

The Role of p16 Expression as a Predictive Marker in HPV-positive Oral SCCHN – A Retrospective Single-center Study

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Abstract. *Background/Aim: Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common type of cancer worldwide; 600,000 new cases are diagnosed every year. Infected with high-risk human papilloma virus (HPV) types are particularly linked to oropharyngeal cancer. Among over 100 different HPV types, HPV-16 and HPV-18 are detected in the majority of HPV-positive SCCHNs. The p16 gene is often mutated in SCCHN, its overexpression is caused by the viral E7 protein. Consequently, p16 is assumed to be an indirect marker of HPV-induced SCCHN. The aim of the present study was to determine the role of p16 expression as a predictive marker of HPV infection in SCCHN tumors in a retrospective single-center study. Materials and Methods: Oropharyngeal tumor samples from 45 patients (34 males, 11 females) were analyzed. Tumor samples were examined for HPV infection using a two-step PCR. p16 staining by immunohistochemistry was then performed. Results: Samples with strong p16 signal were typed HPV-16-positive. Out of 14 tumor samples with HPV-positive PCR results, 13 samples contained the high risk variant HPV-16. In one sample, HPV-6 DNA was detected. All HPV-16-positive tumors overexpressed p16 (p16⁺⁺⁺), whereas the HPV-6 sample was p16-negative. Conclusion: p16 is not a surrogate marker for replacing PCR testing, but both methods in combination, PCR and immunohistochemistry, could lead to a higher diagnostic validation.*

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common type of cancer worldwide. Every year 600,000 new cases are diagnosed (1). In the past decades, it was determined that along with alcohol and

tobacco consumption, human papilloma virus (HPV) infection is one important risk factor among others for the development of oral cancer (2).

Infection with high-risk HPV types is particularly linked to cancer. HPV-16 and HPV-18 are detected in the majority of HPV-positive SCCHNs. Approximately 26% of all SCCHN cases and 36% of oropharyngeal SCCs are positive for HPV (3). Remarkably, the number of HPV-positive SCCHNs has been increasing over the past 20 years (4).

HPV is transmitted sexually, while infection does not depend on age. Notably, several studies have shown that patients with HPV-positive SCCHN have a significantly better overall survival, due to a smaller primary tumor, higher chemo- and radiosensitivity, and a lower risk of second primary tumor (5).

The identification of HPV-induced SCCHN is a challenge itself. HPV can be detected *via* viral DNA, RNA and proteins. The most sensitive method is the analysis of HPV DNA, which is more stable than RNA by polymerase chain reaction (PCR) holding the advantage of HPV typing. But in fact clinically-irrelevant transient HPV infections are also recognized. It is necessary to check the PCR test results by an HPV activity marker.

The p16/Rb/cyclin-D1 pathway is a key regulator of the cell cycle, which controls the passage of cells from G₁ to S phase (6). Consequently, its inactivation is a frequent event in all types of cancer (7). The cyclin-dependent kinase (CDK) inhibitor p16 targets CDK4 and prevents Rb phosphorylation (8). On one hand, the p16 gene is often mutated or epigenetically-silenced in SCCHN (9, 10). On the other hand, p16 protein overexpression is caused by viral E7 protein (11). Consequently, p16 is assumed to be an indirect marker of HPV-induced SCCHN. Several studies describe p16 protein detection as a useful marker of HPV (especially HPV-16) activity (12-15).

The aim of the present study was to determine the role of p16 expression as a predictive marker of HPV infection in SCCHN tumors in a retrospective single-center study. We performed a retrospective analysis and correlation of 26 HPV-positive SCCHN cases, with history of oropharyngeal cancer and p16-evidence.

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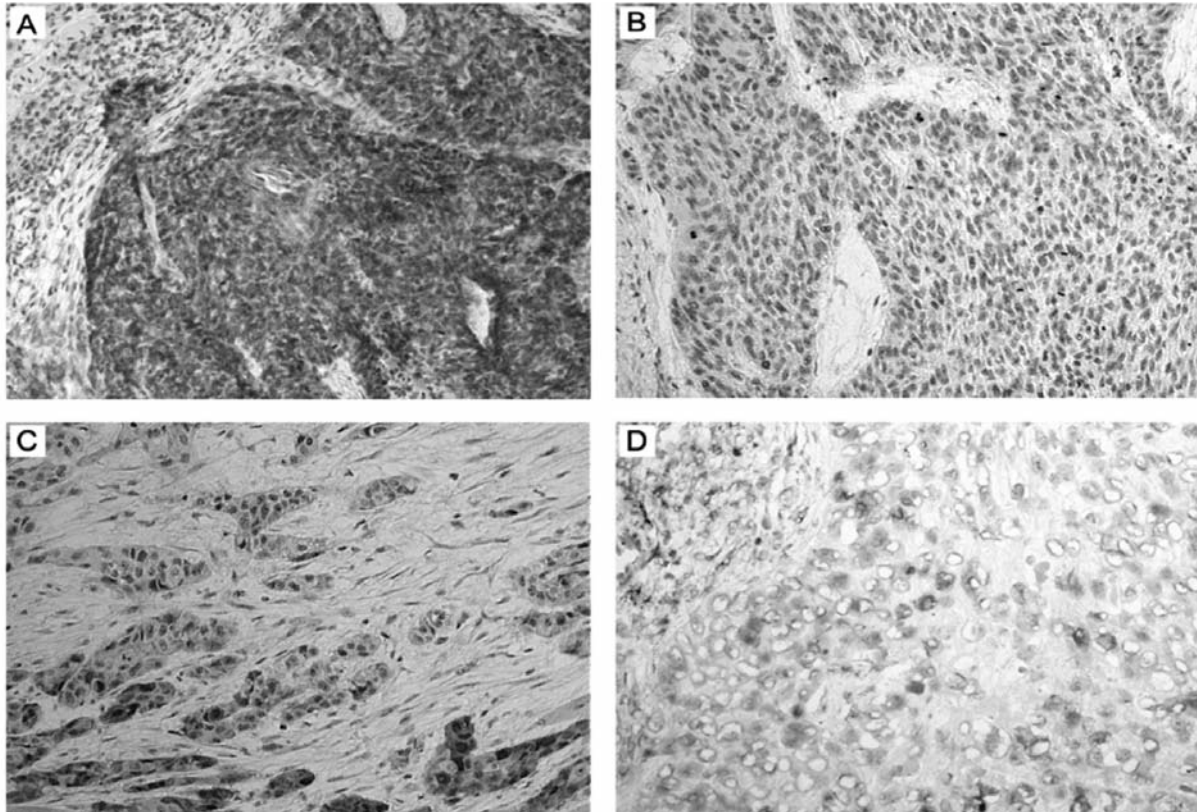


Figure 1. *p16* and HPV staining in SCCHN: *p16* staining of head and neck squamous cell carcinoma tumor samples; HPV⁺/p16⁺⁺⁺ (A), HPV⁻/p16⁻ (B), HPV⁺/p16⁺⁺ (C), HPV⁺/p16⁻ (D), $\times 20$ magnification.

Materials and Methods

Patients and DNA isolation. Oropharyngeal tumor samples of 45 patients (34 males, 11 females) were analyzed. The tumor material was taken in a standard surgery procedure and kept frozen at -80°C . The samples were collected between 1988 and 2008. Experiments were permitted by the Ethics Committee of the Department of Medicine of the Johann Wolfgang Goethe-University, Frankfurt am Main.

The samples were crushed by TissueLyser LT (Qiagen, Hilden, Germany). DNA purification was performed with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 200 μl elution buffer.

The DNA concentration and purity was measured with UV cuvettes in a Helios alpha spectrometer (Spectronic Unicam, Leeds, UK) at wavelengths of 280 nm and 260 nm.

Polymerase chain reaction. The tumor samples were examined for HPV infection using a two-step PCR. General HPV DNA presence was determined with the degenerate 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 fragment of the viral L1 gene (16).

The second step was typing of the HPV-positive samples for HPV-16 and HPV-18 using specific primers. For HPV-16 detection, the primer set 5'-GTC AAA AGC CAC TGT GTC CT-3', 5'-CCA TCC ATT ACA TCC CGT AC-3' amplifies a 499 bp fragment within the E7, E6 and E1 genes. HPV-18-specific primers (5'-CCG AGC ACG ACA GGA ACG ACT-3', 5'-TCG TTT TCT TCC TCT GAG TCG CTT-3') attach in the E6 and E7 gene region and generate a PCR product of 172 bp (17).

If the tumor contained neither HPV-16 nor HPV-18 DNA, sequencing was carried out. The detection of β -globin gene (5'-ACA CAA CTG TGT TCA CTA GC-3', 5'-CAA CTT CAT CCA CGT TCA CC-3', 110bp) served as control (18). All primers were customized by Invitrogen (Darmstadt, Germany).

The PCR was performed with Platinum Blue PCR SuperMix (Invitrogen). For amplification, 40 ng of purified DNA and 200 nM of each primer were added and hot start PCR was accomplished on a Mastercycler (Eppendorf, Hamburg, Germany). After pre-heating on 95°C , the PCR samples were processed 30 s at 95°C , 30 s at 50°C and 60 s at 72°C for 45 cycles.

The DNA fragments were separated on 2% agarose gel in 0.5x Tris/Borate/EDTA (TBE) buffer at 150 V. The gel was stained with 3x GelRed (Biotrend, Cologne, Germany) diluted in water and bands were analyzed with a Kodak Image Station 440CF (Boston, MA USA).

Table I. Comparison of HPV detection methods: HPV DNA detection by PCR and p16 staining.

HPV DNA status	p16 staining			
	+	++	+++	-
HPV+	0	0	13	1
HPV16+	0	0	13	0
HPV16+	0	0	0	1
HPV-	2	2	0	27

p16 immunohistochemistry. Paraffin or cryosections of tumor tissue were stained for p16 using the CINtec Histology Kit (mtm, Heidelberg, Germany) according to the manufacturer's instructions. DAB staining of tumor cells was classified as strong (p16+++), moderate (p16++), weak (p16+) staining and uncolored (p16-) samples.

Results

HPV DNA-containing tumor samples overexpress p16. We stained 45 tumor specimens for p16 protein presence. In this sample group, 14 tumor samples were HPV-positive (Table I). Thirteen of these HPV-positive samples overexpressed p16 (p16+++; Figure 1A) and only one HPV DNA-containing tumor was p16-negative (Figure 1D). p16 was localized in the nucleus and/or cytoplasm. In tumor samples with strong p16 staining, single-p16-negative cells were also found. The majority of HPV-negative samples were stained p16-negative (Figure 1B). We observed a moderate p16 staining (p16++) in one HPV-negative tumor (Figure 1C). Weak p16 staining (p16+) was also seen in one sample without HPV infection.

Taken together, the rate of false-positive cases was 13% (4/31) and 4% of the tumor samples (1/14) were false-negatives.

Samples with strong p16 signals were typed as being HPV-16-positive. Out of 14 tumor samples with HPV-positive PCR results, 13 samples contained the high-risk variant HPV-16. In one sample, HPV-6 DNA was detected. All HPV-16-positive tumors overexpressed p16 (p16+++), whereas the HPV-6 sample was p16-negative.

Discussion

HPV is known to cause SCCHN of the oral cavity. Ten percent of the general population are infected with HPV in the upper aerodigestive tract and this infection is cleared in most cases by the immune system (5). PCR is a very sensitive test that can detect virus presence in very few copy numbers. Consequently, the possibility of a marginal

infections exists. To distinguish between the clinically relevant HPV infection which is associated with tumor formation and a transient one, PCR-alone is not sufficient. The PCR results should be confirmed in a subsequent test by immunohistochemistry.

In this study we checked the HPV DNA-positive tumors immunohistochemically, by p16 staining. In the majority of HPV DNA-containing tumors (92%), the level of p16 was increased and all HPV-16-positive tumors highly expressed p16. These results are consistent with the findings of Heath and colleagues (15).

As a high-risk HPV-type, HPV-16 is known to cause tumor progression, it is the most frequently found type in SCCHN and is detected in 87% of HPV-positive samples (3). It is likely that the HPV-16/p16 double-positive tumors are HPV-induced.

One tested sample contained HPV-6 DNA and was p16-negative. HPV-6 is a low-risk type which is related to lesions and warts (19). The p16 staining of this sample may indicate that in this case, the HPV infection was clinically irrelevant and tumor development was triggered by other factors.

The overexpression of p16 is a negative feedback loop caused by the viral protein E7, which binds directly to Rb and bridges the p16/Rb/cyclin-D1 pathway.(4) McLaughlin claims that p16 overexpression is independent of Rb inactivation and caused by lysine (K)-specific demethylase 6B (KDM6B) histone deacetylase function (20).

When the HPV DNA integrates into host DNA, the viral E2 gene is disrupted. As an inhibitor, the E2 protein controls the promoter of E7 (21). Therefore p16 overexpression could be a potential marker for HPV DNA integration.

The detection of *E6/E7* transcripts to determine the presence of active HPV is difficult because of RNA instability. Here, we show that p16 protein verification is a reliable test for checking HPV-positive PCR results.

In four cases, p16 was weakly up to moderately expressed in HPV-negative tumors. A low rate of false-positive results was also detected by Hoffmann and colleagues (22). Furthermore, p16 expression of HPV-negative tumors was less and the number of p16-negative tumor cells was higher compared to that of HPV-positive samples.

Conclusion

Consequently, we propose that p16 is not a surrogate marker for replacing PCR testing. But both methods, PCR and immunohistochemistry, in combination, could be meaningful for diagnostic purposes.

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