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ORIGINAL ARTICLE

Promoter DNA Methylation and mRNA Expression Level of p16 Gene in Oral Squamous Cell Carcinoma: Correlation with Clinicopathological Characteristics

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Abstract

The aim of this study was to investigate the relationship between p16 methylation and its expression in oral squamous cell carcinoma (OSCC). Also the contribution of clinicopathological factors, HPV infection and smoking in p16 expression and promoter methylation has been investigated. In this study 67 consecutive OSCC patients and 59 normal individuals were enrolled. All patients were candidates for surgery of oral cavity and fresh tumor biopsies were collected and processed for DNA and RNA extraction. Normal gingival tissues were collected from individuals referred to dentistry clinic and considered as controls. All the cases and controls were checked for HPV infection and then promoter methylation and expression of p16 gene were determined using Methylation-specific PCR (MSP) and real-time PCR (QPCR), respectively. Methylation of p16 in tumors and normal tissues were 59.7 and 38.9%, respectively. Most of hypermethylated samples (>82%) were in high grades. P16 methylation was comparable in HPV+ and HPV- patients or smokers. P16 was overexpressed (~3 fold; $p = 0.044$) in HPV+ tumors, but it was significantly down-regulated in smoker patients (40% of all tumors). Comparison of P16 expression in OSCC tumors with different degrees of promoter methylation further suggest the relationship of methylation rate and down-regulation of P16 expression. The p16 methylation and expression was differentially affected in patients with HPV infection and the smoker cases. Regardless of the influence of environmental factors, it appears that P16 status is useful for classifying patients with OSCC and for influencing treatment strategies in accordance with this classification. Moreover, targeting the upregulation of p16 could be a promising therapeutic option.

Keywords OSCC · HPV · Smoking · P16 promoter methylation · P16 gene expression · Iran

Introduction

Oral squamous cell carcinoma (OSCC) is one of the 10 most common cancers with a delayed clinical detection, poor prognosis, without specific biomarkers for the disease and expensive therapeutic alternatives [1]. OSCC arises through multistep carcinogenic pathways, which include cumulative genetic alterations and epigenetic changes leading to a loss of cell cycle control [2, 3].

In addition to genetic predisposition of people to OSCC, there are several environmental and lifestyle factors that can contribute to development of this type of cancer [4]. Among these, human Papillomavirus (HPV) infection, smoking and alcohol drinking are major promotors of cancer development [5]. For instance, it has been reported that the prevalence of HPV is ranging from 16 to 33% oral cavity cancer patients, worldwide [6].

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p16, also known as cyclin-dependent kinase inhibitor 2A is a tumor suppressor protein that in humans is encoded by the CDKN2A gene [7, 8]. p16 is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6. These latter kinases phosphorylate retinoblastoma protein (pRB) which eventually results in progression from G1 phase to S phase. The CDKN2A gene is frequently mutated or deleted in a wide variety of tumors. p16 was originally found in an "open reading frame of 148 amino acids encoding a protein of molecular weight 15,845 comprising four ankyrin repeats [9]. $p16^{Ink4A}$ is named after its molecular weight and its role in inhibiting CDK4 [9]. Mutations in the CDKN2A gene are associated with increased risk of various cancers and alterations of the gene are frequently seen in cancer cell lines [10]. Hypermethylation of tumor suppressor genes is an alternative process to gene mutations which has been implicated in various cancers. Studies show that tissue samples of primary OSCC display methylation in the promoter regions of p16. This epigenetic changes leading to the loss of tumor suppressor gene function can occur through two possible mechanisms. Methylation can either physically inhibit the transcription of the gene or can cause recruitment of transcription factors that repress transcription. Down-regulation of p16 gene leads to decreased levels of the p16 protein which plays an important part in development of various cancers [11–16].

The tumor suppressor gene p16 maps on 9p21, and its protein product $p16^{INK4A}$ is implicated in the regulatory mechanism known as the pRB pathway [17]. Reduced $p16^{INK4A}$ expression with consequent loss of function is often observed in head and neck squamous cell carcinoma [18, 19]. The p16 gene is epigenetically silenced in human cancers such as gastric cancer [20], cervical cancer [21], breast cancer [22], head and neck cancers [16]. Accordingly in various cancer conditions the expression of p16 is suppressed. Paradoxically, p16 overexpression has sometimes been reported in OSCC, suggesting that p16 overexpression may be implicated in the pathogenesis of a subset of OSCC [23, 24].

Association of cigarette smoking and HPV infection with OSCC is a debating issue. On one hand there is an association between p16 expression and its promoter methylation, on the other hands, factors affecting p16 expression may not necessity change the rate of p16 methylation. The present study was carried out in a sample of Iranian patients to assess the association between p16 methylation and its expression level in progression of OSCC. Also the impact of variables such as clinicopathological factors, HPV infection and smoking on expression and promoter methylation of p16 has been studies.

Materials and Methods

Patients and Sample Collection

In this study tumor specimens from 67 consecutive patients with oral cavity squamous cell cancer (OSCC) were selected from the the patients admitted to Imam Khomeini Hospital for surgical operation during January 2013 to September 2015. These patients underwent a surgical biopsy for the first time without adjuvant chemotherapy or radiotherapy. The control group selected of apparently healthy individuals that referred to dentistry for tooth extraction and who agreed to a gingival biopsy.

The demographic characteristic of the patients and controls are shown in Table 1. A written informed consent was obtained from all subjects before sample collection. This study was approved by the Ethics Committee of the Cancer Institute of Imam Khomeini hospital as well as the Medical Ethics Committee of Tarbiat Modares Univerity. The study was conducted in accordance with the Helsinki declaration. All the patients were proved to have oral cavity SCC after histological examination by pathologists and patients undergoing any radiotherapy and/or chemotherapy were excluded from this study. The pathological grade and clinical staging of tumors was determined by pathologist according to standard protocols [25].

Table 1 Demographic characteristics, tumor characteristics and HPV infection among patients and controls

Characteristics	patients	controls
Age (Mean ± SD)	59.8 ± 13.6	44.1 ± 13.5
Gender (Male / Female)	39 / 28	35/ 24
Tobacco smoking	36 (53.7)	31(52.5)
Tumor grade		
Well differentiated	39 (58.2)	
Moderate differentiated	19 (28.4)	N/A
Poor differentiated	9 (13.4)	
Tumor stage		
I	24 (35.8)	
II	27 (40.3)	N/A
III	11 (16.4)	
IV	5 (7.5)	
Tumor site		
Lips	5 (7.5)	
Tongue	28 (41.8)	N/A
Floor of mouth	2 (3.0)	
Buccal Mucosa	14 (20.9)	
Gingiva	8 (11.9)	
Hard Palate	10 (14.9)	
HPV 16/18 positive	10 (14.9)	4 (6.7)

DNA Extraction from Tissue Biopsies

High molecular weight genomic DNA was extracted from one portion of the tumor biopsy as well as from gingival tissues obtained from normal individuals (dentistry samples). DNA was isolated using standard method of phenol chloroform extraction procedure [26]. The final concentration of genomic DNA was determined using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Detection of HPV Infection in Tissue Samples

A PCR assay was adopted to detect the major HPV subtypes (HPV16 and HPV 18) in tumor and non-tumor biopsies. Detection of HPV was performed using specific primers in E6 region of HPV16 (ACCACAGTTATGCACAGA and CCACCGACCCCTTATATT; amplicon size = 361 bp) and HPV18 (CCCTACAAGCTACCTGAT and CTTGTGTT TCTCTGCGTC; amplicon size = 442 bp). The PCR reaction mixture comprised of 100 ng genomic DNA, 5 pmol of each primer, and 10 μl of 2x Taq DNA Polymerase Master Mix RED (Ampliqon, Herlev, Denmark) in a total volume of 20 μl. The PCR protocol involved preheating at 95 °C for 5 min, followed by 30 cycles of amplification in a thermocycler (MyCycler, BioRad, USA). The reaction included a denaturation step at 95 °C for 30 s, primer annealing step at 57 °C for 30 s and chain elongation step at 72 °C for 40 s, followed by a final elongation step at 72 °C for 10 min. Finally, the PCR products were separated by electrophoresis on 2% agarose gel stained with gel red (Biotium, UK) to visualize DNA fragments.

Methylation-Specific PCR (MSP) for p16 Gene

The P16 gene promoter methylation status was analyzed by Methylation-Specific PCR (MSP). The bisulfite conversion of DNA was performed using commercially available kit (EpiJET™ Bisulfite Conversion Kit; Thermo Scientific) in accordance with the manufacturer's instructions. In this experiment, the bisulfite-modified DNA was amplified using two sets of primers specific for methylated and unmethylated p16 sequences. The specific primers for the methylated sequence were TTATTAGAGGGTGGGGCGGATCGC and GACCCCGAACCGCGACCGTAA (product size 150 bp). The specific primers for the unmethylated p16 sequence were TTATTAGGGGTGGGGTGGATTGT and CAACCCAA ACCACAACCATAA, (product size 151 bp).

The PCR reaction mixture comprised of 2 μl bisulfite-treated DNA, 5 pmol of each methylated primer or unmethylated primer, 10 μl of Hot Start 2x master mix blue (Ampliqon, Herlev, Denmark) in a total volume of 20 μl. The PCR protocol involved preheating at 95 °C for 15 min, followed by 35 cycles of amplification in a thermocycler (MyCycler, Biorad, USA). Each cycle included a denaturation step at 95 °C for 30 s, primer annealing step at 63 °C for 30 s and chain elongation step at 72 °C for 40 s, followed by a final elongation step at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized DNA fragments by gelred (Biotium, UK).

p16 Gene Expression by Quantitative Real-Time PCR (QPCR)

Total RNA was isolated from frozen tumor and matched normal tissue biopsies using the RNA extraction kit (Hybrid-R, GeneAll Biotechnology, Republic of Korea), according to manufacturer instructions. The concentration of total RNA in the final eluent was determined using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Two micrograms of total RNA from tissues were reverse-transcribed with oligo (dT) primer using the First Strand cDNA Synthesis Kit (HyperScript RT master mix, GeneAll Biotechnology, Republic of Korea) in accordance with the manufacturer's instructions.

We quantified the mRNA expression of the target gene (p16) and an internal control gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) by real-time PCR using the SYBR Green master mix (Ampliqon; Odense, Denmark) on the ABI 7500 Real time PCR system (Applied Biosystems).

The sequences of the primers for p16 gene were AGCCTTCGGCTGACTGGCTGG and CTGCCCAT CATCATGACCTGGA (product size 139 bp) and the sequences of the primers for HPRT1 gene were CCTGGCGT CGTGATTAGTG and TCAGTCCTGTCCATAATTAGTCC (product size 125 bp). For each qPCR reaction we used 2 μl of the diluted cDNA, 5 pmol each of primers, 10 μl of 2x SYBR green master mix (Ampliqon; Odense, Denmark) in a total volume of 20 μl. The PCR cycle conditions were set as follow: a pre-incubation step for 15 min at 95 °C followed by 40 cycles; each cycle included 15 s at 95 °C, 60 s at 60 °C. A melting curve was generated by linear heating from 60 °C to 95 °C.

All amplification reactions were performed in triplicate. The formula $2^{-\Delta\Delta Ct}$ was used to calculate relative quantitative values from data of an individual sample to normalize with its housekeeping gene for comparing with the normal tissue to show the expression differences in folds.

Using these quantitative methods requires that the PCR efficiencies of all genes be similar and preferably ≥90%. Efficiency was measured using a standard curve generated by serial dilutions of the RNA. Consequently, the initial RNA concentration of 100 ng/μl was serially diluted 5-fold (100 ng, 20 ng, 4 ng, 0.8 ng and 0 ng) for the real-time PCR assay according to the standard protocol of Applied Biosystems. Standard curve assays showed an efficient amplification >90% for both genes and the specificity was shown

by a single peak at the expected temperature on melting curve analyses.

Statistical Analysis

Data analyses performed using SPSS software version 16. Fisher's exact test was selected to compare the HPV infection and promoter methylation between cases and controls. In addition, promoter methylation status in cases was compared among different demographic and clinicopathological groups using Chi-square or Fisher's exact test. The correlation between the expression of p16 and clinicopathological factors were analyses by Welch's t-test and one-way ANOVA. The p values less than 0.05 were considered to be significant.

Results

In this study, tumor biopsies from a group of Iranian patients diagnosed with OSCC and gingival biopsies from normal controls were taken. The samples were analyzed for possible infection with types 16 and 18 by Polymerase Chain Reaction (PCR).

The samples were then processed for p16 expression at mRNA level using Real-time PCR. In addition, methylation status of p16 promoter gene was detected by Methylation Specific Polymerase Chain Reaction (MSP). The demographic and clinical characteristics of the cases and controls have been summarized in Table 1.

Detection of HPV DNA in the Tissue Samples (HPV DNA)

Based on PCR assay performed on oral cavity squamous cell carcinoma (OSCC) samples and healthy gingival biopsies it was shown that only 14.9% of tumors (10/67) and 6.8% of the normal gum samples (4/59) were HPV DNA positive. The incidence of HPV16/18 was not significantly different between case and control samples.

Methylation Status of p16 Promoter in Tissue Biopsies

The result of promoter DNA methylation pattern of p16 gene is shown in Table 2. As shown in Table 2, the methylation status of p16 gene promoter in tumor tissues and healthy tissues was found to be 59.7% (40/67) and 38.9% (23/59) respectively ($p = 0.032$, odds ratio = 2.32).

Table 2 Promoter methylation status of P16 gene in patients with OSCC and healthy control subjects

Methylation status	Patients n (%)	Controls n (%)	OR (95% CI)	P value
Methylated	40 (59.7)	23 (38.9)	2.32 (1.1–4.7)	0.032*
Unmethylated	27 (40.3)	36 (61.0)		

*: $p \leq 0.05$, Fisher's exact test

The p16 methylation status in relation to the expression levels of p16 gene in tumor tissues of patients has been shown in Fig. 4. Comparison of the P16 expression level in tumor samples with promoter methylation in three forms of epigenotypes (MM = Methylated/Methylated; UM = Unmethylated/Methylated and UU = Unmethylated/Unmethylated) clearly explain the impact of methylation on the gene expression. The P^{16} expression level was significantly decreased ($P = 0.013$) in UM and MM epigenotypes compared to samples with UU. The expression level of P^{16} was also significantly different between UM and MM groups ($P = 0.039$).

The p16 methylation status in relation to the clinicopathological characteristics are summarized in Table 3. The rate of p16 methylation in pathologically well differentiated (WD) tumors and in moderately or poorly differentiated (MD/PD) tumor grades was found to be 48.5% (17/35) and 82.1% (23/28), respectively ($P < 0.0022$).

As shown in Table 3, there was no significant relationship between p16 promoter methylation and other demographic criteria of the patients. Likewise, there was no significant relationship between methylation status and HPV16/HPV18 infection in tumor samples.

Comparison of p16 Expression in Oral Cavity SCC and Control Samples

Total RNA was used to perform quantitative real-time PCR analysis of p16 expression in OSCC as well in healthy control samples. The calculated expression of the gene was normalized to HPRT1 and expressed as relative units. The p16 expression was decreased by approximately 40% ($p = 0.007$) in tumor tissues compared to normal gingival samples (Fig. 1a). As shown in Fig. 1b, the efficiency of the PCR method was >90% for target genes viz. p16 and HPRT genes.

Expression of p16 at mRNA level in terms of tumor grade and tumor stage revealed that p16 expression in tumors diagnosed as MD/PD is reduced by 62% compared to control samples ($P = 0.0002$) (Fig. 1c). As shown in Fig. 1d, Likewise the p16 expression was decreased by 61% in tumors diagnosed as stage III/IV compared to controls ($P = 0.003$).

Comparison of p16 Promoter Methylation and P16 Gene Expression between Smoking and Non-smoking Patients

As shown in Fig. 2a, there was a significant decrease in p16 gene expression in smoker patients compared to non-smoker

Table 3 Relationship between promoter methylation status of P16 gene and clinicopathological parameters in patients with oral cavity squamous cell cancer

Characteristic	All patients	Methylation status		P value
		Methylated n (%)	Unmethylated n (%)	
Age (year)				0.441
< 50	17	11 (64.7)	6 (35.3)	
50–59	12	5 (41.7)	7 (58.3)	
60–69	21	12 (57.1)	9 (42.9)	
> 69	17	12 (70.6)	5 (29.4)	
Gender				
Male	39	23 (59.0)	16 (41.0)	1.0
Female	28	17 (60.7)	11 (39.3)	
Tobacco exposure				
Smoking	36	22 (61.1)	14 (38.9)	0.808
Never	31	18 (58.1)	13 (41.9)	
Tumor Grade				
WD	39	17 (43.6)	22 (56.4)	0.0022*
MD/PD	28	23 (82.1)	5 (17.9)	
Tumor Stage				
I/II	51	27 (52.9)	24 (47.1)	0.077
III/IV	16	13 (81.2)	3 (18.8)	
Tumor Site				
Lip	5	3 (60.0)	2 (40.0)	0.695
Tongue	28	15 (53.6)	13 (46.4)	
Floor of mouth	2	1 (50.0)	1 (50.0)	
Buccal mucosa	14	11 (78.6)	3 (21.4)	
Gingiva	8	4 (50.0)	4 (50.0)	
Hard palate	10	6 (60.0)	4 (40.0)	
HPV 16/18 infection				
Positive	10	6 (60.0)	4 (40.0)	1.0
Negative	57	34 (59.6)	23 (40.4)	

Fisher's exact test or Chi-square test

WD well differentiated; *MD* Moderately differentiated; *PD* Poorly differentiated

*: $p \leq 0.05$

patients ($p = 0.03$). Nevertheless, the number of p16 methylated samples was significantly more among smoker patients compared to non-smoker cases (Fig. 2b).

Comparison of p16 Promoter Methylation and P16 Gene Expression in HPV Infection Tumor Samples

The expression of p16 mRNA in HPV-positive tumor samples was significantly increased ($p = 0.044$) when compared to HPV-negative samples (Fig. 3a). However, the methylation status of p16 gene in tumors was unaffected due to HPV infection (Fig. 3b).

Discussion

In the present study, we observed that the expression of p16 at mRNA levels is significantly down-regulated in tumor tissues from OSCC patients. There was a significant association between p16 expression and the pathological grade and stage of the tumors. P16 expression was significantly higher in HPV positive cases; however, smoking has been associated with significant decrease in p16 expression.

The rate of methylation of p16 has been significantly increased in OSCC patients compared to controls ($p = 0.032$). However, methylation was unaffected in cases having smoking habit or HPV infection. Loss of p16 expression has been associated with methylation of promoter of p16 gene. Down-regulation of p16 expression in patients with smoking history is probably independent of p16 methylation status, since the rate of p16 methylation/unmethylation was comparable in smoking and non-smoking cases.

There are a number of reports showing the contribution of p16 methylation in precancerous oral lesions with and without epithelial dysplasia [27, 28]. The association of p16 methylation with advanced OSCC has also been established [29–31]. However, the influence of HPV infection and smoking habit of patients on p16 gene expression and methylation is not well understood. Studies on patients with social and cultures diversity in different geographical regions may help identification of risk factors of OSCC. Iran is a unique society but with different life styles. Epidemiological reports in Iran show that in the last two decades the rate of oral cancer is increasing. A survey shows that OSCC patients in this country are less dependent on alcohol drinking, abnormal sex habits and tobacco chewing. In a small population enrolled in the present study, approximately about 15% of OSCC patients were HPV DNA positive, suggesting that HPV infection is not a major risk factor for OSCC in Iranian patients.

The methylation status of p16 promoter assessed by MSP was significantly higher in tumors (59.7%) compared to control samples (normal gingival). Hypermethylation of p16 was associated with down-regulation of p16 gene in tumors (Fig. 1a). Comparison of the P16 expression level in tumor samples with different degress of promoter methylation (MM; UM and UU) clearly explain the impact of methylation on the gene expression (Fig. 4). This data is in agreement with reports from other laboratories showing decreased p16 expression is associated with consequent loss of its function in OSCC tumors [32, 33]. There are also reports showing overexpression of p16 in human subjects with OSCC [23, 24]. This controversial data could be explained by differences in the pathogenesis of a subset of OSCCs. In this connection we observed that more than 80% of tumors were in higher pathological grade and stage showed p16 methylation (Table 3).

Despite the inverse relationship between p16 gene expression and its methylation status in head and neck SCC [16, 34],

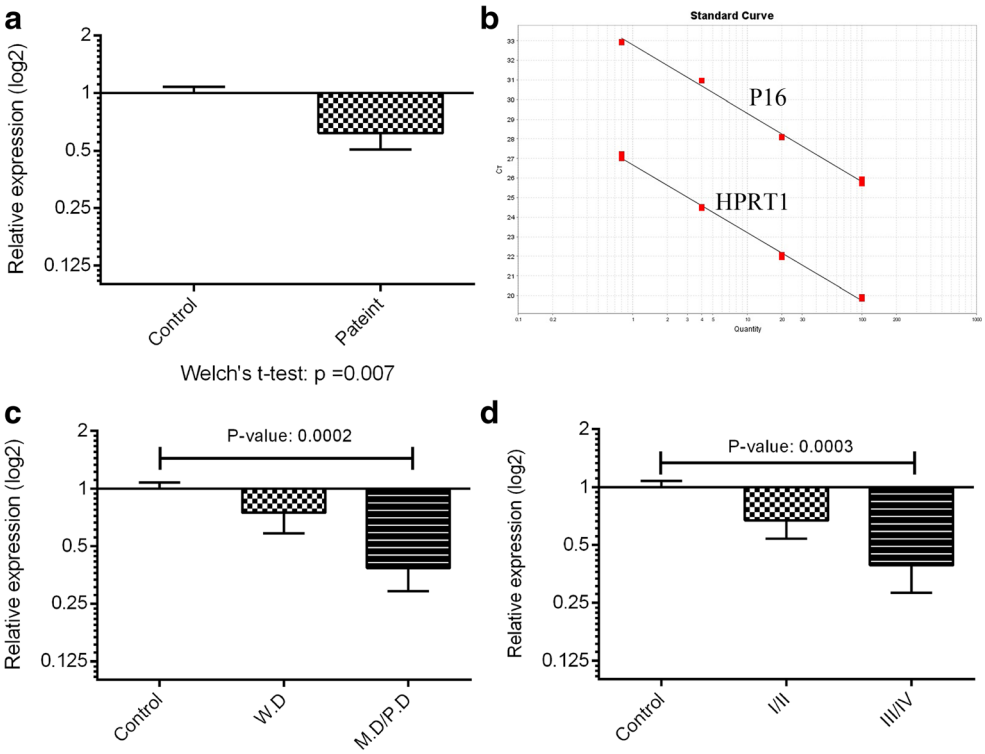

Fig. 1 Comparison of p16 specific mRNA expression in OSCC tumors and normal tissues. **a** p16 expression in OSCC patients and healthy controls. **b** The efficiency of primers used in QPCR from 4 different cDNA template prepared by serial dilution was checked. Sections C and D) show p16 mRNA expression in pathological grade/ stages

the contribution of other factors such as gene mutation [35], life style (smoking) [5] and infection with HPV [36] in regulation of expression of tumor suppressor genes cannot be ruled out.

Both the HPV status and p16 expression are believed to be important parameters influencing the survival of cancer patients [5]. But it appears that the influence of cigarette smoking on this process varies depending on other factors [5, 37]. Our study shows that down-regulation of P16 mRNA expression in smokers (40% of all tumors) is associated with the methylation of p16 promoter region. This finding justifies that smoking exert its deteriorative and carcinogenic effects at least partially by means of the inhibition of p16 protein expression. Cigarette smoke has considerable oxidative stress potential [38], which can predispose tissues to cancer. Exposure to cigarette smoke can also lead to genetic and epigenetic alterations which are considered as major hallmarks of cancer and carcinogenesis process.

The relationship of smoking habit with p16 status in OSCC revealed that the difference in p16 expression in smoker and non-smoker patients was not correlated with p16 expression levels (Fig. 2a, b). Likewise, p16 expression was not consistent with HPV infection in OSCC patients, as it was demonstrated that p16 expression in HPV DNA positive samples was significantly higher ($p = 0.044$) than that detected in HPV-negative tumors (Fig. 3a).

Despite the contribution of HPV infection in p16 expression, it appears that HPV-related changes in p16 expression occurs independent of p16 promoter

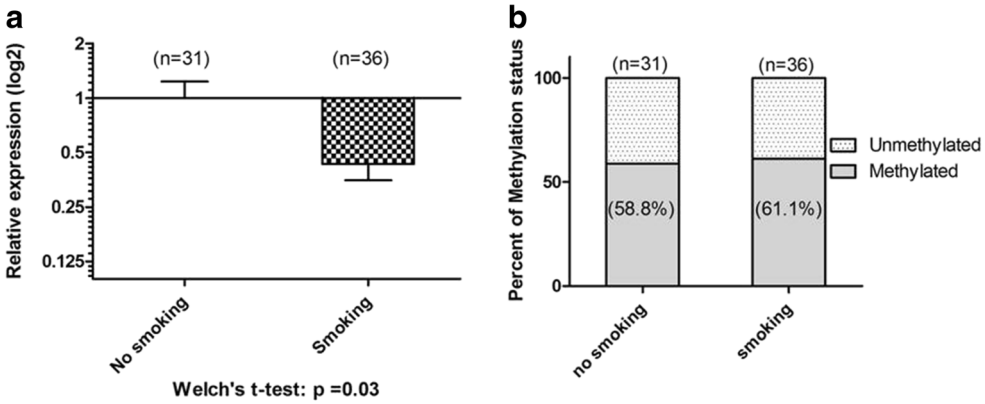

Fig. 2 Comparison of p16 expression and its methylation status in smoker and non-smoker OSCC patients. Patients with smoking habit $n = 36$ (61.1%) and non-smokers $n = 31$ (58.8%). **a** p16 expression was measured by quantitative Real-time PCR. **b** Methylation of p16 promoter was assessed using MSP method. $P < 0.05$ is considered significantly difference between the two groups

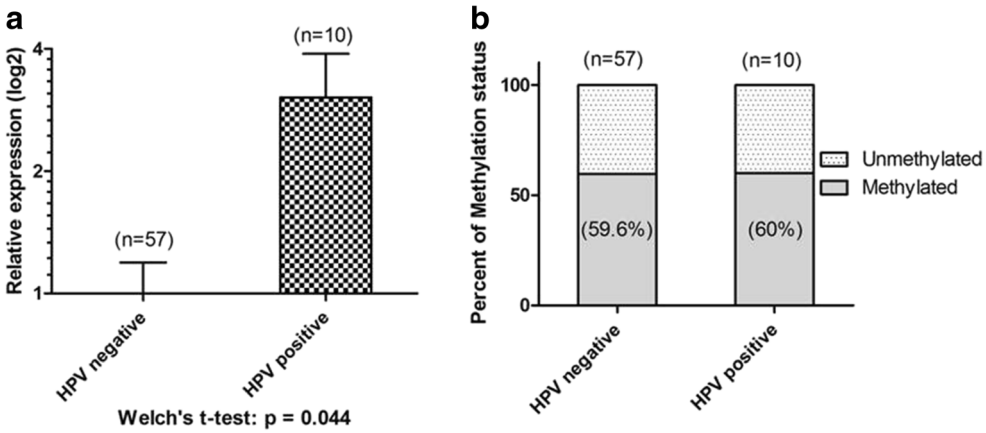

Fig. 3 The impact of HPV infection on expression and methylation status of p16 in OSCC and normal tissues. Section A) expression of P16 specific mRNA in OSCC tumors and gingival biopsies in HPV-DNA positive ($n = 10$) and HPV-DNA negative (n = 31) samples. Section B) compares the effect of HPV infection on p16 promoter methylation status in OSCC tumors and gingival tissues

methylation status (Fig. 3). Lack of influence of HPV infection on methylation status of p16 gene in tumors attest to this finding (Fig. 3b).

These data suggest that HPV16/HPV18 may play important role in the pathogenesis of OSCC by activation of p16 expression [23]. The role of HPV in development of OSCC is justified by showing that the over expression of p16 is due to the presence of transformed HPV genome in tissues [39]. The association of p16 expression with HPV infection in oral and cervical cancers revealed that HPV acts through E7 oncoprotein from high risk HPV which can bind retinoblastoma (Rb) leading to inhibition of Rb-E2F complex formation [40, 41]). As a consequence, the free E2F in absence of the complex with Rb can facilitate p16 gene transcription in malignant tissues [42]. The impact of different genetic mutations in p16 gene on changes in the rate of p16 expression in HNSCC with emphasize on HPV infection has been outlined in The Cancer Genome Atlas Network [43].

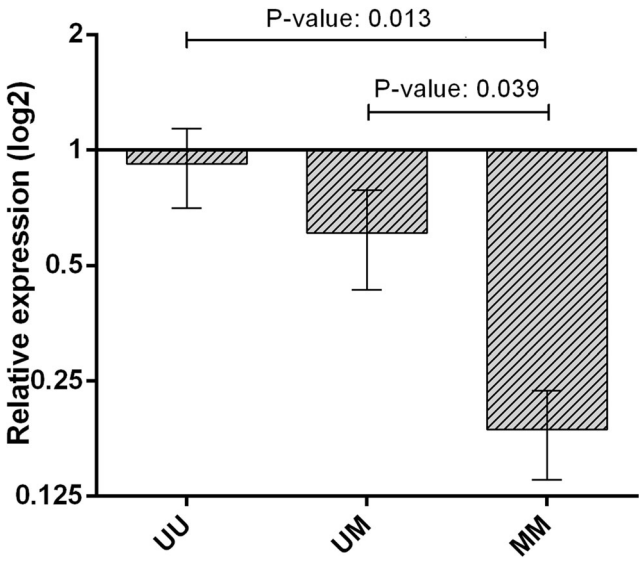

Fig. 4 Relationship of P16 gene expression with the rate of P16 methylation in tumors from OSCC patients. Details about the P^{16} expression and determination of promoter methylation is as described in methods section. MM = Methylated/Methylated; UM = Unmethylated/Methylated and UU = Unmethylated/Unmethylated. The expression data are presented as relative percentage (R%)

In regard to the tumor progression and the p16 expression and methylation in OSCC, it was demonstrated that the p16 gene expression and p16 promoter methylation status were dependent on tumor stage (III/IV) and pathological grade (MD/PD) (Table 3 and Fig. 1c). The expression of p16 gene (QPCR assay) was significantly lower (60%, $P < 0.05$) in higher histological grade of tumors (stage III/IV and grade MD/PD) compared to that measured in biopsies from normal gum tissues (Fig. 1c, d). As it was expected, p16 promoter methylation status in MD and PD tumors was significantly higher than that of normal samples, suggesting that higher grades of tumors are more vulnerable to hypermethylation. This data support the finding of Shaw et al. (2013) which showed that higher frequency of p16 methylation in OSCC patient occurs mainly in MD/PD tumors [44].

In conclusion, our results clearly showed a correlation between p16 expression and promoter methylation in low and high grades OSCC. The influence of factors such as HPV infection and smoking habit is not ruled out in this event. It appears that tumor progression is partially attributed to down-regulation of the p16, and it appears that the influence of HPV infection and cigarette smoking on p16 expression is independent of its promoter methylation.

Despite the significant difference in p16 methylation and expression in HPV positive patients and the smoker cases, this study further suggest that P16 status is useful for classifying patients with OSCC and for influencing treatment strategies in accordance with this classification. Moreover, targeting the upregulation of p16 in cancer patients could be a promising therapeutic option.

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Compliance with Ethical Standards

Conflict of Interest The authors have declared that no potential conflict of interest exists.

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