HPV Status and Overall Survival of Patients with Oropharyngeal Squamous Cell Carcinoma – A Retrospective Study of a German Head and Neck Cancer Center

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Abstract. *Background/Aim: A change in epidemiology of head and neck squamous cell carcinoma (HNSCC) has been noticed: while overall incidence has decreased, the incidence of oropharyngeal SCC (OSCC) has been increasing over the past decades. A growing body of evidence suggests a causative role of the human papillomavirus (HPV) as an independent risk factor in development of OSCC. The aim of this study was to determine the HPV status in all OSCC specimens collected in our biological database since 1988, correlating the results with overall survival, and to compare them with the current literature data. Patients and Methods: A total of 104 tumor samples were obtained and included in this study. Patient records were reviewed. HPV status was determined by a two-step polymerase chain reaction (PCR) combined with p16 immunohistochemistry. Statistical analysis was performed with BiAS™. Results: Overall 12 (12%) of the 104 tumor samples were HPV-positive. Most of the patients had advanced disease [(UICC) stage III or IV)]: 91.7 % in the HPV-positive group versus 78.2% in the HPV-negative group. Multivariate analysis showed that HPV status (p=0.04), UICC stage (p=0.01) and age at initial diagnosis (p=0.0006) were all independent determinants of overall survival. A positive HPV status (hazard ratio=0.52; 95%) was associated with a 48% increase of overall survival compared to patients with HPV-negative tumors. Conclusion: Our findings confirm a prevalence of HPV-positive tumors within OSCC. Due to its epidemiologic and prognostic relevance, HPV status should be considered an important part of tumor staging. For this purpose, HPV detection via two-step PCR combined with p16 immunohistochemistry seems reliable.*

Over the past decades, the epidemiology of head and neck squamous cell carcinoma (HNSCC) has changed dramatically. While a consistent decrease in incidence of HNSCC has been noticed, the incidence of oropharyngeal squamous cell carcinoma (OSCC) in younger patients without alcohol or tobacco abuse history has increased (1-3). The causative role of the human papillomavirus (HPV) is discussed as one possible reason for the growing number of OSCCs. It is estimated that approximately 25-36% of all OSCC cases are associated with high-risk HPV infection (4, 5). Furthermore several studies suggest that HPV is an independent risk factor for OSCC development (6).

Nearly 200 different HPV types have been identified to date. Based on clinical manifestation, they are classified into cutaneous and mucosal HPV. Both groups are further subdivided into high-risk and low-risk HPV according to their carcinogenic potential. In more than 90% of cases, high-risk HPV type 16 can be found in OSCC. With regard to their anatomical site, most HPV-positive OSCCs were located in the tonsils and in the base of the tongue (4, 7). HPV can be detected in the oropharyngeal tumor localized in cell nuclei, transcriptionally active, and is not found in the surrounding benign tissue (8). The expression of the high-risk HPV oncogenes E6 and E7 plays a crucial role in HPV-induced oncogenesis. E6 leads to ubiquitination and proteolytic degradation of the tumour suppressor protein p53, whereas E7 causes a functional inactivation of the tumour-suppressor retinoblastoma protein (pRb). This in turn results in overexpression of the p16 tumour-suppressor protein (9-12). Therefore p16 expression is a valuable predictive marker in HPV-positive OSCC (13).

Generally speaking the overall survival of patients with HPV-positive HNSCC is notably better than that of patients

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with HPV-negative disease (14-16). In particular, HPV-positive OSCC exhibits enhanced sensitivity to chemoradiation. In the future, these findings could lead to a modulation and reduction of therapy intensitiy in patients with HPV-positive OSCC, concomitantly reducing therapy-associated side-effects such as mucositis, xerostomia, neuropathy, febrile neutropenia and renal failure. Moreover HPV vaccines could have a preventive effect not only in cervical cancer, but also in OSCC (1, 2, 17).

The aim of this retrospective single-center study was to determine the HPV status in all OSCC specimens collected and stored in our biological database since 1988. The prevalence of HPV-positive tumors and the impact of HPV status on overall survival were compared with data of current literature.

Materials and Methods

Study design. From 1988 to 2008, human tumor samples from patients with HNSCC were collected in the ENT Department of the J.W. Goethe University Hospital in Frankfurt am Main. The specimens were derived from biopsies of resected tumors and were stored at –80˚C for further investigations. Patients with primary tumors of the oropharynx were included in this study. All patients had died at baseline. A total of 104 frozen tumor samples were obtained. All included cases had histologically confirmed diagnosis of squamous cell carcinoma. Medical records and our tumor documentation system 'AdOnco' were reviewed (18, 19). Clinical information, including patients' characteristics, were recorded for statistical analysis. Tumor stage was classified according to the current classification of malignant tumors (TNM) and Union internationale contre le cancer (UICC) staging classification (seventh edition) (20). For HPV detection, the DNA from each tumor specimen was purified first. Subsequently, the HPV status was determined by a two-step polymerase chain reaction (PCR) combined with p16 immunohistochemistry (p16 IHC) as described previously (13). Statistical analysis was performed with BiAS™ (Version 10.02© 1989-2013 epsilon-Verlag, Hochheim, Darmstadt, Hesse, Germany) in cooperation with the Department of Biostatistic and Mathematic Modelling of the J.W. Goethe University Hospital in Frankfurt am Main. The study protocol was approved by the Ethics Committee of the Department of Medicine of the J.W. Goethe University Hospital in Frankfurt am Main.

DNA purification. HPV DNA was purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). A 25 mg piece of frozen tumor tissue was cut and 180 ml tissue lysis buffer (ATL) (Qiagen, Hilden, Germany) containing 0.5% Reagent DX (Qiagen) was added. The disruption was performed with steel beads (5 mm diameter) by Tissue Lyser LT (Qiagen) on 50 Hz for 40 sec. Proteinase K (20 µl) was added and digestion took three hours in a water-bath (56˚C). The samples were shaken three times each hour. AL buffer (Qiagen, Hilden, Germany) (200 µl) was added and mixed samples were incubated for 10 min in a water bath at 70˚C, ethanol was added and the mixture was transferred into QIAamp Mini columns. Following centrifugation (8000 rounds per minute (rpm) for 1 min), the remaining DNA was washed with AW1; 500 µl, 8000 rpm for one minute) and AW2 buffer (500 µl, 14,000 rpm for 3 min) The DNA was eluted with 200 µl AE buffer (Qiagen, Hilden, Germany) (8,000 rpm for 1 min). DNA concentration and purity was measured with Helios α spectrometer (Thermo Scientific, Dreieich, Hesse, Germany; wavelength: 260 nm, 280 nm).

Table I. *Patients' characteristics*.

	All patients n=104 (100%)	HPV-positive n=12 (12%)	HPV-negative n=92 (88%)
Gender			
Male	87 (83.7%)	10 (83.3%)	77 (83.7%)
Female	17 (16.3%)	2 (16.7%)	15 (16.3%)
Age (years)			
Mean	58.2	65.3	57.2
Median	58	68	57
T-Stage			
T1	12 (11.5%)	1 (8.3%)	11 (12%)
T2	37 (35.6%)	4 (33.3%)	33 (35.9%)
T3	16 (15.4%)	3 (25%)	13 (14.1%)
T4	39 (37.5%)	4 (33.3%)	35 (38%)
N-Stage			
N0	28 (26.9%)	3 (25%)	25 (27.2%)
N1	15 (14.4%)	2 (16.7%)	13 (14.1%)
N2	47 (45.2%)	5 (41.7%)	42 (45.7%)
N3	11 (10.6%)	2 (16.7%)	9 (9.8%)
Nx	3 (2.9%)	0 (0%)	3 (3.3%)
UICC-Stage			
I	6 (5.8%)	0 (0%)	6 (6.5%)
II	15 (14.4%)	1 (8.3%)	14 (15.2%)
II	17 (16.3%)	3 (25%)	14 (15.2%)
IV	66 (63.5%)	8 (66.7%)	58 (63%)

Data are numbers of patients unless otherwise indicated. Tumor stage was classified according to the seventh edition of the TNM and UICC staging classification.

HPV detection. HPV status was determined by a two-step PCR combined with p16 IHC (13, 21).

PCR. For PCR, 40 ng DNA was used. Firstly general HPV DNA was detected with the primers MY09 (5'-CGT CCM ARR GGA WAC TGA TC-3') and MY11 (5'-GCM CAG GGW CAT AAY AAT GG-3'), which amplify a 450 bp fragmet of the *HPV L1* gene (12). As an internal control, a fragment of the human *β-globin* gene (110 bp) was amplified by the primers Pr03 (5'-ACA CAA CTG TGT TCA CTA GC-3') and Pr04 (5'-CAA CTT CAT CCA CGT TCA CC-3') (23). In a second step, the HPV DNA-positive cases underwent a type-specific PCR for analysis of the high-risk HPV subtypes HPV-16 and HPV-18. For this reason, the specific primers Pr1 (5'-CCG AGC ACG ACA GGA ACG ACT-3'), Pr2 (5'-TCG TTT TCT TCC TCT GAG TCG CTT-3'), Pr3 (5'-GTC AAA AGC CAC TGT GTC CT-3') and Pr4 (5'-CCA TCC ATT ACA TCC CGT AC-3') were used. The primers Pr1 and Pr2 amplify a 172 bp fragment of the HPV18 genome (E6/7) and primers Pr3 and Pr4 amplify a 499 bp fragment of the HPV16 genome (E7+6/1) (24). All primers were purchased from Invitrogen (Darmstadt, Germany). PCR was prepared with Platinum Blue PCR SuperMix (Invitrogen) and performed on an Eppendorf Mastercycler (Eppendorf AG,

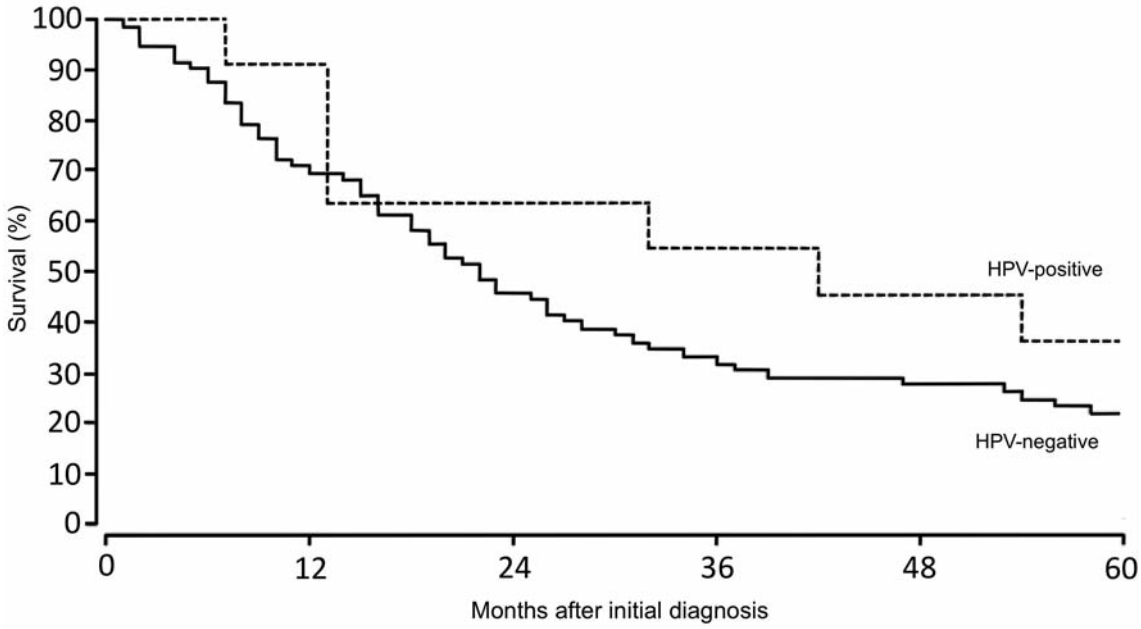

Figure 1. *Kaplan-Meier estimates of 5-year overall survival according to HPV-status.*

Hamburg, Germany). After 2 min heat-shock activation of Taq DNA polymerase, the PCR ran for 45 cycles: 95˚C denaturation for 30 sec, annealing at 50˚C for 30 sec and extension at 72˚C for 1 min. Previously examined samples positive for HPV 16 and HPV 18 were used as controls. The PCR products were analyzed on a 2% agarose gel in 0.5 × TRIS-Borat-EDTA-Puffer (TBE) (Qiagen, Hilden, Germany). The Gel electrophoresis run 45 min at 150 V. The gel was stained in 3×GelRed (Biotrend, Cologne, Germany) for 30 min. The bands were detected by Kodak Image Station 44C by exposing for 30 sec.

p16 IHC. Detection of p16 protein by IHC was performed with CINTec Histology Kit (mtm laboratories AG, Heidelberg, Germany). The slices were cut by Cryotome (Reichert-Jung 2800 Frigocut-E, Depew, NY, USA), fixed in methanol for 10 min and stored at –20˚C. For the staining procedure, the sections were rehydrated and blocked against peroxidase for five minutes. All incubation steps were performed in a moist chamber. After washing (5 min), the sections were incubated with antibody to $p16^{INK4a}$ (mtm laboratories AG, Heidelberg, Germany) (or negative control) for 30 min. The Antibody solution was removed with wash buffer and sections were incubated with visualization reagent for 30 min. After a wash step, the sections were stained with substrate-chromogen solution, 3,3'-diaminobenzidine tetrahydrochloride (DAB) (DCS, Hamburg, Germany) for 10 min. The Reaction was stopped in water. Hematoxylin (AppliChem, Darmstadt, Hesse, Germany) counterstaing was performed for 30 sec followed by blueing for 2 min. The sections were then dehydrated by incubation in ascending ethanol series (70%, 95%, 100%, ProTaqs Clear (quartett)) and mounted with ProTaqs Paramount (quartett). The microscopy images were taken with an AxioCam ICc1 (Zeiss, Oberkochen, Germany).

Results

A total of 104 tumor specimens were included and analyzed in this study. Out of these, 12 (12%) were HPV-positive and 92 (88%) were HPV-negative. Patients' characteristics were well balanced between the two groups (Table I). A tumor specimen was defined as HPV-positive when HPV DNA was deteced *via* PCR. All HPV-positive cases exhibited a positive immunostaining for p16 indicating not only the presence of HPV but also a biologically relevant infection and thus a possible causative correlation. Most of the patients had an advanced disease (79.8% UICC stage III or IV overall; 91.7% in the HPV-positive group and 78.2% in the HPV-negative group).

Statistical analysis. The primary end-point of this study was overall survival. Overall survival was defined as the time from date of histological proof of the OSCC to death from any cause. We used the Kaplan-Meier method to estimate five-year overall survival. Univariate und multivariate analysis were used to asses differences in overall survival with respect to HPV status, UICC stage and age at initial diagnosis. Kaplan-Meier estimates stratified by HPV status (HPV-positive *vs*. HPV-negative) are displayed in Figure 1. Univariate analysis (log-rank test) showed no significant difference in overall survival between HPV-positive and cases with HPV-negative tumors (p=0.28). In contrast multivariate analysis showed that HPV status (p=0.04),

UICC stage (p=0.01) and age at initial diagnosis (p=0.0006) were all significant determinants of overall survival. According to the relative hazard ratios, a high UICC stage (hazard ratio=1.31) and a high age at initial diagnosis (hazard ratio=1.03) was found to be associated with a poor overall survival, while a positive HPV status (hazard ratio=0.52; 95% CI=0.28 to 0.99) indicated a 48% increase of overall survival compared to those with HPV-negative status.

Discussion

Among accepted risk factors such as alcohol and tobacco use nowadays HPV is being more and more regarded as one major cause of oropharyngeal cancer (6). Dramatically, especially younger patients are affected. According to the literature it is estimated that 25-36% of all OSCC are HPV-positive (4, 5). HPV-positive OSCC leads to a better overall survival compared to matched HPV-negative tumors. Our findings show a prevalence of 12% HPV-positive OSCC in our biological tumor database. Although the percentage of cases with advanced tumor (UICC stage III and IV) is higher in the HPV-positive group (91.7%) compared to the HPV-negative group (78.2%) multivariate analysis confirms a significant advantage in overall survival of the presence of HPV-positive tumors as described in the literature. HPV status (p=0.04), UICC stage (p=0.01) and age at initial diagnosis (p=0.0006) appear to be independent determinants of overall survival, a positive HPV status being associated with a 48% increase in overall survival compared to HPV-negative tumors (14-16) (hazard ratio=0.52; 95% CI, 0.28 to 0.99). Due to its epidemiological and prognostic relevance the HPV status should be considered as an important part of the tumor staging for future therapy comparison studies, as well as for the individualized therapy concepts with the intention of reducing therapy-associated morbidity for patients. For this purpose, HPV detection *via* two-step PCR followed by p16 IHC seems reliable. The consistent growing incidence of HPV-positive OSCC may also require new therapy strategies. Several studies suggest that oral HPV-infection is sexually transmitted. For example a study of D'Souzza *et al*. demonstrated that OSCC is associated with a high-lifetime number of vaginal-sex partners (26 or more) and oral-sex partners (6 or more). The degree of association increased with the number of sex partners (6). For this reason, HPV vaccines might not only have a preventive effect in cervical cancer but also in OSCC (1, 2, 17).

Conclusion

Our findings confirm a relevant rate of HPV-positive OSCC (12%). A positive HPV-status was associated with a better 5-year overall survival compared to HPV-negative OSCC. HPV status should be determined and implemented within future staging classification systems for clinical trials, as well as for individualized therapy protocols for OSCC. For this purpose, HPV detection *via* two-step PCR combined with p16 immunostaining seems reliable.

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