ANTICANCER RESEARCH *39*: 1293-1300 (2019)
doi:10.21873/anticanres.13241

Combined Testing of p16 Tumour-suppressor Protein and Human Papillomavirus in Patients With Oral Leukoplakia and Oral Squamous Cell Carcinoma

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Abstract. *Background: Oral leukoplakia (OL) is a potentially malignant oral mucosal disorder. A casual association between OL, oral squamous cell carcinoma (OSCC) and human papillomavirus (HPV) infection has been suggested, but no conclusive evidence has been presented. p16, a tumour-suppressor protein, is used as a surrogate marker for HPV infection. The aim of this study was to investigate how overexpression of p16 correlates with HPV infection in OL and in OSCC. Patients and Methods: Seventy-four patients with OL and 13 with OSCC with p16 overexpressed, were analyzed by immunohistochemistry visualizing p16 and a real-time polymerase chain reaction (PCR) assay targeting HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 52, 56, 58 and 59. Results: Overexpression of p16 was observed in 18% of patients with OL. None of the HPV subtypes were detected by PCR analysis in patients with OL. In the p16-positive OSCC specimens, 38% were also HPV16-positive. Conclusion: Overexpression of p16 was not found to be a reliable biomarker for HPV infection in patients with OL and OSCC.*

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Key Words: Potentially malignant oral disorders, oral cancer, tumour suppressor protein, virus infection, biomarker.

Oral squamous cell carcinoma (OSCC) is the result of a multi-step process during which genetic alterations transform normal cells into cancer cells (1). The appearance of tumours is often preceded by a potentially malignant oral disorder (2, 3). Oral leukoplakia (OL) is one of the most prevalent potentially malignant oral disorders, with the potential to turn into OSCC. Depending on the clinical presentation, leukoplakia can be divided into homogeneous and non-homogeneous forms (Figure 1), where the latter has been attributed a higher risk of malignant transformation (4).

Warnakulasuriya and Ariyawardana reported an estimated overall malignant transformation rate for OL of 3.5%, with a wide range from 0.13% to 34.0% (4). The annual transformation rate for OL is estimated to be in the range of 1.0-2.6% (5, 6). Suggested treatment protocols for OL are surgical excision and surveillance (7-10). Even after radical surgical removal, OL recurrence rates are reported to be in the range of 10-20% and despite surgical intervention, cancer transformation occurs in 3-9% of cases at the site of the excised lesion (7, 9).

Many signalling pathways and genes have been implicated in both cell dysplasia and malignant transformation in OL. Genetic alterations, including changes in genes that regulate genomic stability, the cell cycle, the cytoskeleton, angiogenesis, and apoptosis, have been suggested to be drivers of tumourigenesis in cases of OL (11-13).

The exogenous factors that cause DNA damage include viral infection, which can promote progression to malignancy by different mechanisms. The capacity of human papillomavirus (HPV), especially of HPV type 16, to become incorporated into the host DNA of infected epithelial cells is believed to increase the risk of cancer development (14-16). The associations of cancer of the *cervix uteri* and squamous cell carcinoma in the oropharynx with high-risk HPV (HR-HPV) infection are well known (17-20). Over the past two decades, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) related to HPV infection has increased alarmingly (21-23). In the oral cavity, a casual association between HPV and OL, as well as between HPV and OSCC, has been suggested by several groups, although no conclusive evidence for such an association has been presented (24-32).

The tumour-suppressor protein p16 (also known as $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2A) inhibits the binding of the cyclin-dependent kinases 4 and 6 to cyclin D1. This inhibits the phosphorylation of retinoblastoma protein (RB), which is needed for release of the E2F transcription factor to enable E2F to enter the cell cycle (33, 34). This pathway can be disturbed by a HR-HPV infection by the viral oncoprotein E7. E7 binds to and inactivates pRB, allowing E2F to induce cell-cycle progression and to promote the transcription of the *p16* gene. Thus, p16 can serve as a surrogate marker for HR-HPV infection (33, 35).

In OPSCC, the overexpression of p16 in tumour tissue is a well-established surrogate marker for HR-HPV infection, and is used both in clinical and scientific protocols (36, 37). The expression patterns of p16 in OL and OSCC have been investigated in several studies. No conclusive evidence of a correlation between p16 expression, HPV infection and malignant transformation has been presented. Some studies have also suggested that p16 expression differs between anatomical sites in the oral cavity (38-41).

Thus, based on current knowledge, the correlation between p16 and HPV infection in OL and OSCC is uncertain. The aim of this study was to explore the correlation between the overexpression of p16 and HPV infection in both OL and OSCC.

Table I. *Clinical characteristics of the patients with oral leukoplakia (N=81).*

Characteristic	N (%)
Gender	
Male	39 (48)
Female	42 (52)
Age (at first appointment), years	
<50.0	11 (13)
50.0-59	25 (31)
≥60.0	45 (56)
Site of lesion	
Floor of the mouth	5 (6)
Buccal mucosa	17 (21)
Lateral tongue	19 (24)
Ventral tongue	5 (6)
Dorsal tongue	1 (1)
Soft palate	1 (1)
Hard palate	3 (4)
Alveolar gingiva	27 (33)
Lip	3 (4)
Clinical diagnosis	
Homogenous leukoplakia	54 (67)
Non-homogenous leukoplakia	27 (33)
Histopathological diagnosis	
Benign hyperkeratosis	63 (78)
Lichenoid reaction	1 (1)
Mild dysplasia	13 (16)
Moderate dysplasia	3 (4)
Severe dysplasia	1 (1)
Smoker	
Yes	25 (31)
No	55 (68)
No data	1 (1)
Previous smoker	
Yes	45 (56)
No	30 (37)
No data	6 (7)
Use of snuff	
Yes	7 (6)
No	73 (89)
No data	1 (5)
Previous use of snuff	
Yes	14 (17)
No	57 (70)
No data	10 (13)
Alcohol consumption	
Daily	0 (0)
Several times per week	12 (15)
Once a week	29 (36)
Never/rarely	34 (42)
No data	6 (7)

Patients and Methods

Patients. Ninety-five patients who participated in a prospective longitudinal multi-centre study in Sweden (ORA-LEU-CAN Study) between 2011 and 2017, were included. The inclusion criterion was a clinically and histopathologically verified diagnosis of OL. Data on medical and dental histories, tobacco habits, clinical appearance (homogenous and non-homogenous) OL, and localisation and size of the lesion, together with clinical photographs and the results of biopsy analysis were collected. Histopathological diagnoses were established by Board-certified specialists both in general pathology and subspecialized in oral pathology.

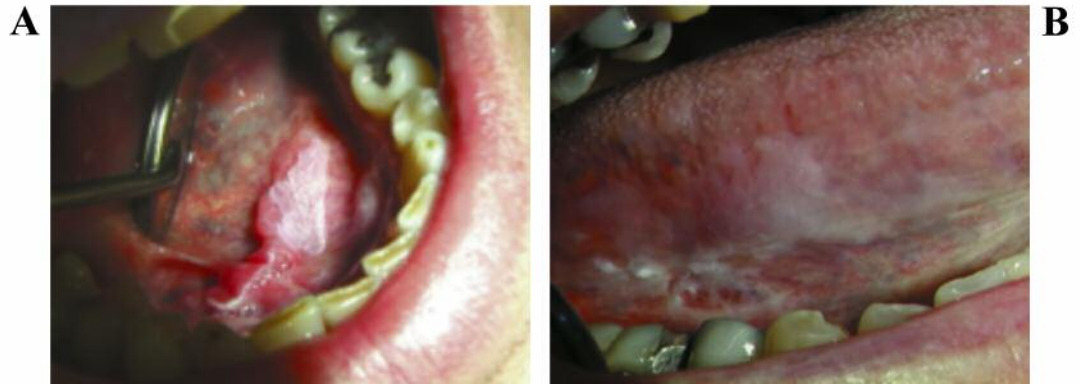

Figure 1. *Clinical presentation of homogenous leukoplakia in the floor of the mouth (A) and non-homogenous leukoplakia at the lateral border of the tongue (B).*

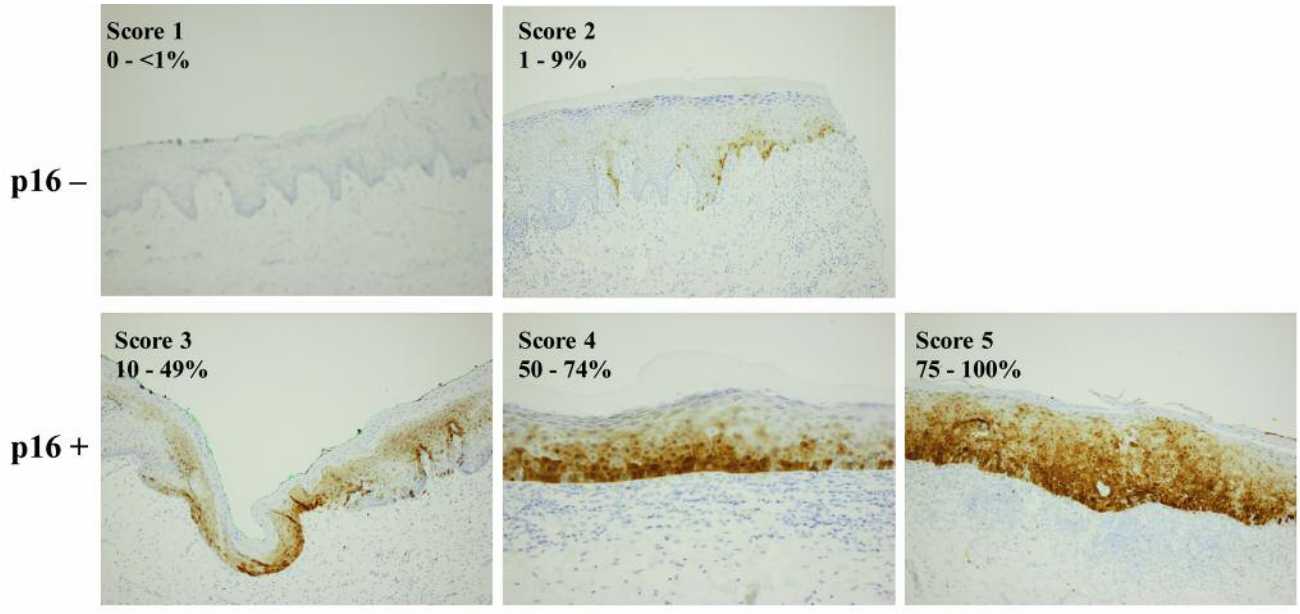

Figure 2. *Semi-quantitative assessment of p16 expression based on the percentage of p16 expression detected in the epithelial layer. Samples with scores in the range of 3-5 were regarded as p16-positive. The magnification in pictures 1, 2, 3 and 5 is ×100 and in picture 4 it is ×200.*

The clinical diagnosis was re-reviewed by two specialists in oral medicine. If there was a discordant opinion regarding a diagnosis, a discussion was initiated until consensus was reached. Of the 95 recruited patients, 14 were excluded: 10 because the diagnosis was revised and four because the diagnosis was uncertain. Thus, 81 patients were included in the final analysis.

For comparison, 15 patients with a clinical and histopathological diagnosis of OSCC and five patients with cervical carcinomas were included, because previous examinations had classified them as p16-positive. Paraffin-embedded samples from these cases were retrieved from the Diagnostic Biobank at the Department of Clinical Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden. Re-evaluation of p16 positivity was carried out by a senior pathologist (AK). Two patients with OSCC that were previously considered to be p16-positive were by re-assessment classified as p16-negative and therefore excluded. The five patients with cervical carcinoma were confirmed to be p16-positive. This latter group of patients formed the positive control group for the polymerase chain reaction (PCR) analysis. The characteristics of the patients with OL are listed in Table I and those of the patients with OSCC are listed in Table II.

Immunohistochemistry. Sections from paraffin-embedded biopsies were prepared, and immunohistochemistry was performed. Formalin-fixed and paraffin-embedded (FFPE) blocks were used to prepare 4-μm-thick sections that were placed onto positively charged slides (Flex IHC Microscope Slides; DAKO, Glostrup, Denmark). Subsequently, the tissue sections were subjected to

deparaffinisation and rehydration followed by heat-induced epitope retrieval in Tris/EDTA buffer (pH 9.0) for 20 min at 97˚C using a PT Link instrument (DakoCytomation, DAKO).

The tissue sections were immunostained with mouse monoclonal antibody to p16 (clone E6H4, CINtec Histology Kit; Roche Diagnostics GmbH, Mannheim, Germany;) using the DAKO visualisation system [EnVision FLEX, High pH (Link); DAKO] and DAKO Autostainer Plus for IHC (DakoCytomation, DAKO) following the manufacturer's instructions. Peroxidase-catalysed diaminobenzidine tetrahydrochloride was used as the chromogen to determine the protein expression levels in tumours from head and neck cancer of unknown primary, and then the slides were counterstained with haematoxylin (EnVision FLEX Hematoxylin; DAKO). The stained slides were rinsed with deionised water followed by a dehydration series in 70% ethanol, 95% ethanol, and absolute ethanol, and then cleared in xylene and a cover-glass (Coverslipper; DAKO) added. Sections from tonsillar cancer tissues served as positive controls, while omission of the primary antibodies served as negative controls.

Semi-quantitative analysis of p16 expression. Expression of p16 was recorded and evaluated using a semi-quantitative scale based on the percentage of p16 expression detected in the epithelial layer, using the following scoring scheme: 1: 0-1%; 2: 1-9%; 3: 10-49%; 4: 50-74%; and 5: 75-100% (Figure 2). Samples with scores in the range of 3-5 were regarded as p16-positive.

HPV DNA analysis. Formalin-fixed, paraffin-embedded tissue samples were sectioned (30×5 μm) using a microtome, with precautions taken to ensure that there was no contamination between cases. After each set of sections, the microtome was cleaned with Invitrogen DNAZap, (Thermo Fisher Scientific, Waltham, MA, USA), to avoid cross-contamination between the samples.

Xylene was used for deparaffinisation. The DNA extractions were performed using QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The concentration and purity of the extracted DNA were analysed in a spectrophotometer.

A TaqMan real-time PCR assay targeting 12 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and two low-risk HPV (LR-HPV) types (6 and 11), including the primers and probes presented in Table III, was used for identification of HPV DNA (42). As quality control, real-time PCR for the human betaglobin gene was run in parallel, and only samples with a betaglobin Ct-value of below 40 were included in the study. Seven patients from the OL group were accordingly excluded, resulting in 74 patients remaining in the OL group.

The Ethical Review Board in Gothenburg approved the study (Dnr. 673-10/T872-18).

Statistical analysis. Statistical analyses were carried out using the Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Analyses of differences between groups were performed using Fisher's exact test. A *p*-value of less than 0.05 was considered statistically significant.

Results

p16 expression and PCR analysis of samples of OL. Among the 74 patients in the OL group, p16 overexpression was observed in 13 patients (18%). None of the 74 patients with OL showed evidence of infection with any of the HPV sub-types targeted by PCR (Table II).

Table II. *Patterns of p16 expression in the patients with oral leukoplakia (OL) and patients with oral squamous cell carcinoma (OSCC) according to characteristics of the cancer in these patients.*

	OL, N (%)		OSCC, N (%)
Patients	p16-Positive (N=13)	p16-Negative (N=61)	p16-Positive (N=13)
Gender			
Male	7 (54%)	29 (48%)	9 (69%)
Female	6 (46 %)	32 (52%)	4 (31%)
Cancer transformation	0 (0%)	4 (7%)	-
HPV PCR result			
Positive	0 (0%)	0 (0%)	5 (38%)
Negative	13 (100%)	61 (100%)	8 (62%)
Clinical diagnosis			
Homogenous	10 (77%)	42 (69%)	-
Non-homogenous	3 (23%)	19 (31%)	-
Dysplasia			
Yes	4 (31%)	10 (16%)	-
No	9 (69%)	51 (84%)	-
Site of lesion			
Tongue	2 (15%)	19 (31%)	2 (15%)
Buccal mucosa	3 (23%)	12 (20%)	0 (0%)
Gingiva	8 (62%)	19 (31%)	8 (62%)
Floor of the mouth	0 (0%)	5 (8%)	1 (8%)
Palate	0 (0%)	3 (5%)	2 (15%)
Lip	0 (0%)	3 (5%)	0 (0%)

The clinical diagnosis of OL (homogenous or non-homogenous) and overexpression of p16 were not significantly correlated (p=0.74); the sensitivity was 19% and the specificity was 86% (Table II).

Overexpression of p16 was found in four out of 14 patients with dysplasia compared to nine out of 60 without dysplasia (Table II; p=0.25). Four patients (31%) out of the 13 p16-positive patients displayed dysplasia and 10 patients (16%) out of 61 patients with p16-negative samples had dysplasia (Table II). A slightly higher incidence of overexpression of p16 was observed among those with OL with dysplasia than OL without dysplasia, although no statistical significance was observed for this difference (p=0.25). This corresponds with a sensitivity of 29%, specificity of 85%, positive predictive value of 31% and negative predictive value of 84% for using p16 to identify dysplasia.

In four patients (7%) with p16-negative samples, lesions progressed from OL to OSCC, while none of the p16-positive cases of OL progressed to OSCC (Table II).

Eight patients (62%) who were adjudged to be p16-positive had OL located in the gingiva and 19 samples (31%) from p16-negative patients had OL in the gingiva (p=0.057).

Table III. *Primers and probes used for the detection of 14 human papillomavirus (HPV) types by real-time polymerase chain reaction.*

Target	Oligo	Sequence
HPV-6	F	RCGGTTYATAAAGCTAAATTGTACGT
	R	AGGGTAACATGTCTTCCATGCA
	P	AAGGGTCGCTGCCTACACTGCTGG
HPV-11	F	GCTTCATAAAACTAAATAACCAGTGGAA
	R	GTCAGGAGGCTGCAGGTCTAGTA
	P	CTATATCCTTTAGGGTAACAAGTCTTCCATGCATGTTG
HPV-16	F	TTGCAGATCATCAAGAACACGTAGA
	R	CAGTAGAGATCAGTTGTCTCTGGTTGC
	P	AATCATGCATGGAGATACACCTACATTGCATGA
HPV-18	F	AGAGGCCAGTGCCATTCGT
	R	GTTTCTCTGCGTCGTTGGAGT
	P	TCCTGTCGTGCTCGGTTGCAGC*
HPV-31	F	ATTCCACAACATAGGAGGAAGGTG
	R	CACTTGGGTTTCAGTACGAGGTCT
	P	ACAGGACGTTGCATAGCATGTTGGA
HPV-33	F	ATATTTCGGGTCGTTGGGCA
	R	ACGTCACAGTGCAGTTTCTCTACGT
	P	GGACCTCCAACACGCCGCACA*
HPV-35	F	TCGGTGTATGTCCTGTTGGAAAC
	R	CATAGTCTTGCAATGTAGTTATTTCTCCA
	P	TGCATGATTACACCTCGGTTTCTCTACGTG
HPV-39	F	GCAGGAAGCTATACAGGACAGTGTC
	R	CTTGGGTTTCTCTTCGTGTTAGTCT
	P	CCCGTTTTGTGGTCCAGCACCG*
HPV45	F	GGACAGTACCGAGGGCAGTGTAA
	R	TCCCTACGTCTGCGAAGTCTTTC
	P	CATGTTGTGACCAGGCACGGCA
HPV-51	F	AAAGCAAAAATTGGTGGACGA
	R	TGCCAGCAATTAGCGCATT
	P	CATGAAATAGCGGGACGTTGGACG
HPV-52	F	GACATGTTAATGCAAACAAGCGAT
	R	CATGACGTTACACTTGGGTCACA
	P	TGTTCAGAGTGTTGGAGACCCCGACC
HPV-56	F	TGCATTGTGACAGAAAAAGACGAT
	R	CTCCAGCACCCCAAACATG
	P	CCCGGTCCAACCATGTGCTATTAGATGA*
HPV-58	F	GGCATGTGGATTTAAACAAAAGGT
	R	TCTCATGGCGTTGTTACAGGTTAC
	P	TGGAGACCCCGACGTAGACAAACACAA
HPV-59	F	TGTATGGAGAAACATTAGAGGCTGAA
	R	TGGACATAGAGGTTTTAGGCATCTATAA
	P	AGACACCGTTACATGAGCTGCTGATACGC
β-Globin	F	CAGGTACGGCTGTCATCACTTAGA
	R	CATGGTGTCTGTTTGAGGTTGCTA
	P	GCCCTGACTTTTATGCCCAGCCCTG

F: Forward-primer; R: reverse-primer; P: probe.

The overexpression of p16 and the gender of the patients did not correlate (p=0.76), nor did smoking (p=0.52) or use of snuff (p=0.28) with the overexpression of p16.

p16 expression and HPV infection in patients with OSCC. Among the 13 p16-positive patients with OSCC, five (38%) were positive for HR-HPV (sub-type16) (Table II). Four of these five (80%) HPV-positive patients had OSCC in the gingiva and one (20%) had OSCC in the floor of the mouth. In the eight HPV-negative patients, the tumour was located in the gingiva in six (75%), and the tongue in two patients (25%).

HPV infection in patients with cervical carcinoma. In the control group, four out of five patients (80%) with p16-positive cervical carcinomas were positive for HR-HPV (sub-type 16, 31, 16+31, 51) in the PCR analysis.

Discussion

One of the main findings of the present study is that none of the 74 analysed cases of OL were infected with any of the 12 HR-HPV sub-types or either of the two LR-HPV sub-types that were addressed.

This finding was surprising because several previous studies have reported the presence of HPV in OSCC, OL and other potentially malignant oral disorders (29, 30, 43), and Syrjanän *et al*., concluded that HPV likely is causally associated with potentially malignant oral disorders and OSCC (24).

Pierangeli *et al*. reported that 53.2% of patients with potentially malignant oral disorders were HPV-infected, with HPV types 16 and 18 being the most frequently detected types (43). Isayeva *et al*. reported the weighted prevalence of HPV DNA was 41.4% in the 144 cases of potentially malignant oral disorder from 10 different studies, which was significantly higher than in controls (44).

Explanations for the discrepancy between our findings and these of previous studies could be differences in the quality of the samples, in the methodology used, or the geographical region (44, 45). Kreimer *et al*. analyzed the global distribution of HPV genotypes in head and neck squamous cell carcinomas, both in cases of OPSCC and OSCC, and found a higher HPV prevalence of OSCC in Asia than in Europe and the USA (44, 45). Nonetheless, we expected to find some HPV-positive cases of OL in our cohort, given that HPV prevalence in the healthy oral mucosa is reported to be 4.5%, including a 3.5% prevalence of HPV-16 (46).

Another possible explanation for the finding of no HPV infection in our patients with OL is that the mean age of our cohort was rather high at 61 years, with an age range of 28-82 years, which is higher than in studies that found a higher prevalence of HPV infection (47, 48).

Of the 74 patients with OL, 18% showed overexpression of p16 but HPV DNA was not detected in any of them. In contrast, HPV-16 was detected in 38% of the patients in the OSCC group with overexpression of p16, and 80% of the patients with cervical carcinoma showed HPV-16 infection. These findings suggest that in patients with OL, overexpression of p16 does not correlate with HPV infection in the same manner as in OSCC and cervical carcinoma.

Angiero *et al*. have shown that the expression of p16 increases with the presence of dysplasia (49). In line with this, we found that p16 was more often overexpressed in OL with dysplasia. The sensitivity was 29% and the specificity 84%, which differs from the 83% sensitivity and 40% specificity reported by Pathak *et al*. (50).

The relatively low sensitivity of p16 in identifying dysplasia, and the finding that none of the four patients with OL who developed OSCC were p16-positive indicates that analysis of p16 is not useful for predicting malignant progression of OL. This seems to fit with our finding that HPV infection, at least of the types targeted by our assay, is not important for the pathogenesis of OL. Furthermore, overexpression of p16 was found not to be a reliable biomarker for HPV infection in patients with OL and OSCC.

Acknowledgements

This study was supported by grants from The Healthcare Board, Region Västra Götaland (Hälso-och sjukvårdsstyrelsen), TUA Research Funding; The Sahlgrenska Academy at University of Gothenburg/Region Västra Götaland, Assar Gabrielsson Foundation, Adlerbertska Foundation, Swedish Dental Society and Gothenburg Dental Society, Sweden. The Authors acknowledge the valuable assistance of Dr Vincent Collins for English proofreading of the manuscript.

Conflicts of Interest

The Authors declared no conflicts of interests in regard to this study.

Authors' Contributions

Jonas Sundberg: participated in the design, planning, analysed and interpreted data and drafted the manuscript; Magdalena Korytowska: analysed and interpreted data, critically reviewed the manuscript; Patricia Miranda Burgos: clinical data collection and follow-up of study patients, critically reviewed the manuscript; Johan Blomgren: clinical data collection and follow-up of study patients, critically reviewed the manuscript; Lena Blomstrand: clinical data collection and follow-up of study patients, critically reviewed the manuscript; Shahin De Lara: performed immunohistochemical staining, co-drafted the immunohistochemistry method part in the manuscript, critically reviewed the manuscript; Lars Sand: participated in the design and study planning, clinical data collection, critically reviewed the manuscript; Jan-Michäel Hirsch: participated in the design and study planning, clinical data collection, critically reviewed the manuscript; Erik Holmberg: statistical analyses, critically reviewed the manuscript; Daniel Giglio: analysed and interpreted data and co-drafted the manuscript; Jenny Öhman: participated in the study design, planning, analyses, data interpretation and co-drafted the manuscript; Anikó Kóvacs: histopathological diagnoses, planning, data interpretation and co-drafted the manuscript; Peter Horal: participated in the study design, planning and interpretation of PCR analyses, critically reviewed the manuscript; Magnus Lindh: participated in the study design, planning and interpretation of PCR analyses, co-drafted the manuscript; Göran Kjeller: participated in the design and study planning, clinical data collection, critically reviewed the manuscript; Bengt Hasséus: participated in the design and study planning, clinical data collection, analyses, co-drafted the manuscript.

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Received December 31, 2018
Revised January 25, 2019
Accepted February 6, 2019