognostic significance.

In the western world, head and neck squamous cell carcinomas account for ~5% of all cancers. Particularly the annual incidence of cancers developing in the oropharynx, oropharyngeal squamous cell carcinomas (OSCC), is still increasing, whereas the incidence at other sites is declining (1, 2). An estimated rate of >35,000 new cases of OSCC are expected in the United States in 2008, and ~7,000 patients are expected to die from the disease (3). Despite the implementation of multimodal treatment regimes in recent years, the prognosis of OSCC has only slightly been improved (4, 5). Identification of the molecular mechanisms underlying the carcinogenesis of OSCC, therefore, seems crucial to enable the development of new therapeutic agents.

OSCC form an interesting group of head and neck cancers to focus on, because in the past decades molecular and epidemiologic studies have revealed that 25% to 50% of these tumors are associated with oncogenic human papillomavirus (HPV; refs. 6–8). High-risk sexual behavior has particularly been related to the development of this group of OSCC (9). The remaining group of OSCC is predominantly associated with the risk factors of tobacco smoking and alcohol consumption, also referred to as chemical/toxin-induced OSCC. In contrast to the finding that the majority of patients harboring HPV-DNA-containing carcinomas present with an advanced stage of the disease, there is growing evidence that these patients have a favorable prognosis (10–13). In addition, differences in the expression of cell cycle and survival proteins (14–16), and TP53 gene mutation status (17–19) have been identified.
Translational Relevance

In this study we have identified specific chromosomal alterations which are associated with HPV- or chemically-induced oropharyngeal squamous cell carcinomas (OSCC). This underlines the biological differences between both tumor subgroups. Due to its prognostic implication, this finding already has clinical relevance.

Today a clinical study on OSCC without differentiating between HPV-related and HPV-unrelated cancer cannot be recommended. There is evidence that HPV-related OSCC might be more radiosensitive than the HPV-unrelated OSCC caused by smoking and alcohol. This, however, has to be shown in prospective clinical trials. To distinguish HPV-related and HPV-unrelated OSCC is even more compelling as surgery with postoperative radiation versus definitive chemoradiation are competing strategies to treat OSCC.

Furthermore, our study provides insight into some crucial genetic events in these different tumor entities, which might be important for the improvement of treatment decisions, prediction of response to therapy, as well as the development of new target therapies in the future, as so far the followed therapeutical strategies are largely dependent on tumor stage.

Materials and Methods

Subjects and material. For this study we selected from previous studies 29 HPV16-DNA- positive OSCC and 31 HPV-negative tumors from patients treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery of the University of Cologne from 1997 to December 2005 on the basis of the following criteria: (a) availability of sufficient fresh-frozen tumor tissue with ≥70% tumor cells and high quality and enough tumor DNA, (b) availability of clinical follow-up for ≥2 y, and (c) assessment of the presence or absence of HPV sequences. HPV DNA was detected with a highly sensitive group-specific nested PCR with degenerate primers A5/A10 and A6/A8 (36). HPV typing was done as described previously (8). A subset of 20 HPV16-positive cases was confirmed by fluorescence in situ hybridization using HPV16-specific DNA probes as previously described (Fig 1A; refs. 12, 17).

Patient ages ranged from 43 to 79 y (median, 60 y). Forty-seven patients were males (78%) and 13 were females (22%). Written, informed consent was obtained from all patients. Tumor staging was assessed according to the 2002 American Joint Committee on Cancer staging criteria (37). Three percent of the patients presented at stage I, 12% at stage II, 15% at stage III, 53% at stage IVA, and 12% at stage IVC. Three patients (5%) had distant metastases (stage IVc) at the time of diagnosis. Histologic grading was done in a blind manner following the WHO criteria for squamous cell carcinomas of the oral mucosa (38). Patients were treated with multimodal treatment regime as previously reported (5). Stratification of tumor stage, patient’s age, and treatment within HPV-related and -unrelated tumors revealed equal distribution and no significant differences. To include other major risk factors for OSCC in our analysis we evaluated history of tobacco smoking and alcohol consumption from chart reviews. Total cumulative tobacco exposure was expressed in pack-years, where one pack-year is equivalent to smoking one pack of cigarettes a day for one year. To dichotomize this parameter patients were divided into the categories smoker and non-smoker having history of more or less than 1 pack-year in the last 10 years. Alcohol exposure was defined as the average number of drinks per week. Follow-up data were collected at periodic visits in 4- to 6-mo intervals at our outpatient departments.

Immunohistochemical staining. To confirm PCR-based HPV status p16INK4A immunohistochemical detection of expression was done as described previously (18). Two- to three-micrometer sections of formalin-fixed, paraffin-embedded biopsy samples were processed by the avidin-biotin-peroxidase method (ChemMate Detection kit: Dako Cytomation) using a primary antibody against p16INK4A (Ab-4, clone 16P04 Neo Markers). HPV16-positive cervical carcinoma specimens were used as positive control. Strong and diffuse nuclear as well as cytoplasmic staining in ≥60% of the tumor cells was regarded as overexpression. Only cases with positive HPV-PCR and p16 overexpression in their tumors were regarded as HPV-related OSCC (see below).

CGH. CGH was done with DNA isolated from tumor samples as previously described (39). Briefly, 2 μg tumor DNA was labeled with Spectrum Green-dUTPs (Abbott Molecular) by nick translation (BioNick labeling kit; Life Technologies). Spectrum Red-labeled normal and sex-matched reference DNA (Life technologies) was used for hybridization. The hybridization mixture consisted of 800 ng Spectrum Green-labeled tumor DNA, 800 ng Spectrum-Red labeled reference DNA, and 10 μg human Cot-1 DNA (Life Technologies) dissolved in 12 μL of hybridization buffer (50% formamide, 2×SSC, 10% dextran sulfate, pH 7.0). Hybridization was carried out for 3 d at 37°C to denature (5 min at 75°C in 2×SSC, pH 7.0), followed by 5 min in 70% formamide/2×SSC, pH 7.0) normal male human metaphase spreads (Abbott Molecular). Slides were washed twice at 45°C for 5 min in 50% formamide/2×SSC, pH 7.0, followed by dehydration in an ethanol series. The chromosomes were counterstained with 0.2 μg 4,6-diamidino-2-phenylindole per μL Vectashield (Vector laboratories) for identification.

Comparing HPV-positive with HPV-negative OSCC. Recently, our group was able to show that p16INK4A (CDKN2A gene product) is highly expressed in HPV-related OSCC (12, 20, 21) and is a reliable surrogate marker to identify these tumors (11). Therefore, p16INK4A immunostaining and subsequent HPV-PCR have been recommended to detect HPV-related OSCC (22). All together these findings lead to the hypothesis that at least two genetic routes underlie the development of OSCC (23, 24).

The development of chemical/toxin-induced OSCC seems to occur via accumulation of distinct (epi)genetic changes. Deletions at chromosomes 3p and 9p21 are already found in premalignant lesions (25, 26), whereas further carcinogenesis is associated with losses at 4q, 5q, 7q, 8p, 13q, 17p, 18q, 21q, and 22q, and gains at 1q, 2q, 3q26, 5p, 7p, 8q, 9q, 11q13, and 20q (27–29). The target genes for some alterations are known, such as the CDKN2A gene at 9p21, TP53 at 17p13, and SMAD4 at 18q21. Together these findings lead to the hypothesis that at least two genetic routes underlie the development of OSCC (23, 24).

In contrast, HPV-related cancers harbor gains of 1q in early stages of tumor development and gains of 3q, 5p, and 8q, and losses of 2q, 3p, 4q, 11q, and 19p later in tumor progression (32–34). Gain of 3q has been correlated to integration of HPV DNA into the cellular genome and progression to cancer in uterine cervical premalignant lesions (35). Therefore, it can be speculated that the genetic signature of chemical/toxin-induced and HPV-related OSCC also differ with respect to quantity and chromosomal location of genetic changes.

In this study, we analyzed a series of 60 OSCC, of which the HPV status and the clinical follow-up were available, for DNA copy number changes by genome-wide comparative genomic hybridization (CGH). Results were correlated with clinicopathologic characteristics, smoking and alcohol intake, and disease outcome.
Digital images were collected from at least 10 metaphases using the Metasystems Image Pro System black and white CCD camera (Altluessheim) mounted on top of a Leica DMRE fluorescence microscope, equipped with 4’6-Diamidino-2-phenylindole, Spectrum Green, and Spectrum Red filter sets. The software Metasystems ISIS 4.4.25 program was used to calculate average green-to-red ratio profiles for each chromosome. At least 10 observations per autosome and 5 observations per sex chromosome were included in each analysis. Gains and losses of DNA sequences were defined as chromosomal regions where the mean green-to-red fluorescence ratio was >1.20 and <0.80, respectively. Overrepresentations were considered amplifications when the fluorescence ratio values in a subregion of a chromosomal arm exceeded 1.5. In negative control hybridizations, the mean green-to-red ratio occasionally exceeded the fixed 1.2 cutoff level at the following chromosomal regions: 1p32-pter, 16p, 19, and 22. Gains of these G-C-rich regions were therefore excluded from all analyses.

Statistical analysis. Contingency table analysis was used to analyze the relationship between HPV/p16 INK4A-status and genomic alterations with the SPSS Base System, version 14.0 (SPSS). Student’s t test and ANOVA were applied to compare the number of genomic alterations in different groups. A significance level of $P \leq 0.05$ was chosen. Multivariate logistic regression was done to assess the association between HPV/p16 INK4A status and genomic alterations while controlling for clinicopathologic factors and potential confounders.

Disease-free and overall survival rates were estimated using the Kaplan-Meier algorithm for incomplete observations. Three patients were excluded from survival analysis due to having stage IVc disease. The overall survival time was defined as the interval between the date of diagnosis and the last date when the patient was known to be alive (censored) or date of death for any reason (uncensored). The disease-free survival was measured as the period of time between the date of diagnosis and the date of the last follow-up examination, where the patient was disease-free (censored), or the date of first recurrence independently if it was a local, regional, or distant recurrence (uncensored). The log rank test was used to test for differences between subgroups. All $P$ values were considered statistically significant if $P < 0.05$ in two-sided tests.

Results

Clinicopathological data and p16 INK4A expression. To further determine HPV-association p16 INK4A immunostaining was done on paraffin-embedded tissue sections of 57 OSCC, of which 33 (55%) were positive (Fig. 1B). As expected there was a highly significant correlation of HPV-DNA detection by PCR and p16 INK4A expression ($P < 0.001$). Twenty-eight (97%) of the HPV-positive tumors showed strong p16 INK4A expression and these HPV/p16 INK4A-positive tumors were classified as HPV-related tumors for further analysis. The remaining 32 cases were summarized as HPV-unrelated. There was no significant

Fig. 1. Representative examples of fluorescence in situ hybridization analysis showing one HPV16-specific punctate signal per nucleus indicating viral integration in the cellular genome (A) and p16 INK4A immunostaining (B) with strong p16 INK4A overexpression on paraffin-embedded tissue sections of OSCC. DNA copy number changes in HPV-related (C) and unrelated (D) OSCC as identified by CGH. Chromosome ideograms: right, gains; left, losses. Bold lines, amplifications.
difference of staging between both groups, but there was a significant association of the HPV-related cancers with poorer differentiation of the tumor \( (P = 0.05; \text{Table 1}) \). HPV-related tumors were more likely located in the tonsils or in the base of the tongue compared with other parts of the oropharynx \( (P < 0.001; \text{Table 1}) \). Evaluation of the risk factors of smoking and alcohol use as categorized variables showed a significant lower exposure for nicotine and alcohol for patients with HPV-related OSCC \( (P = 0.006 \text{ and } P < 0.001, \text{respectively}; \text{Table 1}) \).

**CGH analysis.** The CGH results are presented in Fig. 1C and D and Table 1. The total number of chromosomal aberrations per tumor was significantly lower in the HPV-related \( (8.9 \pm 4.9) \) than in the HPV-unrelated group \( (12.1 \pm 5.9; P = 0.03) \), as was also observed for amplifications \( (14\% \text{ versus } 38\%; P = 0.039) \). The HPV-unrelated tumors particularly harbored amplifications at 11q13 and 18p, whereas amplifications at chromosome 3q were detected in both groups \( (3q26.3-qter \text{ as the smallest region of involvement}) \). Gain of 3q was the most frequent chromosomal alteration in OSCC \( (72\%) \) independent of HPV status and was significantly associated with advanced T- and tumor stage in the total group \( (T_1/T_2 \text{ versus } T_3/T_4, P = 0.042; \text{I/II versus III/IV, } P = 0.035) \) and in the HPV-unrelated group of tumors \( (T_1/T_2 \text{ versus } T_3/T_4, P = 0.028; \text{I/II versus III/IV, } P = 0.013) \).

Losses of 3p, 4q, 9p, and 13q, and gains of 3q, 8q, and 17q were found in \( >33\% \) of all OSCC. The most frequent genetic changes in \( >30\% \) HPV-unrelated cancers were losses at 3p, 4q, 5q, 9p, 13q, 18q, and Y, and gains at 3q, 8q, 11q13, 12q, 17q, and 20q. In the HPV-related group losses at 3p, 4q, 11q14-qter, and 13q, and gains at 3q, 8q, 17q, and 20q were identified in \( >30\% \) of tumors. Significant differences in the occurrence of chromosomal changes between HPV/p16 \(^{\text{INK4A}} \) -positive and -negative tumors were observed for gains at 11q13 and Xp and for losses at 3p, 5q, 9p, 11q14-qter, 15q, 16q, and 18q \( (\text{Table 1}) \). To determine possible confounders we analyzed the DNA copy number changes comparing HPV-related and HPV-unrelated OSCC while correcting for tumor stage, age, and gender (model 1) and for tumor stage, age, gender, and tobacco and alcohol consumption (model 2) by using multivariate logistic regression. In model 1 differences remained significant for most chromosomal aberrations \( (\text{Table 1}) \). Adjusting in addition for tobacco and alcohol use revealed still significant differences for 3p, 9p, and 18q loss \( (\text{Table 1}) \) but excluded 11q13 gain as a discriminating genetic alteration because it strongly correlated with exposure to these carcinogens \( (\text{data not shown}) \).

**Survival analysis.** The median follow-up time was 27.5 months with a maximum of 111 months. Tumor relapse developed in 38\% of the study population and 38\% of the patients died during observation time. The mean disease-free survival time after first diagnosis was 31.3 \pm 3.5 months and the mean overall survival time was 37.0 \pm 3.8 months. HPV-related OSCC showed a significantly better 5-year disease-free survival rate compared with HPV-unrelated OSCC \( (71\% \text{ versus } 46\%; P = 0.02; \text{Table 2, Fig. 2A}) \). Overall survival did not significantly differ between both OSCC groups \( (\text{Fig. 2B}) \). Using HPV-DNA detection by PCR for stratification yielded similar results \( (\text{data not shown}) \).

**Table 1. Clinical and genomic differences between HPV-unrelated and related OSCC**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total ( n (%) )</th>
<th>HPV-unrelated ( n (%) )</th>
<th>HPV-related ( n (%) )</th>
<th>( P^* )</th>
<th>( P^\dagger )</th>
<th>( P^\ddagger )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
<td>60 (100)</td>
<td>32 (53)</td>
<td>28 (47)</td>
<td>&lt;0.001</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Alcohol use(^1)</td>
<td>24 (40)</td>
<td>20 (63)</td>
<td>4 (14)</td>
<td>&lt;0.001</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Tobacco use(^2)</td>
<td>48 (80)</td>
<td>30 (94)</td>
<td>18 (64)</td>
<td>0.006</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Tumor grading(^3)</td>
<td>30 (51)</td>
<td>12 (39)</td>
<td>18 (64)</td>
<td>0.05</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Tumor location**(^4)</td>
<td>46 (77)</td>
<td>19 (59)</td>
<td>27 (96)</td>
<td>&lt;0.001</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Genetic alterations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of at least one amplification</td>
<td>16 (27)</td>
<td>12 (38)</td>
<td>4 (14)</td>
<td>0.04</td>
<td>0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td>Gains or amplifications</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11q13</td>
<td>16 (27)</td>
<td>14 (44)</td>
<td>2 (7)</td>
<td>0.001</td>
<td>0.003</td>
<td>N.S.</td>
</tr>
<tr>
<td>Xp</td>
<td>7 (12)</td>
<td>1 (3)</td>
<td>6 (21)</td>
<td>0.03</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Losses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3p</td>
<td>34 (57)</td>
<td>24 (75)</td>
<td>10 (36)</td>
<td>0.002</td>
<td>0.008</td>
<td>0.02</td>
</tr>
<tr>
<td>5q</td>
<td>19 (32)</td>
<td>14 (44)</td>
<td>5 (18)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>9p</td>
<td>23 (38)</td>
<td>20 (63)</td>
<td>3 (11)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>11q14-qter</td>
<td>16 (27)</td>
<td>5 (16)</td>
<td>11 (39)</td>
<td>0.04</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>15q</td>
<td>5 (8)</td>
<td>5 (16)</td>
<td>0 (0)</td>
<td>0.02</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>16q</td>
<td>10 (17)</td>
<td>2 (6)</td>
<td>8 (29)</td>
<td>0.02</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>18q</td>
<td>11 (18)</td>
<td>10 (31)</td>
<td>1 (4)</td>
<td>0.004</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Abbreviations: N.D., not done; N.S., not significant.

*Univariate analysis.

†Multiple regression adjusting for age, gender, and TNM stage.

‡Multiple regression adjusting for age, gender, TNM stage, and alcohol and tobacco use.

\(^\dagger\)Alcohol exposure includes beer, wine, and liquor. Alcohol equivalents (drinks) were defined as one 12-ounce can/bottle of beer = one 4-ounce glass of wine = one 1 1/2 ounce shot of liquor \( \approx 12 \text{ g alcohol} \). Variable was categorized > 5 drinks/wk.

\(^\ddagger\)1 pack-year in the last 10 y.

**Tumor located in tonsils or base of tongue compared to other parts of the oropharynx.**
To determine the impact of genetic changes on prognosis in OSCC patients all chromosomal alterations were used for stratification in Kaplan-Meier analysis. Chromosome amplification independent of its location was a significant predictor for a decreased 5-year disease-free and overall survival in all patients ($P = 0.01$ versus $P = 0.05$; Table 2; Fig. 2C and D) and within the HPV-related cancers ($P = 0.03$ versus $P = 0.04$). Detection of a 11q13 amplification was an indicator for unfavorable overall survival (29% versus 62%; $P = 0.02$; Table 2), which was found with one exception in HPV-unrelated cancers (Fig. 1C and D).

Patients with 16q loss in their tumor showed a strongly improved 5-year disease-free and overall survival ($P = 0.008$ and $P = 0.01$, respectively; Fig. 2E and F), which was also significant in HPV-related cancers ($P = 0.05$ and $P = 0.04$). Interestingly, 9p loss also proved to be an indicator for favorable prognosis in this group, despite the small number of tumors exhibiting this alteration ($P = 0.04$ and $P < 0.0015$ for 5-year disease-free and overall survival, respectively). Within the group of HPV-unrelated cancers 3p loss was an indicator for an improved 5-year disease-free survival rate (55% versus 19%; $P = 0.04$). In addition, patients with OSCC containing 11p loss or Xp gain and nonsmoker tended to show a favorable prognosis, which, however, did not reach statistical significance (data not shown).

### Discussion

Head and neck cancers are a heterogeneous group of malignancies in terms of etiology, biological behavior, and prognosis (40). Tobacco and alcohol consumption are the main risk factors for these cancers, and models of sequential accumulation of (epi)genetic changes have been introduced (41). There is also molecular evidence that a significant proportion of these cancers are associated with oncogenic HPV, particularly those originating from the oropharynx. Our study represents the most comprehensive assessment of chromosomal alterations in relation to HPV status and prognosis in tumors detected in this anatomical site. HPV-related cancers were defined as being HPV-DNA–positive using PCR and p16 INK4A immunostaining, as recommended recently (22).

We found that per tumor the total number of chromosomal alterations as well as amplifications was significantly lower in the HPV-related than in the HPV-unrelated OSCC. This finding is in accordance with previous molecular genetic reports (42, 43) and with the assumption that due to the inactivation of the tumor suppressor proteins p53 and pRb by the viral E6 and E7 oncoproteins, respectively, the number of required genetic alterations for a malignant phenotype is lower in HPV-driven tumorigenesis. Moreover, a recent study using uterine cervical cancer specimens has pointed out that a substantial number of lesions show integrated HPV DNA in (near) diploid rather than aneuploid cells (44). Preliminary data on a series of 20 tumors suggest that this is also the case in most HPV-related OSCC when compared with HPV-unrelated cases.7

Although in a previous CGH study on 25 tonsillar carcinomas 3q gain was associated with HPV presence (43), in our series of 60 tumors and a recently reported series of 24 tumors (42) this alteration was found to be the most frequent in both HPV-related and HPV-unrelated tumors. Gains in 3q copy number are frequently found in squamous cell carcinomas, including those originating from the head and neck, lung, vulva, and uterine cervical mucosa (27, 32, 44–46). This alteration has been implicated as a key event in the

---

7 Unpublished results.
progression from severe dysplasia to invasive cancer in lung and uterine cervical tumors (32, 46). For OSCC, however, it remains to be determined if 3q gain is a marker for progression (12). Our data indicate that 3q gain is a late event in HPV-unrelated OSCC, because 3q gain did correlate with advanced T- and tumor stage in this subgroup. The regions of copy number gain ranged in size from the whole 3q arm to the smallest region of involvement at 3q26.3-qter. Of the numerous genes on chromosome 3q that have been implicated in cancer, ATR at 3q23 is a candidate. ATR has been shown to induce loss of
Genetic Signatures of HPV-related and Unrelated OSCC

differentiation, aneuploidy, and to eliminate radiation-induced G_{1} arrest upon duplication in rhabdomyosarcomas (47), features also recognized in OSCC. Most reported candidate genes, however, reside in the 3q26.3-qter region, which we found amplified in six tumors, and is also frequently amplified in lung tumors (46). These include, among others, PKCgCA, TP63, TERC, DCUN1D1, LAMP3, and RPL35A (44, 46, 48–51). More studies are needed to indicate which of these genes are pivotal in HPV-related and HPV-unrelated OSCC pathogenesis.

We noticed that the most significant genetic differences between HPV-related and HPV-unrelated OSCC included 11q13 gains and 3p, 9p, and 18q losses, which occurred more frequently in the latter group. Increased 11q13 copy numbers were identified in 44% of the HPV-unrelated OSCC, a frequency which is often detected in chemical/toxin-induced head and neck cancer (28, 31, 42, 52, 53). In our study population 64% of the patients with HPV-related cancers were smokers, which could confound our results, but only 6 (21%) of these patients were heavy smokers with the lifetime history of more than 24 pack-years and none of them had a 11q13 gain/amplification in their tumor. In contrast, in the HPV-unrelated OSCC we did find a significant association between 11q13 gain and tobacco and alcohol consumption. The best candidate genes in the gained 11q13 region, whose products have been shown to be overexpressed, include CCND1, CTTN, and FADD resulting in deregulation of cell cycle control and migration. Because cyclin D1 up-regulation is ineffective in inactivating pRb in virus-driven tumorigenesis, which uses E7 for this purpose, 11q13 amplification is seldom observed in HPV-related OSCC. Some studies suggest that 11q13 amplification is caused by breakage-fusion-bridge cycles initiated at the chromosomal fragile site FRA11F (54) and is often associated with loss of material of distal 11q (55). Fragile sites have also been frequently observed as sites for HPV-DNA integration (56) resulting in alterations of genomic structure. For example, in cervical cancer a high percentage of 11q22 loss has been reported (57, 58). In our study we also found 11q losses more frequent in the HPV-related OSCC and most were distal to 11q22. This difference could not be detected in either of two previous CGH studies (42, 43). Several DNA repair genes, such as ATM and CHEK1, are located on chromosome 11q22-q24, and gene inactivation might underlie the genomic instability observed in HPV-related cancers.

Deletions at 3p and 9p have been observed in more than two thirds of head and neck squamous cell carcinomas (25, 28) and were in our study predominantly detected in the HPV-unrelated OSCC. Therefore, the suggestion that in HPV-negative OSCC 3p and 9p loss is sporadically detected by CGH, as stated previously (43), cannot be confirmed. Among the target tumor suppressor genes for these loci are FHIT at 3p14.3 and CDKN2A at 9p21. FHIT is located at the frequently altered fragile site FRA3B and its inactivation has been linked to deregulation of cell signaling pathways, including nuclear factor-x-β, AKT-Survivin, and Src. The observation that 3p loss is also detected in a subgroup of HPV-related OSCC might relate to the fact that FRA3B is an integration hot spot for HPV (33). Inactivation of p16INK4A is an early and frequent event in head and neck squamous cell carcinoma leading to the disruption of pRb cell cycle control. In our study 9p loss coincided with 11q13 gain/amplification (P = 0.003) underscoring deregulation of this pathway in chemical/toxin-induced OSCC. In the HPV-related tumors HPV-E7 interferes with this pathway and as a consequence, p16INK4A inactivation is not required and overexpression of the protein is usually not detected (11, 20, 21).

In accordance with previous reports by us and others (11–13, 20), HPV-related OSCC showed a favorable prognosis. Interestingly, different genetic changes also proved to be significant indicators of clinical outcome, such as chromosome amplification at 3q, 11q13, and 18p predictive for unfavorable and 16q deletions predictive for favorable patient survival. The latter finding has also been reported for breast and prostate cancer (59, 60). Although it is unlikely that E-cadherin inactivation is related to 16q deletion, because of its correlation with poor prognosis (61), our finding might be linked to the common fragile site FRA16D, located at 16q23.2 and containing the WWOX gene (62). The relatively high occurrence of 16q loss in HPV-positive tumors suggests the use of FRA16D as a HPV integration site that, as described above, might lead to extended loss of 16q DNA. In our study population we also noticed a strong correlation between a worse disease-free survival and (a) 3p loss in HPV-unrelated OSCC, and (b) 9p loss in HPV-related tumors. Although the number of tumors harboring these deletions in the different subsets was small, both alterations have been associated with an increased risk of malignancy in head and neck precursor lesions (25). Future studies are needed to validate these findings in larger series of OSCC in relation to HPV.

In conclusion, we have identified specific DNA copy number changes that are associated with HPV- or chemically-induced OSCC, and with prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Genetic Signatures of HPV-related and Unrelated Oropharyngeal Carcinoma and Their Prognostic Implications

Jens P. Klussmann, Jeroen J. Mooren, Martin Lehnen, et al.


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/15/5/1779

Cited articles  This article cites 58 articles, 11 of which you can access for free at: http://clincancerres.aacrjournals.org/content/15/5/1779.full.html#ref-list-1

Citing articles  This article has been cited by 9 HighWire-hosted articles. Access the articles at: /content/15/5/1779.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.