Relation between Human Papillomavirus Positivity and p16 Expression in Head and Neck Carcinomas – A Tissue Microarray Study

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Abstract. Overexpression of p16 has been demonstrated to be strongly related to the presence of HPV16,18. Squamous cell carcinoma of the head and neck has been shown to be associated with human papillomavirus (HPV) infection. The main aim of this study was to investigate the relationship between HPV presence and p16 expression in a representative collection of 60 head and neck carcinomas by tissue microarrays. The presence of HPV (HPV6,11-low risk, HPV16,18-high risk) was detected by applying in situ hybridisation. P-16 protein expression was detected immuno histochemically. HPV6,11 positivity was observed in 10 out of 60 carcinomas. HPV16,18 presence was found in 30 out of 60 tumours. P-16 expression was detected in 35 out of 60 tumours. A statistically significant relationship was demonstrated between HPV16,18 presence and increased expression of p16. Also the HPV6,11 presence was significantly correlated with p16 immunoreactivity. Additionally, this study demonstrates that it is possible to analyse p16 expression and HPV presence by tissue microarrays.

The importance of human papillomavirus (HPV) detection lies not in identifying the infection but in identifying the risk of cervical neoplasia (1). HPV comprises a group of more than 75 closely related but genetically distinct viral types (2, 3). At least 30 types infect the genital tract, and also indirectly, the oral cavity, and are grouped into high or low risk based on the frequency of their association with malignant lesions. HPV6,11 belong to the low risk HPV types, HPV16,18 to the high risk and HPV31,33 to the intermediate risk type (4, 5). HPV DNA has been shown to be present in cervical cancer (2, 3). Various molecular studies have demonstrated not only a close epidemiological link between cervical neoplasias, but also plausible biological mechanisms of viral oncogenesis. This knowledge has led to the widespread expectation that testing for HPV could improve cervical cancer screening or aid in the management of patients with abnormal cytology results.

p16 is a gene with the second most frequently altered expression after p53. Its function is to regulate negatively the retinoblastoma (Rb) gene by eliminating the regulation of the cell cycle and attenuating apoptosis (6, 7). The alteration of p16, located on chromosome 9p21, is often caused by aberrant methylation as opposed to gene mutation. Alterations of this gene have been shown in gliomas, mesotheliomas, nasopharyngeal and bladder carcinomas (8-10). Mutations of p16 have been reported to be present in breast cancer, oesophageal cancer, gall bladder tumours and bladder tumours, as well as in prostate and ovarian cancers. In the current scientific literature it has been postulated that HPV presence can be connected to an altered expression of p16 (6, 7).

A recent study by Bohmer et al. (2003) reported the presence of HPV DNA in 100% of confirmed cervical intraepithelial neoplasia grade 3 and primary cervical cancers. HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins, E6 and E7. HPV infection is manifested in changes at the expression level of host cell cycle regulatory genes. Such altered host genes may have a role as molecular biomarkers.
and could potentially be used in cervical cancer screening and diagnosis (7, 11, 12).

p16 has been implicated as a target of the chromosome 9 deletions in many human cancers. The p16 promoter has recently been shown to be methylated in several human tumours. The p16 protein binds to cyclin-dependent kinases (CDK)-4 and -6 and inhibits their ability to interact with cyclin D1, thus, blocking a cycling cell’s passage through the G1-phase by negatively regulating the phosphorylation of Rb. Deletions and mutations in the p16 gene seem to affect the balance between functional p16 and cyclin D1, thus, leading to abnormal growth, which can be suppressed by in vitro transfection of the p16 gene into p16 deleted tumour cells. Point mutations of p16 have been reported for melanoma cell lines, in the germline of several melanoma kindreds linked to 9p21 and in a number of carcinomas including pancreatic tumours (13, 14). Overexpression of p16 in cervical cancers is believed to be due to the inactivation of Rb by the HPV E7 oncoprotein. It has been suggested that p16 transcription may be directly induced by the transcription factor E2F released from pRb after binding of HPV E7 (15).

The main aim of this study was to detect HPV presence in a tissue array consisting of carcinomas from the oral cavity and larynx. Additionally, p16 protein expression was determined to identify whether an increased immunoreactivity for p16 can be used as a screening criterion for HPV investigation in head and neck carcinomas.

Materials and Methods

Patients. The 60 patients analyzed had squamous cell carcinoma of the oral cavity and larynx with the following tumour size (pT) distribution (16, 17): five patients represented pT1, eight pT2, 20 pT3 and 27 pT4. Forty-one tumours were located in the larynx and 19 in the oral cavity. Thirty six patients demonstrated lymphatic metastases and two of these patients demonstrated the presence of distant metastases. Among the patients were 52 males and eight females. Their ages ranged between 28 and 87 years, averaging 62 years.

Construction of tissue arrays. Normal and tumour tissue was embedded in paraffin and five-micrometers sections were cut and stained in hematoxylin and eosin. This method made it possible to identify viable, morphologically representative areas of the specimen from which core biopsies were then taken. Three tissue cores with a diameter of 0.2 cm were punched from each specimen and arrayed on a recipient paraffin block (Figure 1D). Sections of five micrometers thick were cut from these tissue array blocks and placed on charged poly-lisine-coated slides. These sections were used for immunohistochemistry and hybridization in situ.

In situ hybridization. In situ hybridization was performed according to the method described by Breitschopf et al. (18) applying probes against HPV6,11 (low risk) and HPV16,18 (high risk) (Zytomed, Berlin, Germany). In brief, the tissue sections were deparaffinized, rehydrated in serial dilutions of ethanol, and postfixed in 4% TBS buffered paraformaldehyde. Samples were permeabilized using proteinase K (10 µg/ml) at 37°C for 30 min. Digestion was stopped by washing the samples in phosphate-buffered saline (pH 7.4). The samples were then dehydrated in serial dilutions of ethanol. Digoxigenine-labeled riboprobes were diluted in hybridization buffer (Amersham, Braunschweig, Germany). After sense or antisense probes were applied, the samples were covered with sterile coverslips and placed on a hot plate at 85°C for 5 min in order to denature the probe. Hybridization was performed overnight at 45°C in a sealed humidified chamber containing 50% formamide. Non-specific binding or unbound probes were removed by the following post-hybridization washes: 1xSSC/0.1%SDS at room temperature (2x5 min) and 0.2xSSC/0.1%SDS at 60°C (2x10 min) followed by RNase digestion (20 µg/ml, Gibco, Karlsruhe, Germany) at 37°C for 30 min. Finally the sections were washed in TBS containing 0.1% Tween-20 (Boehringer, Mannheim, Germany).

Hybridization signals were detected using a sheep polyclonal antibody F(ab)2-fragment against Digoxigenine conjugated with horseradish peroxidase (1:300, Boehringer). After washing in Tris-buffered saline Tween-20 (TBST), the slides were incubated in streptavidin-horseradish peroxidase (HRP) complex (diluted 1:100 in trinitrobenzol-TNB) for an additional 30 min. Three washes with TBS were followed by a 15 min application of biotin-tyramide diluted 1:50 in amplification diluent. Reactive sites were detected with streptavidin-HRP (1:100 in TBS, 30 min, RT). Peroxidase activity was visualized by using diaminobenzidine (DAB) as chromogen. Sense riboprobes served as controls for each tissue section.

Immunohistochemistry. Immunohistochemistry was performed by using 5 µm paraffin sections which were deparaffinized in xylene (three times for five minutes) and rehydrated in decreasing concentrations of ethanol (100%, 96%; two times each for ten minutes) followed by washing in deionised H2O for one minute. To unmask the p16 antigen the slides were covered with 0.01 M sodium citrate buffer (pH 6.0) and placed on a hot plate (95°C) for 10 minutes. After cooling down the specimens were rinsed briefly in deionised H2O (three times). The specific primary (mouse monoclonal) antibody (p16, clone E6H4, Dako, Carpintera, USA) was applied in a dilution of 1:50 overnight at 4°C. The reagents I-V used on the second day were supplied in the Immunocruz Staining System (Santa Cruz Biotechnology, Santa Cruz, USA). Each of these reagents was pre-diluted and ready to use at room temperature. After extensive washing with 0.02M Tris/phosphate buffer (TPBS, pH 7.2) the immunoreactivity was detected with a biotinylated secondary antibody (I) by incubating the specimens for 30 min at room temperature. Slides were rinsed with TPBS for 5 min before a horseradish peroxidase (HRP)-streptavidin complex (II) was added for 30 min. Washing in TPBS for 5 min followed. Subsequently colour was developed by using a HRP substrate (mixture of 1.6 ml deionised H2O, 250 µl 10x substrate buffer (III), 50 µl 50x DAB chromogen (IV) and 50 µl 50x peroxidase substrate(V) which was applied on the sections until light brown staining was visible (approx. 10 min). Samples were washed again in deionised H2O, then counterstained with hematoxylin, dehydrated (increasing concentrations of ethanol: 96% and 100%, followed by xylene, each two times for 10 sec) and mounted in DePeX (Merck, Whitehouse Station, USA). For negative control staining the primary antibodies were omitted. For quantification of the tumour cells with positive staining the
CAS200 image analyzer (Becton-Dickinson, Hamburg, Germany) was used and the results were expressed as percentages of positive cells (indices).

Statistics. The data were analyzed using the statistical analysis system (SAS, version 7.5) on an IBM-compatible PC running on Windows XP. First, they were scanned into a spreadsheet (Microsoft Excel XP); from Excel, they were fed to the statistics program via an ODBC driver (open database connectivity).

The average of the index values were obtained from three single cores. In general no means or standard deviations were calculated because the parameters investigated did not involve metrical data. Medians, quartiles, minima and maxima were calculated for all parameters. The Mann-Whitney U-test was used for group comparisons.

Results

Distribution of HPV6,11 positive cases in all tumours investigated. Cells with the presence of HPV6,11 were found in 10 out of 60 tumours investigated. Tumour cells with a positive reaction product for HPV6,11 are demonstrated in Figure 1A.

The HPV6,11 index (percentage of HPV6,11 positive tumour cells) collectively in the group of HPV6,11 positive tumours ranged between 1 and 15%.

Among HPV6,11 positive cases one case was stage II, two cases were stage III and seven cases were classified as stage IV. Tumour size demonstrated the following distribution: one case was classified as pT2, five cases as pT3 and four cases as pT4. Three of the HPV6,11 positive cases did not show any involvement of lymph nodes in the metastatic process (N=0) while in seven lymphatic metastases were present (N>0). All the HPV6,11 positive cases did not demonstrate any involvement of distant organs in the metastatic process (M=0).

Distribution of HPV16,18 positive cases in all tumours investigated. Cells with the presence of HPV16,18 were found in 30 out of 60 tumours investigated. Tumour cells with HPV


16,18 positivity are demonstrated in Figure 1B. The HPV16,18 index (percentage of HPV16,18 positive tumour cells) in the group of HPV16,18 positive tumours ranged between 3 and 40%. Among HPV16,18 positive cases one case was stage I, two cases were stage II, nine tumours were stage III and 18 cases were classified as stage IV. Tumour size demonstrated the following distribution: two cases were classified as pT1, four cases as pT2, 13 cases as pT3 and 11 cases as pT4. Twelve HPV16,18 positive cases did not show any involvement of lymph nodes in the metastatic process (N=0). In 18 HPV16,18 positive cases lymphatic metastases were present (N>0). Twenty-nine out of 30 HPV16,18 positive cases did not demonstrate any involvement of distant organs in the metastatic process (M=0). In one case distant organs were involved in metastatic tumour disease.

Distribution of p16 positive cases in all tumours investigated. Cells with p16 expression were found in 35 out of 60 tumours investigated. Immunoreactivity for p16 is demonstrated in Figure 1C. The p16 index (percentage of p16 positive tumour cells) in the group of p16 positive tumours ranged between 2 and 60%.

Among the p16 positive cases one case was stage I, one case was stage II, 11 cases were stage III and 22 cases were classified as stage IV. Tumour size demonstrated the following distribution: two cases were classified as pT1, six cases as pT2, 12 cases as pT3 and 15 cases as pT4. Twelve p16 positive cases did not show any involvement of lymph nodes in the metastatic process (N=0). In 23 p16 positive cases lymphatic metastases were present (N>0). Thirty-two out of the 35 p16 positive cases did not demonstrate any involvement of distant organs in the metastatic process (M=0). In three cases distant organs were involved in metastatic tumour disease.

Comparison of p16 expression in HPV6,11 positive and negative head and neck carcinomas. Values of p16 immunoreactivity in HPV6,11 positive and negative head and neck carcinomas are demonstrated in Table I. Statistical comparison of p16 expression in HPV6,11 positive and negative tumours demonstrated a significant result (p<0.05).

Comparison of p16 expression in HPV16,18 positive and negative head and neck carcinomas. Values of p16 immunoreactivity in HPV16,18 positive and negative head and neck carcinomas are demonstrated in Table I. Statistical comparison of p16 expression in HPV16,18 positive and negative tumours demonstrated a significant result (p<0.05).

Discussion

In this study, an association between altered expression of p16 and the presence of HPV in oral and laryngeal carcinomas has been demonstrated. Both factors especially p16 expression and positivity for high risk HPV16,18 were correlated significantly. A statistical relationship between p16 immunoreactivity and HPV6,11 presence was also found. Such a relationship has been reported previously for cervical carcinomas, but in head and neck carcinomas series it had not been demonstrated.

It is well known that the loss of p16 expression is either connected with mutation or with the methylation of the p16 promoter region. Mutations that disable the p16 pathway are common in human cancers. Cells with an altered retinoblastoma gene pathway have produced aberrant Mad2 expression and mitotic defects leading to aneuploidy (19). These mutations promote tumour development by deregulating the E2F family transcription factors leading to uncontrolled cell proliferation.

Generally the problem of HPV infection should be discussed in two separate groups of tumours, cervical and head and neck carcinomas. In cervical lesions immunostaining for p16 and detection of HPV is already a diagnostic procedure. An increased expression of p16, determined by immunohistochemistry is a screening method for the selection of patients for HPV subtyping (20-23). Human papillomaviruses are causally involved in the genesis of cervical carcinomas and their precursors. In cervical carcinomas there is a strong relationship between the cyclin dependent kinase inhibitor-p16/INK4a and HPV infection mostly of high risk type. p16, however, has not only a screening function for HPV subtyping, but also functions as a putative molecular biomarker that consistently discriminates uterine cervix adenocarcinomas from benign lesions and from endometrioid adenocarcinomas of the uterine corpus (20). The p16 positivity pattern was defined. In cervical lesions positivity for p16 can be regarded as a surrogate marker for the presence of high-risk human papillomavirus (21). In the vulva, p16 is positive in HPV associated vulval intraepithelial neoplasias. From a molecular genetic point of view, overexpression of p16

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occurs when the retinoblastoma protein is inactivated by high risk human papillomavirus oncoprotein e7 (22). Expression of e6 and e7 oncoproteins of high risk HPV affects the Rb-p16 pathway leading to p16 up-regulation (23). p16 is not only up-regulated in malignant cells of the cervix but also in dysplastic cells (24). This fact suggests the hypothesis that up-regulation of p16 expression is a very early phenomenon in the progression of cervical carcinomas and that analysis of p16 protein expression is a very sensitive tool to detect dysplastic changes not yet morphologically visible.

From the point of view of molecular pathology it is important to find out whether the relationship between p16 immunoreactivity and HPV infection found primarily in cervical carcinomas can be also confirmed in other sites of HPV infection for example in squamous cell carcinomas of the head and neck. For this purpose, a representative panel of oral and laryngeal carcinomas was investigated using tissue microarrays which are a very useful tool for the rapid and efficient analysis of large numbers of paraffin embedded tissues (25-26).

The results obtained in this study demonstrated significant differences not only between HPV16,18 positive and negative tumours, but also between HPV6,11 positive and negative head and neck carcinomas.

The advantage of molecular methods for this study was the possibility of determining whether the same population of cells which were HPV positive were also p16 immunoreactive. In the majority of the cases investigated this relationship was evident. A previous study using DNA chip technology (28) has identified several cell cycle regulators and transcription factors differentially expressed in HPV positive and negative oral carcinomas including not only p16, but also p18, CDC7, tat7l, rfc4, rpa2 and tfdp2 (29). As one can see, HPV infection in head and neck carcinomas dysregulates several pathways of cell cycle control and transcription leading to acceleration of malignant transformation and tumour progression. Weinberger et al. (29) proposed a molecular classification of oropharyngeal carcinomas. Using this system of classification the molecular profile (HPV positive with high p16 expression) has been defined and may have value for patient stratification for clinical trials testing HPV-targeted therapy (30). For example 94% of the highly positive p16 tumours had a complete response to radiotherapy. The highly p16 and HPV positive cases had a favourable prognosis (30). In addition HPV-positive tonsillar carcinomas exhibited nonkeratinising basaloid morphology and a characteristic immunophenotype in contrast to HPV negative cases (27).

Our study has demonstrated that it is possible to apply p16 immunostaining as a prescreening method for HPV subtyping in head and neck carcinomas. p16 immunoreactivity seems to correlate not only with the presence of HPV16,18 (high risk) but also with HPV6,11 (low risk).

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References


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