Basaloid Lesions of Oral Squamous Epithelial Cells and their Association with HPV Infection and P16 Expression

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Abstract. Basaloid squamous cell carcinoma is a variant of squamous cell carcinoma, preferentially arising in the head and neck region. Current reports point to an association of basaloid squamous cell carcinoma (BSCC) and human papilloma virus (HPV) infection. This virus is supposed to be an aetiological factor in this entity. The aim of this study was to analyse the HPV infection status in different entities of oral neoplasms or dysplasias with basaloid differentiation of epithelial cells. Materials and Methods: The study comprised data from 34 oral lesions of squamous epithelial origin: 17 BSCCs, 10 papillomas and 7 hyperplasias/dysplasias of oral epithelia. HPV DNA was detected by means of a hybrid capture technique. HPV types were identified by direct sequencing. Immunohistochemical investigation of the specimens with anti-p16 antibody was performed in order to elucidate the putative role of p16 as a surrogate marker of HPV infection. Results: The rate of HPV-infected BSCC was extraordinarily high. About two thirds of the cases (61%, 11/17) were infected with HPV high-risk types, predominantly with HPV genotype 16 (>90%, 10/11). The infection status differed significantly between BSCC and other oral lesions in terms of frequency of HPV infection and HPV genotypes. p16 expression proved not to be a suitable surrogate marker of HPV high-risk infection in oral lesions, in particular in BSCC. This was an essential difference of this collective compared to genital carcinomas with HPV high-risk infections. Conclusion: This study revealed a high association between BSCC and HPV type 16. This close phenotype-genotype correlation could be of diagnostic value. Type-specific analysis of HPV infection in head and neck cancer may be important in the differential diagnosis of malignancies in the head and neck region with a basaloid growth pattern. However, the investigation is technically demanding, including hybridisation and sequencing techniques. A simplified test for HPV in BSCC of the oral cavity using the immunohistochemical proof of p16 expression as a surrogate marker is non-effective.

The basaloid squamous cell carcinoma (BSCC) is a rare variant of squamous cell carcinoma. BSCC constitutes about 1% of all squamous cell carcinomas. BSCC is a highly malignant, bimorphic tumour. This entity was first described for the head and neck region by Wain et al. (1), followed by approximately 200 cases reported in the literature to date. In the head and neck region, this variant is diagnosed predominantly in the hypopharynx, base of the tongue, larynx, parapharyngeal and subglottic regions. The histological characteristic of this entity is a basaloid component of epithelial cells. The term basaloid describes conglomerated cells with hyperchromatic nuclei and sparse cytoplasm. Small cystic spaces and necrotic areas lie in between these conglomerates (1). The grade of malignancy is generally high in BSCC. This entity is often diagnosed in advanced stages of disease and has a poor prognosis (2, 3). The basaloid cell type is not restricted to squamous cell carcinoma (SCC) but can also be found in some other carcinomas, dysplasias and focal epithelial differentiations of squamous epithelia.

The aetiology and pathogenesis of head and neck cancer points to a variety of carcinogens and co-carcinogens, with cigarette smoking and alcohol abuse as the main risk factors. Epidemiological studies support evermore the aetiological role of human papilloma virus (HPV) infection as a further risk factor, at least in some histologically defined subgroups of squamous cell carcinoma (4, 5). The results of these studies support the conclusion that HPV may be used as a
prognostic marker in head and neck carcinoma. However, despite strong evidence for HPV expression in head and neck cancer gained from epidemiological and experimental studies, the mode of action of the virus in carcinogenesis of the squamous cell carcinoma in the head and neck region remains unclear.

The differential diagnosis of BSCC from SCC is usually performed by immunohistochemical analyses of representative specimens. Initially, BSCC, as a subtype of SCC, has to be delineated from undifferentiated small cell lung cancer and poorly differentiated adenoid cystic carcinoma (6-8). A detailed classification of BSCC is lacking owing to the deficiency of a grading system (9). Studies on the protein expression and analysis of molecular genetics to elucidate the pathogenesis of BSCC are rare. Recently, certain genetic differences between BSCC and SCC were detected (9, 10). A peculiar finding in BSCC is the association of HPV infection with basaloid morphology in oropharyngeal carcinoma (5). Further studies should clarify the association of oral SCC with a basaloid phenotype and an HPV-associated pathogenesis. The demonstration of morphological alterations in precancerous lesions associated with HPV could be a valuable tool in differential diagnosis and a targeted therapy. HPV types 16 and 18 are frequently found in head and neck cancer. This association resembles the pattern of HPV infection associated with cervical cancer.

The aim of this study was to demonstrate HPV high-risk subtypes as causative agents in BSCC and the identification of these subtypes in lesions of the oral cavity characterized by a basaloid phenotype of epithelia. Further, the putative role of p16 expression in these lesions as a surrogate marker of HPV infection was investigated.

Materials and Methods

Thirty-four formalin-fixed and paraffin-embedded lesions of the head and neck region were investigated, which were collected and processed in the Department of Oral Pathology, Eppendorf University Hospital. The diagnosis of BSCC was made between 2000 and 2006 (Department of Oral Pathology, Eppendorf University Hospital). The lesions were diagnosed as BSCC (n=17), papilloma papillomas and oral dysplasias or hyperplasias served as a control group. The lesions were diagnosed as BSCC (n=17), pa-pilla (n=10) and dysplasia (n=7).

Oligonucleotides. Well-defined oligonucleotides were used for sequencing (primer GPS+: 5'-TTGTTACTGTGTTAGATACACTAC-3'; primer GPS6+: 3'-CTTATCTAAATGTCAAATAAAAAG-5'; control primer B-globin-F: 5'-GCCATCTAAGGACCGAGC-3'). The primer sequence was described by Jacobs et al. (11) and supplied by MWG Biotech AG (Ebersberg, Germany).

Hybrid capture and direct sequencing. All samples were screened for HPV infection and selected for high risk and low risk types, using the hybrid capture method (Digene Hybrid-Capture HPV Kit; Digene, Dreieich, Germany). The technique has been described else-where in detail (12). In cases with identification of a HPV high-risk infection by means of a hybrid capture test and in cases with dubious negative results, namely p16 overexpression, the HPV type was identified with consensus primers via direct sequencing (12).

DNA extraction. The Qiagen Mini Kit was used for DNA extraction (QIAamp DNA Mini Kit, No. 51306; Qiagen, Hilden, Germany; Taq PCR Core Kit, No. 201225, Qiagen). The test was performed according to the manufacturer’s instructions. The technique is described elsewhere in detail (13).

PCR. Several hybridisation probes were used to detect HPV [ HPV Test Dual Probe, Digene (5101-1096); HPV Test High Risk, Digene (5101-1190); HPV Test Panel 6 member, Digene (5101-1024); HPV Validation Panel, Digene (5101-1042); Agarose (Nusieve 3:1) Biozym, Hess. Oldendorf, Germany, No. 850090; Gene Ruler, MBI Fermentas, Vilnius, Lithuania, Cat. No. SM0241]. Qiagen Taq PCR Core Kit was used for the amplification of HPV-DNA (1 ng DNA). PCR was performed in a thermocycler (Biometra, Göttingen, Germany) according to the manufacturer’s instructions and following a standardised program. In all PCR assays, controls were run under identical conditions. An HPV-positive, high-risk type, anal squamous cell carcinoma was used as a control specimen for HPV sequencing (negative control: aqua sterile). PCR products were revealed and identified following electrophoresis on prepared agarose gels (MBI Fermentas, Gene Ruler). Evaluation of the electrophoresis was performed with an ultraviolet transilluminator. In cases of weak or inconclusive results, PCR was repeated.

Antibodies. The antibodies and kits were applied according to the manufacturer’s instructions (Mouse anti-human p16; clone G175-405, Pharmingen, U.S.A., No. 554070; rabbit serum, No. X0902, Dako, Hamburg, Germany; rabbit anti-mouse, IgG biotin-labelled, Dako, E0413).

Immunohistology. A specimen with proven intense p16 expression was used as a positive control. For negative control, the immunohistological protocol was performed with the primary antibody being omitted. Incubation with anti-p16-antibody was performed on 4 μm thick slices, placed on APES-coated slides. A positive and a negative control with established p16 expression and non-expression respectively, underwent the same protocol. Diaminobenzidine (DAB) was used to visualise the immunological reactions, counterstained with hemalaun. Microwave pretreatment of slices was performed in order to enhance antigen presentation. The reactions were semiquantitatively evaluated. Although p16 is a nuclear protein, a cytoplasmatic staining can be observed occasionally, in particular in high-risk HPV infections. Therefore, both nuclear and cytoplasmatic staining patterns were evaluated separately. p16 immunoreactivity was diagnosed in cases with staining of both compartments (nuclear and cytoplasmatic).

Histology BSCC. BSCC is characterized by predominant basaloid cells, frequently associated with a marked nuclear pleomorphism and necrosis. Besides these characteristics, transitions to non-basaloid carcinomatous areas may be found (14).

Papilloma. The papilloma is characterized by benign epithelial proliferations. These epithelial proliferations show a branching growth pattern with basal atypia.

Hyperplasia/Dysplasia. The squamous epithelia show marked structural changes in terms of a papillomatous-endophytical, partially parakeratotic and akanthotic broadening of epithelia. Mitotic figures are increasingly found in basal and parabasal areas, sometimes even in suprabasal layers.

Results

Clinical characteristics. In all locations, the number of males with tumour was higher than that of females. The age distribution was even in the papilloma and dysplasia/hyperplasia groups. However, among the patients with BSCC, an extraordinarily high number of patients were in their sixth decade (i.e. aged 60 to 69 years), followed by the group of patients in their fifth decade of life. Table I summarizes the results that are detailed as follows:

HPV detection. HPV capture. All samples were subjected to hybrid capture analysis for HPV infection. This method allows the identification of high-risk and low-risk HPV groups without further specification.

Out of 34 samples, 13 were HPV negative (38%) and 21 were HPV positive (62%). Subgrouping of HPV-positive cases recorded 15 of HPV high-risk (44%) and 6 of low risk infection (18%). The distribution of HPV infection differed and was dependent on the type of oral lesion. Six out of 17 BSCC were HPV negative (35%). Eleven cases were identified as carrying an HPV high-risk infection (65%). Three out of 10 papillomas were HPV negative (30%); in 2 cases, an HPV high-risk infection (20%) was verified. Five papillomas proved to be infected with an HPV low-risk type (50%). Four out of 7 hyperplasias/dysplasias were HPV negative (57%); an HPV high-risk infection was revealed in 2 cases (29%), one of them with a further HPV low-risk infection. One case was HPV low-risk type-positive (14%).

Amplification and sequencing. The sequence analysis of purified PCR products was performed in an automated system (ABI). The sequences were compared to the HPV gene bank [Gene-Bank HPV sequences (http://www.ncbi.nlm.nih.gov)] following the FASTA Sequence and BLAST programs. Sequence analysis revealed a 99% overlapping of each identified HPV type. The sequence analysis allowed the determination of specific HPV types. This allowed both the assignment to high- and low-risk groups and further specifications to be assessed (Figures 1 and 2).

Analysis related to risk type of HPV infection. Out of 15 HPV high-risk cases, 14 (93%) were identified as HPV type 16. One of these cases was shown to be infected also with HPV type 6 (low risk type). One further case was infected with HPV type 35 (7%).

Four out of 6 HPV low risk cases were infected by both HPV 6 and 11 (67%). One case proved to be infected solely by HPV type 6 and one by type 11 (each 17%).

Analysis related to histology and diagnosis. The sample of oral lesions overall was predominantly infected by HPV high-risk infections, followed by the group of non-infected tissues. Interestingly, all SCC were infected with HPV high-risk types. Papillomas were predominantly infected by low-risk HPV. The majority of dysplasias were not associated with HPV infection. In the case of HPV-positive dysplasias, the high-risk types predominated.

Out of 11 HPV high-risk infected BSCC cases, 10 were identified as type 16 (91%). Only one case showed an HPV type 35 infection (9%).

Both the HPV high-risk infected papillomas were proven to carry type 16 (100%). HPV low-risk infected papillomas (5 cases) were types 6 and 11 (60%); the remaining were infected with type 6 or 11 (each 20%).

Both HPV high-risk infected dysplasias contained type 16 (100%). One further HPV low-risk infection was associated with HPV type 6. In one HPV low-risk case, both types 6 and 11 were identified (100%).

HPV-positive BSCC were always identified as high-risk infections (type 16 and 1 type 35). HPV-positive papillomas showed one case of high-risk type 16 infection. In HPV low-risk infections, both single infections with type 6 and type 11 and combinations thereof occurred. HPV-positive dysplasias were alternatively infected with type 16 or type 6/11.
**p16 immunohistology.** The putative correlation of HPV infection and p16 expression was studied immunohistochemically.

**BSCC.** In BSCC, p16 expression was not found in 4 out of 17 cases (24%). Out of 13 p16-immunoreactive cases (76%), the staining was moderate in 4 (++) and strong in 9 (+++++) (31% and 69%, respectively). The moderately immunoreactive cases were predominantly cytoplasmatically stained, leaving one case with nuclear staining. Out of nine strongly stained cases (++++), 3 showed a cytoplasmatic and 6 a nuclear staining.

**Papilloma.** Four out of 10 papillomas showed no p16 expression (40%). All six p16-positive cases showed a strong expression (60%); four of them nuclear and 2 cytoplasmatic.

**Hyperplasia/Dysplasia.** Five out of 7 hyperplasias/dysplasias failed to express p16 (71%). In two cases, a strong nuclear p16 expression was seen (29%).

**p16 expression and HPV infection.** The correlation of HPV infection and p16 expression is summarised in Table I. Specifically: **BSCC.** Three HPV-negative cases showed a moderate (++) p16 expression (50%); two of them with cytoplasmatic and one with nuclear staining. An HPV-negative case showed strong p16 expression (17%), confined to the cytoplasm. Two of the HPV high-risk cases were p16 negative (18%). One case showed a moderate cytoplasmatic p16 expression (9%). Eight cases showed a strong p16 expression (73%); 2 with cytoplasmatic and 6 with nuclear staining.

**Papilloma.** Out of 10 papillomas, 3 were HPV negative (30%); one of them was associated with the lack of p16 expression. The other 2 HPV-negative cases showed p16 overexpression; each of them nuclear or cytoplasmatic. Five cases showed an HPV low-risk infection (50%); 2 of them with no p16 expression. The other 3 cases showed a p16 overexpression: 2 cytoplasmatic and 1 nuclear. In 2 cases (20%), an HPV high-risk infection type was identified: 1 with no p16 expression and 1 with p16 overexpression, confined to the nucleus.

**Hyperplasia/Dysplasia.** Four (57%) out of 7 cases of hyperplasia/dysplasia were HPV negative; two of them also did not express p16. An HPV-negative case showed nuclear p16 overexpression. Three cases (43%) were HPV positive. In one case, the HPV high-risk type 16 infection was demonstrated; however, there was no p16 expression. One case was infected by both HPV high-risk type 16 and HPV low-risk type 6. This case showed also no p16 expression. The third case showed an HPV low-risk type 6 and 11 infection associated with nuclear p16 overexpression.

In the majority of cases, the epithelia with no HPV infection did not express the p16 protein (in particular in dysplasia). About 2 out of 3 HPV-negative papillomas showed p16 overexpression; the remainder were p16 negative. In the group of HPV-negative BSCC, no preference for p16 expression was detectable.

An HPV low-risk infection was found only in the groups of papilloma and hyperplasia. In about 33% of the cases in these groups, this infection was associated with p16 overexpression. All other cases of these groups were p16 negative. In the dysplasia group, all cases with HPV low-risk infection showed a p16 overexpression.

The majority of cases with HPV high-risk infection were associated with p16 overexpression, excluding the dysplasia group. In the dysplasia group, an HPV high-risk infection was always associated with no p16 expression. In the papilloma group, the lack of p16 expression and p16-overexpression were equally distributed, associated with an HPV high-risk infection. Many BSCCs with HPV high-risk infection showed p16 overexpression. However, moderate p16 expression, and negativity for p16 expression was also noted.

These findings led to the conclusion that there is no strong association of p16 expression with HPV infection status.

**Discussion**

This study described the prevalence of HPV infection and p16 expression in 34 oral lesions (17 BSCC, 10 papillomas and 7 hyperplasias/dysplasias). At first glance, the sample size of BSCC was small. However, similar BSCC group sizes have been used in several published studies; this is due to the fact that BSCC is a rare subgroup of SCC. Both Poetsch et al. and Coppola et al. were able to study 8 cases (15, 16). Up to 1994 about 86 cases were reported, summarised by Campman et al. (17). These data were added to 45 further cases by Ide et al. (18). The current NIH study of Begum et al. is clearly the largest study on this item, based on 53 cases (19).

**BSCC.** The BSCC as a subgroup of SCC is clinically characterised by a high rate of metastases at the time of...
diagnosis and poor survival (20). Campman et al., in their review, reported a metastasis rate of 70% (17), supporting the judgement of Coppola et al. that BSCC is a very aggressive variant of SCC (16). Based on the study by Poetsch et al. (15), the current study aimed to provide evidence for the hypothesis that the basaloid morphology of this entity is the microscopic equivalent of an HPV infection in tumour cells. In this study HPV DNA was demonstrated in 65% of BSCC. Poetsch et al. (15) demonstrated an HPV high-risk infection in all cases and postulated the association of HPV infection with the basaloid morphology in BSCC.

The BSCCs of this study were predominantly located in the anterior and lower parts of the oral cavity (floor, mandible and base of the tongue). However, in some cases, organs of the neck or salivary glands gave rise to BSCC. About half of the cases were found to be located in the oropharynx, while the others were distributed in the hypopharynx and larynx (15). Possibly these differences are more than anecdotal: Begum et al. classified their cases of BSCCs according to location into oropharyngeal and non-oropharyngeal BSCC (19).

The mean age of the present patient group was 62 years and did not differ from the mean ages of a recent study (61 years, (18)). The gender ratio of this study was in favour of men (10:7) and was similar to the ratios detailed by Ide et al. (18) and Luna et al. (20). In contrast, El-Mofty recorded a male to female ratio of 4:1 (21).

An HPV high-risk infection was determined in 65% of BSCC. This rate was identical to the total HPV infection rate. However, Miller et al. found high-risk HPV infections in 84.4% of their cases, suggesting an even higher

<table>
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<tr>
<th>No.</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Histology</th>
<th>Localisation</th>
<th>HPV Infection</th>
<th>p16 Type</th>
</tr>
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<tbody>
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<td>+16+++</td>
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<td>Parotid</td>
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**Papilloma**

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<th>Histology</th>
<th>Localisation</th>
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<th>p16 Type</th>
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<td>+6</td>
<td>+++</td>
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<td>+++</td>
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<td>Lower lip/cheek</td>
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**Hyperplasia/dysplasia**

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<td>Verrucous</td>
<td>Angle of the mouth</td>
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**Control**

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<th>p16 Type</th>
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</tbody>
</table>
infection rate (22). The total number of HPV-positive cases was as low as 26.2% of SCC in their study (22). Zhang et al. found HPV high-risk infections of oral SCC in 74% (23). In contrast, Nemes et al. detected a low infection rate in oral SCC (48% HPV high-risk infections) (24). The total infection rate in oral SCC is suggested to be between 20 and 30% (5, 25-30). Poetsch et al. investigated oral BSCC only (n=8) and found high-risk HPV infections in all cases (15). The study by Begum et al. was also restricted to BSCC (19). They investigated 53 cases of BSCC for the presence of high-risk type 16 and demonstrated this virus in 34%. The distribution of HPV infection in this collective was remarkable. HPV high-risk type 16 was detected in 76% (16/21) in the oropharynx and only 6% (2/32) outside this region. This correlation of HPV high-risk type 16 infection with BSCC localisation to the oropharynx was also described by El-Mofty (21). Another study supports the assumption that there is a predilection for HPV infection with SCC arising in certain regions of the oral cavity (31).

Out of the 65% HPV high-risk infected BSCCs, 91% were positive for HPV high-risk type 16 (1 case HPV type 35 infected). The simultaneous detection of HPV high-risk types 16 and 35 in the same type of tumour was not extraordinary. In fact, both types share structural homologies and are evolutionary closely related. Other authors described the HPV high-risk type 35 in tonsillary carcinoma (32).

According to Begum et al., BSCC constitutes a mixed group of tumours to be distinguished by their HPV infection state (19). They concluded that BSCC with HPV-16 infection appear to be a biologically less aggressive variant compared to non-virus-associated SCC of the head and neck region. This suggests that the HPV 16 infection state could be a marker of a good prognosis. This conclusion was supported by El-Mofty (21); however, it is not generally accepted (2, 3).

In the control groups of papillomas and dysplasias/hyperplasias, no strong correlation of HPV infection and the entities could be demonstrated. In the group of papillomas, the HPV type 16 was detected in 20%. In contrast, 50% of papillomas were infected with an HPV low-risk type. These results were inconclusive regarding whether there is a relation between papilloma and HPV high-risk type 16. Other studies also confirm the observation that HPV high-risk types play a minor role in the pathogenesis of oral papilloma (33, 34).

Fregonesi et al. concluded that oral papillomas are predominantly associated with an HPV low-risk infection and the prognosis is usually good (29). However, at least 20% of oral papillomas and papillary hyperplasias of the current study were identified as HPV high-risk type 16 infected. These findings are reminiscent of the frequent HPV high-risk type 16 infections in benign papillomas/condylomas of the uterine cervix (12).

Lopez Amado et al. investigated a large group of benign oropharyngeal lesions (234 papillomas) (35). In 8 cases, a metachronous carcinoma developed at the site of the excised papilloma, not addressing the question whether an HPV infection was the cause for a benign papilloma preceding the carcinoma. To date, there are only very limited data regarding the biological behaviour of squamous papilloma of the pharynx, the nose and oral cavity. These papillomas are usually excised and show neither local recurrence nor malignant degeneration (22).

The dysplasias/hyperplasias were found to be associated with an HPV high-risk type 16 infection in 29% of cases. These lesions showed no strong association with this virus. A strong argument for HPV (including high-risk types) being normally found on oral epithelia is the finding that the distribution of HPV in normal tissues, benign leukoplakia and intraepithelial neoplasia show a similar distribution pattern. According to Miller et al., it is likely that these findings are indicative of the long-latency period of HPV in transforming epithelial cells (22). This hypothesis is supported by the low rate of malignant transformation in leukoplakia.

\( p16 \). In addition to a reduced p53 expression in high-risk HPV-transformed cells found in anal (36) and cervical carcinoma (37, 38), further investigations showed that other tumour suppressor genes, in particular \( p16^{INK4A} \), are overexpressed in HPV high-risk-associated carcinomas (39-42). Obviously the p16 expression is up-regulated following Rb inactivation (43-48).

In this study, the BSCC showed no correlation to p16 overexpression (18%: no, 9%: moderate, and 73% strong expression). In contrast, Poetsch et al. postulated a causal nexus between HPV high-risk infection and p16 overexpression (15, 29). This postulation corresponds well to the results of Begum et al., where HPV high-risk type 16 infections correlated positively with p16 overexpression in oropharyngeal BSCC (19). The control group of oral dysplasia/hyperplasia and papilloma showed no overlap between a HPV high-risk type 16 infection and p16 overexpression. However, in the group of oral hyperplasia, the detection of HPV high-risk type 16 in association with p16 overexpression supported the assumption that some of the benign lesions carry a substantial risk of malignant degeneration (29). According to Fregonesi et al., a p16 dysfunction is not strongly HPV dependent (29). Therefore, other mechanisms and cofactors may contribute to the protein regulation.

The dysplasias were predominantly HPV negative. Only in sporadic cases was an HPV high-risk infection detectable. Interestingly, both cases with no HPV infection and with HPV high-risk infection were associated with a lack of p16 expression. Only one dysplasia associated with a HPV low risk infection showed p16 overexpression. Therefore, oral lesions are not comparable to female genital lesions concerning the regulation of p16 expression (13, 40). p16 is currently not suitable as a surrogate marker of an HPV high-risk-associated (pre-) neoplasia.
References


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