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Article

Promoter hypermethylation of tumor suppressor genes, *p16^{INK4a}*, *RASSF1A, TIMP3* and *PCQAP/MED15*, in salivary DNA as a quadruple biomarker panel for early detection of oral and oropharyngeal cancers

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Abstract: Silencing of tumor suppressor genes (TSGs) by DNA promoter hypermethylation is an early event in carcinogenesis; Hence TSGs may serve as early tumor biomarkers. We determined the promoter methylation levels of p16^{INK4a}, RASSF1A, TIMP3 and PCQAP/MED15 TSGs in salivary DNA from oral cancer (OC), and oropharyngeal cancer (OPC) patients, using methylation specific PCR coupled with densitometry analysis. We assessed the association between DNA methylation of individual TSGs with OC and OPC risk factors. We evaluated the performance and the clinical validity of this quadruple methylation marker panel in discriminating OC and OPC patients from healthy controls using CombiROC web tool. Our study reported that RASSF1A, TIMP3 and PCQAP/MED15 TSGs were significantly hypermethylated in OC and OPC cases compared to healthy controls. We found that DNA methylation levels of TSGs were significantly augmented by smoking, alcohol use and betel quid chewing by indicating that the fact that frequent exposure to risk factors may drive oral and oropharyngeal carcinogenesis through TSG promoter hypermethylation. Also, this quadruple-methylation marker panel of p16^{INK4a}, RASSF1A, TIMP3 and PCQAP/MED15 TSGs demonstrated excellent diagnostic accuracy in the early detection of OC at 91.7% sensitivity and 92.3% specificity, and OPC at 99.8% sensitivity and 92.1% specificity, from healthy controls.

Keywords: Oral cancer, Oropharyngeal cancer; Tumor-suppressor genes; Promoter hypermethylation.

1. Introduction

Oral and oropharyngeal squamous cell carcinomas (OSCC and OPSCC) are the most common types of head and neck squamous cell carcinoma (HNSCC) ranked as the 6th most common cancer type and the 8th most common cause of cancer death, worldwide [1, 2]. Annually cancers in lip and oral cavity account for more than 130,900 new cases and 74,500 deaths for males in developing countries [2]. Oral cancer (OC) and oropharyngeal cancer (OPC) record a high age standardized incidence rate (ASIR) in Sri Lanka [3]. Despite advances in treatment, no significant improvement is witnessed in the 5-year survival rate of OC patients in Sri Lanka over the past several decades, where survival remains at 50 to 55% [4, 5]. Tobacco smoking, alcohol consumption and betel quid chewing are considered as established risk factors for OC and OPC, while there is a dramatic increase in the incidence of OPC attributable to human papillomavirus (HPV) infections [6, 7].

Cancer is a disease which evolves from successive accumulation of genetic and epigenetic alterations [8]. Hypermethylation of CpG islands in the promoter regions of tumor suppressor genes (TSGs), cause loss of expression leading to cancer initiation[9].Research has revealed that many TSGs are epigenetically silenced by promoter hypermethylation in the OC cell genome [10-13].

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More importantly, increasing evidence is mounting that age, race, tobacco and alcohol exposure, and betel quid carcinogens are capable of inducing promoter hypermethylation in TSGs which may subsequently induce oral malignancies [14-17].

Successful screening and surveillance approaches consider the collection of genomic material using minimally invasive approaches. Aberrant DNA methylation can be detected in DNA from serum, sputum, bronchial-lavage fluid, urine, ductal fluids from patients with many different types of cancers [10, 18-21]. Saliva is a complex and important body fluid which has already been used for screening cancers in the upper aerodigestive tract as well as OC and OPC[10, 22-26].Methylation array analysis of DNA allows to interrogate cancer-related genes that are specific and sensitive for the early detection of cancer [27]. Early screening of oral lesions using salivary biomarkers is very promising because saliva is in direct contact with the oral mucosa and cancerous lesions with a low background of inhibitory substances [28]. Methylation specific polymerase chain reaction (MS-PCR) method provides a highly sensitive, economical and time efficient method to detect relatively low concentrations of methylated sequences in salivary rinses [29-32].

Early detection efforts of using molecular markers have the potential to decrease the disease burden and play a significant role in successful clinical treatment of any cancer [28]. In the current study, we aimed to determine whether the promoter methylation of *p16^{INK4a}*, *RASSF1A*, *TIMP3* and *PCQAP* TSGs in DNA derived from saliva can serve as a diagnostic maker panel in the early detection of OC and OPC. This study focused on two objectives; Firstly, to determine the promoter methylation levels of TSGs by using MS-PCR method combined with densitometry analysis to assess their association with established and emerging risk factors of OC and OPC. Secondly, to evaluate the clinical performance of this methylation marker panel in discriminating OC and OPC subjects from healthy controls using CombiROC analysis and validation tests.

2. Materials and Methods

Ethical approval

This study received consent of the Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo, Sri Lanka (EC-16-125) for the collection of biological samples, and socio-economic and demographic details of OC and OPC patients, and of healthy controls.

Study subjects and data collection

Newly enrolled, treatment naive cancer patients with a primary cancer in the oral cavity(N=54) and oropharynx (N=34) or patients with loco-regional metastasis with oral/oropharyngeal origin who gave voluntary consent were enrolled in this study as case/test subjects, from the National Cancer Institute, Sri Lanka. An interviewer administered questionnaire was used to collect demographic data and risk factors associated with OC and OPC. Medical information of patients was retrieved from their pathology report, with pathological staging of the tumor and histopathological classification of the tumor grade. Age and gender matched healthy individuals (N=60) with no personal history of cancer were recruited as normal healthy control subjects.

Sample collection

Saliva samples from case and control subjects were collected at baseline. Patients who volunteered to participate in the study were requested to sit in a comfortable upright position and to rinse their mouth with saline water to remove food debris. Next, they were asked to tilt their head down for 5 minutes to pool saliva in the mouth. From each patient, a volume of 2 ml of saliva was collected into sterile containers. All sample containers were then transported to the laboratory on dry ice. Samples were centrifuged at 1200 × g for 10 min at 4 °C to isolate cellular pellet collection [11, 33, 34]. Samples were collected for a period of 12 months. DNA extraction was performed within 7 days from the date of sample collection.

DNA extraction and human DNA confirmation

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The DNeasy Blood and Tissue kit (QIAGEN, Germany; Cat no: 69504) was used according to the manufacturer's instructions to extract DNA from cell pellets of saliva samples. PC03 and KM38 primers were used on extracted DNA samples to amplify a 167-base pair (bp) region of the human β-globin gene to confirm presence of human DNA. PCR positive samples were used for further analyses [35].

HPV-L1DNA detection

GP5+/GP6+ primer sequences were used to amplify a 150 bp region of the HPV L1 gene[36](Table 3). The reaction mixture was prepared with the Promega GoTaq Flexi DNA Polymerase PCR kit. Each PCR reaction contained 2.5 mM of $MgCl_2$, 0.2 mM of dNTP, and 0.5 µM of each primer. PCR conditions were setup as follows; Initial denaturation at 94 °C for 3 minutes, 40 cycles of denaturation at 94 °C for 45 seconds, primer annealing at 42 °C for 45 seconds, primer extension at 72 °C for 45 seconds and final extension at 72 °C for 5 minutes. All reactions included HPV positive and negative controls. The PCR products were visualized on 2% agarose gels with ethidium bromide (EtBr) staining [37].

DNA bisulfite conversion

EpiTect Plus DNA Bisulfite Kit (QIAGEN, Germany; cat no: 59124) was used for the DNA bisulfite conversion of extracted DNA according to the manufacturer's instructions. Each bisulphite reaction mix was prepared at room temperature by using 20 µL (1µg/µL) of thawed DNA. Bisulfite-converted DNA (50 ng/µL) was eluted from the column in elution buffer and immediately used for the MS-PCR or stored at −20°C. DNA purity and the concentrations were determined using NanoDrop™ -1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

Target gene selection

A total of four genes with tumor suppressor activities were selected for the examination of methylation abnormalities in promoter regions. These TSGs were described as targets for epigenetic silencing in diverse human cancers. Among these genes, *p16^{INK4a}* is involved in cell cycle control, *RASSF1A* in apoptosis, *TIMP3* in cell invasion and *PCQAP/MED15* in transcription regulation [10, 12, 13, 26].These TSGs have been already evaluated in other studies and our plan was to validate them in a different population [22, 26, 38]. Thus, we anticipate that the evaluation of epigenetic alterations, such as promoter hypermethylation, would be a useful diagnostic tool for OC and OPC.

Methylation analysis of tumor suppressor genes using MS-PCR assay

The methylation status of the selected TSGs in bisulfite-converted DNA was detected by MS-PCR assay [29]. Specificities of the MS-PCR primer pairs used in this study (Table 1) were validated by previous studies [10, 11, 26, 31, 39]. In our study, both methylation and unmethylation primer pairs for each gene was tested using bisulfite unconverted gDNA samples to determine their specificity, and was found not to amplify. Unmethylation PCRs were used as a normaliser for methylation PCRs. Any sample which showed no unmethylation band was either discarded from the analysis or the PCR was repeated. For each set of MS-PCR, a tumor sample with known hypermethylation was used as a positive control, while DNase/RNase-free distilled water with no DNA template served as a control for contamination.

In this study, we used a previously reported nested MS-PCR method to detect methylation at specific sites located at the promoter regions of the genes *p16^{INK4a}* and *RASSF1A* (31). First methylation-independent primers of *p16^{INK4a}*, and *RASSF1A*TSG*s* were used at 0.5 µM in a standard PCR (25 µl of reaction volume), using Go Taq DNA polymerase (Promega, USA) with 3 µl (~150 ng) of converted DNA template (diluted either three or five folds depending on the DNA concentration) with supplied 1X Go Taq Flexi Buffer, 2.5 mM $MgCl_2$ and 0.6 mM dNTPs (Promega-USA), using the following cycling conditions: Initial denaturation at 94 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 60°C for 30 seconds, primer extension at 72 °C for 30

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seconds and final extension at 72 °C for 5 minutes. To detect unmethylated or methylated alleles for *p16^{INK4a}* and *RASSF1A* genes, stage 2 unmethylated and methylated touchdown gradient PCRs were carried using 1 µL from the stage-1 product as the DNA template. Both touchdown gradient PCRs consisted of an Initial denaturation at 94 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, primer annealing temperature decreasing from 64°C to 58°C in 2°/5-cycle steps, primer extension at 72 °C for 30 seconds and a final extension at 72 °C for 5 minutes.

For *TIMP3*, specific methylated and unmethylated primer sets (0.8 µM) were used in two separate PCR reactions in 25 µL of final reaction volume. For both methylated and unmethylated reactions, a ratio of 25:1 of the total converted DNA template was used. The PCR amplification consisted of initial denaturing stage at 95 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 54 ° C and 30 s at 72 °C before summing up with elongation stage at 72 °C for 5 min. Comparable to *TIMP3, PCQAP* also consisted of two separate setup conditions for the methylated and unmethylated reactions under similar cycling conditions. In contrast, respective methylated and unmethylated primer sets specific for the CpG islands in the main promoter region of *PCQAP* gene/PCQAP 5′ were used at 1 µM concentration. A ratio of 25:1 of total converted DNA template was used for the methylated and unmethylated reactions. The PCR amplification consisted of initial denaturing stage at 95 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 62.5 °C and 1 min at 72 °C before summing up with elongation stage at 72 °C for 5 min.

Table 1. Primer sequences used in the study.

Gene	Primer sense (5′-3′)	Primer Antisense (3′-5′)	PCR Product size(Bp)
β-globin	PC03: ACACAACTGTGTTCACTAGC	KM38: TGGTCTCCTTAAACCTGTCTTG	167
HPV L1	GP5+: TTTGTTACTGTGGTAGATACTAC	GP6+: GAAAAATAAACTGTAAATCATATT	150
p16^{INK4a}(MI)	GAGGAAGAAAGAGGAGGGGTTG	ACAAACCCTCTACCCACCTAAATC	274
p16^{INK4a}(M)	GAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCG	143
p16^{INK4a}(U)	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	145
RASSF1A(MI)	GGAGGGAAGGAAGGGTAAGG	CAACTCAATAAACTCAAACTCCC	260
RASSF1A(M)	GGGGGTTTTGCGAGAGCGC	CCCGATTAAACCCGTACTTCG	203
RASSF1A(U)	GGTTTTGTGAGAGTGTGTTTAG	ACACTAACAAACACAAACCAAAC	172
TIMP3(M)	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAATTACCGTACGCG	116
TIMP3(U)	TGTGTTGGAGGTTAAGGTTGTTTT	ACTCTCCAAAATTACCATACACACC	122
PCQAP 5′(M)	GTTTTGTGATTGAGGYGGCGGC	AAAAATCCCACAATCCAACCC	167
PCQAP 5′(U)	GTTTTGTGATTGAGGYGGTGGT	AAAAATCCCACAATCCAACCC	167

MI: Methylation-independent primers; M: Methylated primers; U: Unmethylated primers; Bp: Base pairs

Agarose gel electrophoresis and determination of gene methylation Levels

For the MS-PCR analysis, 5 µL of each PCR amplicon was visualized on 4% agarose gels. Samples that gave a strong consistent band using the unmethylated allele-specific reference PCR were exclusively used for promoter methylation analysis of these TSGs. Fusion SL gel documentation system (Vilber Lourmat, Marne la Vallee, France) was used to scan gels and visualized using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Band intensities for each sample were determined using ImageJ software. The ratio between methylated and unmethylated band intensities was calculated using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA). Quantification of band intensities was conducted by two independent researchers to minimize observational errors.

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Statistical analyses

Statistical analyses were performed using SPSS version 20 for Windows (IBM-SPSS Inc, USA) software package and Graph-Pad Prism (GraphPad Software, Inc, San Diego, California, USA). Comparisons of normally distributed variables of independent samples were performed using the t-test. Since methylation levels were not normally distributed non-parametric Mann-Whitney U test was used to compare methylation levels of normal healthy controls with those of OC and OPC patients. Chi-square test and binary logistic regression were used to assess the relationships (combined effect and independent effect, respectively) between the presence of OC/OPC and predictor variables/risk factors. Calculated odds ratio (OR) measured the risk of developing OC and OPC. Wald statistic was used to determine parameter significance (*p*-value) in logistic regression analyses. All statistical tests were two-tailed, and $P < 0.05$ was regarded as statistically significant.

CombiROC web tool (http://CombiROC.eu) was used to determine the clinical performance of these four methylation markers in discriminating OC and OPC subjects from healthy controls [40]. Accordingly, optimal marker combinations and their clinical performances were determined through the combinatorial analysis of receiver operating characteristic (ROC) curves provided by the CombiROC tool. Additionally, a 10-fold cross validation (CV)step was conducted in order to attain a reliable estimation of the clinical performance of the best marker combination [40]. This step is crucial as it could avoid the risk of over fitting and show how well the panel translates into clinical diagnosis. As well, analyzing the statistical significance of the area under the curve (AUC) value is imperative as CV procedure could generate over-optimistic results. Therefore, permutation tests were performed in order to assess the statistical significance of AUC values generated by each ROC curve analysis.

3. Results

Population characteristics of the study cohorts

Both patient cohorts and controls were comparable in age and gender ($p > 0.05$). Mean age of OC and OPC subjects and of normal healthy subjects was 62±12.5, 62±10.1and 60±7.1 years, respectively. A male preponderance was evident in both OC (90.7%) and OPC (94.1%) cohorts (Table 2). Majority of the OC (79.7%) and OPC (91.25) subjects were smokers, where most of them consumed more than 5 cigarettes daily (Table 2). Further, higher levels of alcohol consumption (OC=74.1%; OPC=94.1%) and betel quid chewing (OC=77.8%; OPC=76.4%) was recorded in patient cohorts, where most of them were habitual consumers for more than 25 years of their lifespan (Table 2).

Regarding the primary tumor site of cancer subjects, most of the OC cases were cancers on the front 2/3 of the tongue (29.5%), while cancers on the back wall of the throat were common in OPC cases (44.1%). Most of the cancer subjects were reported with grade 1 (well differentiated) tumors (OC=44.4%; OPC=50.0%). Although cancer TNM stage information for all patients was not available, a majority of the recruited patients (OC=42.6%; OPC=38.2%) were in advanced stages (III, IV) of cancer development (Table 2).

Table 2. Socio-demographic and tumor characteristics of the study cohorts.

	OC N=54	OPC N=34	Healthy controls N=60
DEMOGRAPHIC CHARACTERISTICS			
MEAN AGE	62	62	60
<50	7 (13.0)	3 (8.8)	1 (1.6)
50-59	11 (20.3)	13 (38.2)	29 (48.3)
>60	36 (66.7)	18 (53.0)	30 (50)
GENDER			
Male	49 (90.7)	32 (94.1)	55 (91.6)

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Female	5 (9.3)	2 (5.9)	5 (8.4)
SMOKING			
Cigarette/day smoked			
Non-smokers	11 (20.3)	3 (8.8)	39 (65.0)
1 to 5	14 (26.0)	11 (32.4)	8 (13.3)
>5	29 (53.7)	20 (58.8)	13 (21.6)
ALCOHOL USE			
No. Of years drank			
Non-drinkers	14 (25.9)	3 (8.8)	31 (51.6)
1-25	14 (25.9)	12 (35.3)	10 (16.6)
>25	26 (48.2)	19 (55.9)	19 (31.6)
BETEL QUID CHEWING			
No. Of years in use			
Non-consumers	12 (22.2)	8 (23.5)	41 (68.3)
1-25	16 (29.6)	9 (26.5)	7 (11.6)
>25	26 (48.2)	17 (50.0)	12 (20)
HPV INFECTION			
HPV positive	5 (9.2)	3 (8.8)	1 (1.6)
HPV negative	49(90.8)	31(91.2)	59 (98.4)
TUMOR CHARACTERISTICS			
ANATOMIC SITE			
Lips	2 (3.7)		
Tongue (front 2/3rd)	26 (48.1)		
Hard palate	1 (1.9)		
Buccal mucosa	21 (38.9)		
Mouth floor	3 (5.5)		
Retromolar	1 (1.9)		
Tongue (back 1/3rd)		0 (0)	
Soft palate		7 (20.6)	
Tonsillar pillar		12 (35.3)	
Back wall of the throat		15 (44.1)	
TUMOR GRADE			
Well differentiated (1)	24 (44.4)	17 (50.0)	
Moderately differentiated (2)	7 (13.0)	5 (14.8)	
Poorly differentiated (3)	0 (0)	2 (5.9)	
Undifferentiated (4)	19 (35.2)	6 (17.6)	
Unknown	4 (7.4)	4 (11.7)	
TUMOR STAGE			
Early stage (i, ii)	8 (14.8)	2 (5.9)	
Advanced stage (iii, iv)	23 (42.6)	13 (38.2)	
Unknown	23 (42.6)	19(55.9)	

N:Total number of subjects in each cohort.

HPV L1 analysis

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Detecting the presence of the L1 gene in salivary DNA samples provides an accurate output on high-risk HPV strains [36]. In this study, HPV-L1 gene was detected in five OC subjects, three OPC subjects and a single subject from the normal healthy control cohort (Figure 1).

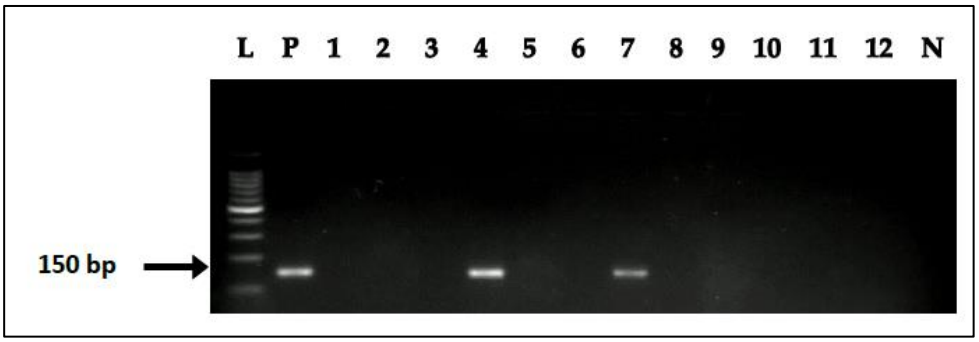

Figure 1. Agarose gel electrophoresis of HPV L1 analysis. L: 100 bp DNA ladder with 500 bp marker; P: Positive control; N: Negative PCR controls.

Combined and independent effect assessment of etiologic agents of OC and OPC

Combined and independent effects of the risk factors of OC and OPC are summarized in Table 3. Examining the effects of established risk factors of OC and OPC revealed that smoking, alcohol use and betel quid chewing had significant combined effects for both cancer types. Smoking demonstrated the strongest combined effect for OC (OR=7.3 [95% CI=2.8-18.6]) as well as for OPC (OR=20.8 [95% CI=2.4-178.2]).In addition, the strongest independent effect was also found to be driven by smoking, for OC and OPC. Though, the combined effect of HPV infection was not significant for both cancer types, it exerted a high independent effect for OPC (OR=19.6 [95%CI=1.0-146.4]) compared to OC development.

Table 3. Combined and independent effects of established and emerging risk factors of OC and OPC.

Cancer type	Predictor variable/ Risk factor	Crude OR (95% CI)	*p*-value[a]	Adjusted OR[R] (95% CI)	*p*-value[b]
OC	Smoking	7.3 (2.8-18.6)	<0.0001	7.8 (2.1-28.4)	<0.05
	Alcohol use	3.1 (1.2-7.3)	<0.05	0.6 (0.1-2.0)	0.385
	Betel quid chewing	7.1 (3.0-19.2)	<0.0001	5.7 (2.2-14.3)	<0.05
	HPV infection	6.0 (0.6-140.9)	0.07	6.7 (0.6-123.8)	<0.05
OPC	Smoking	19.2 (4.7-89.8)	<0.0001	20.8 (2.4-178.2)	<0.05
	Alcohol use	11.0 (2.7-51.0)	<0.0001	0.8 (0.1-6.7)	0.854
	Betel quid chewing	7.0 (2.4-20.7)	<0.0001	3.9 (1.2-12.4)	<0.05
	HPV infection	5.7 (0.4-148.9)	0.133	19.6 (1.0-146.4)	<0.05

Non-consuming/non-infected group was selected as the reference group for each predictor variable/risk factor. Combined effects of risk factors were assessed using Crude OR values derived by Chi-square test[a] while independent effects were assessed using adjusted OR values derived by binary logistic regression analysis[b]. Adjusted OR[R]-adjusted for smoking, alcohol use, betel quid chewing, and HPV infection.

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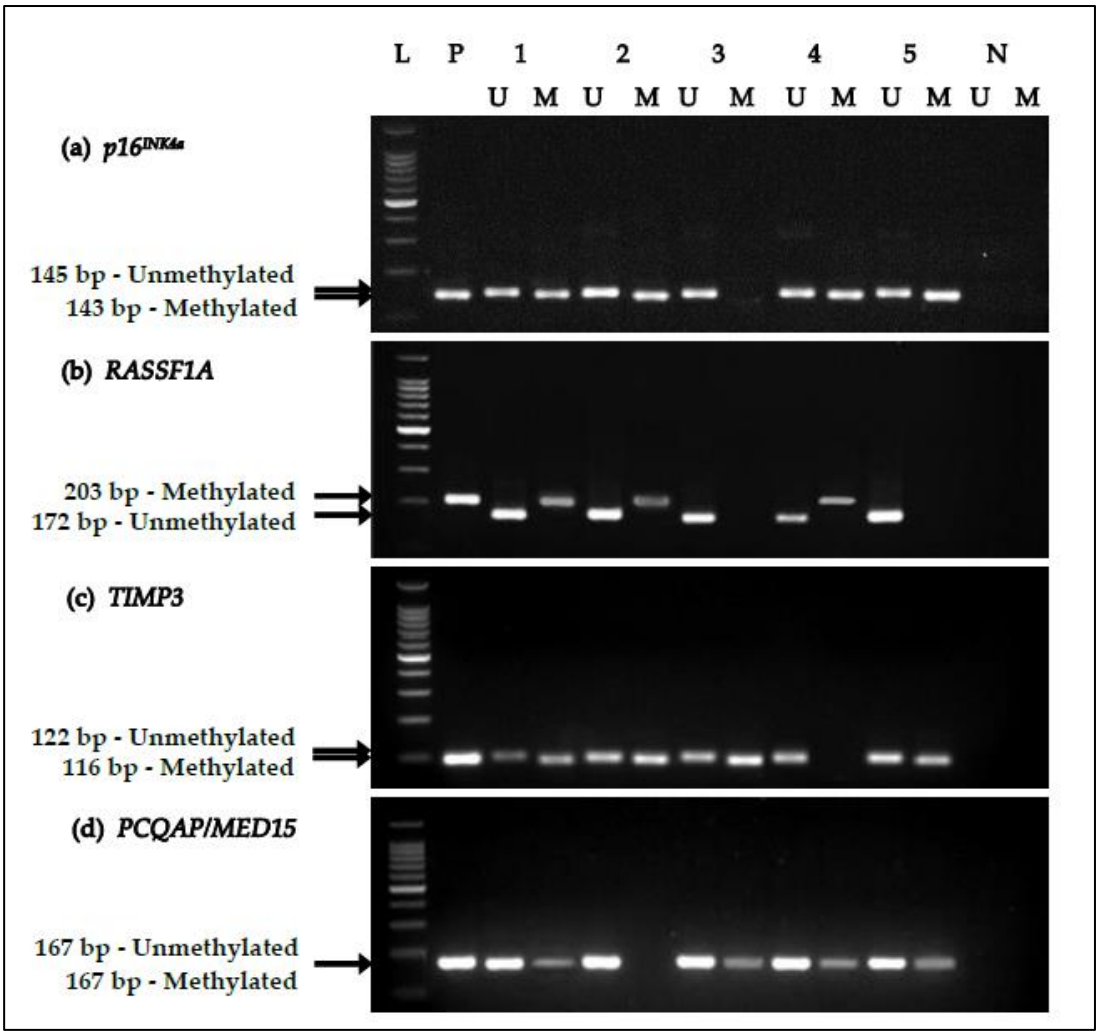

Figure 2. Agarose gel electrophoresis of MS-PCR analysis of TSGs. L: 100 bp DNA ladder with 500 bp marker; P: Positive control; U: Unmethylated amplicon; M: Methylated amplicon; N: Negative PCR controls.

Comparative DNA methylation analysis of individual TSGs

Agarose gels analyzed for the detection of promoter hypermethylation events for all four TSGs are presented in Figure 2. Salivary DNA promoter methylation levels of TSGs were comparatively analyzed among the three study cohorts (Figure 3). Accordingly, only *RASSF1A, TIMP3*and*PCQAP* TSGs showed significant promoter methylation (hypermethylation) in saliva collected from OC subjects ($p<0.0001$; $p<0.05$; $p<0.0001$, respectively) and from OPC subjects ($p<0.0001$; $p<0.001$; $p<0.0001$, respectively), compared to normal healthy controls. Conversely, there was no significant difference in promoter methylation levels between OC subjects and OPC subjects, for any of the four TSGs examined ($p>0.05$).

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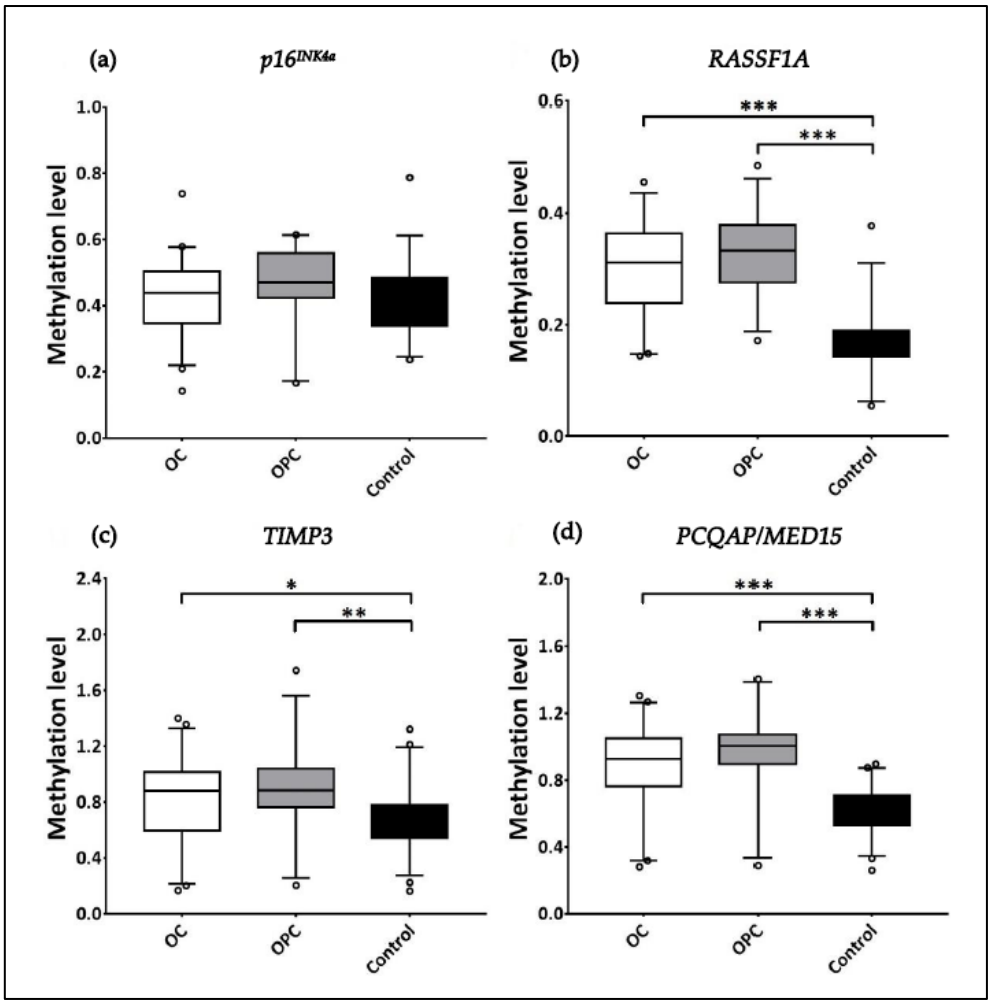

Figure 3. Promoter hypermethylation profiles of individual TSGs among OC, OPC and normal healthy control cohorts. Whisker-box plots were drawn (on 5%-95% percentile) for the methylation signatures of **a***p16*^{INK4a},**b***RASSF1A*,**c***TIMP3*, and **d***PCQAP* in the saliva collected from OC patients (N = 54), OPC patients (N = 34) and normal healthy controls (N = 60), analyzed using Mann-Whitney U-test. Significant differences in promoter methylation between each cohort is marked with *$p< 0.05$, ** $p< 0.01$, *** $p< 0.0001$.

Association between promoter methylation of TSGs and clinicopathological parameters of OC and OPC

Clinicopathological variables of OC and OPC patients and their exposure to risk factors were compared with the promoter methylation levels of individual TSGs (Table 4). Accordingly, promoter methylation of TSGs showed no significant association with respect to age and gender($p>0.05$). However, a significant promoter hypermethylation was seen in*p16*^{INK4a}and *RASSF1A*TSGs in saliva collected from smokers and alcohol consumers, compared to non-consumers ($p< 0.05$; Table4). Also, betel quid chewers demonstrated significant promoter hypermethylation of all four TSGs (*p16*^{INK4a}: $p< 0.05$; *RASSF1A*: $p< 0.05$; *TIMP3*:$p< 0.005$ and *PCQAP*: $p< 0.05$)compared to non-consumers (Table 4). On the other hand, we found that *p16*^{INK4a},*TIMP3*and *PCQAP*TSGs were significantly hypomethylated ($p< 0.005$, $p< 0.005$ and $p< 0.005$, respectively) in saliva of HPV-positive subjects compared to HPV-negative subjects (Table4).

A significant *p16*^{INK4a} and *RASSF1A*promoter hypermethylation ($p< 0.005$, $p< 0.05$, respectively) was observed in advanced OC stages (stage 3 and 4), compared with less advanced OC stages (stage 1 and 2) (Table 4). Similarly, a substantial promoter hypermethylation was noted between *p16*^{INK4a}and *RASSF1A*TSGs($p< 0.05$, $p< 0.05$, respectively)and high grade (3,4)OC tumors compared to low grade (low grade 1 and 2) OC tumors, whereas for OPC, *p16*^{INK4a}, *RASSF1A*and *TIMP3*TSGs

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were significantly hypermethylated ($p< 0.05$, $p< 0.05$ and $p< 0.05$, respectively) in high grade (grade 3 and 4) OPC tumors (Table 4).

Performanceof the methylation marker panelin discriminating OC and OPC from healthy controls

By using CombiROC curve analysis, the best performing marker combination was identified by evaluating its clinical performance in discriminating OC, OPC and healthy control cohorts (Table 5).Remarkably, the marker combination of all four markers (quadruple-methylation marker panel) performed well in discriminating OC patients from healthy controls with an AUC of 0.92, accuracy (ACC) of 0.92, sensitivity of 91.7 % and 92.3 % of specificity (Table 5; Figure 4.a). Furthermore, this marker panel performed exceptionally well with an AUC of 0.97, ACC of 0.96, sensitivity of 99.8 % and 92.1 % of specificity when discriminating OPC patients from healthy controls (Table 5; Figure 4.b). Next, the performance of the quadruple-methylation marker panel was investigated using 10-fold CV and permutation test, aiming for the effective clinical validation of the marker-panel. The results of CV procedure suggested that the quadruple-methylation marker panel is a perfect fit for the diagnosis of OC and OPC patients from healthy controls, as the overall accuracy, sensitivity and specificity were least affected by the imposed likelihood (Table 5, Figure4.a and b).In addition, we obtained the "real AUC value" outside the permutated AUC distribution which signified the high validity of this marker panel in discriminating OC and OPC subjects from normal healthy controls (Figure4.c and d).

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Table4. Association of promoter hypermethylation of TSGs with demographic factors, risk factors and clinicopathological characteristics.

Variable		Category	N	*p16*^{INK4a}		*RASSF1A*		*TIMP3*		*PCQAP/MED15*	
				Meth	*p*-value[b]	Meth	*p*-value[b]	Meth	*p*-value[b]	Meth	*p*-value[b]
Age		≥62[a]	28	13	0.455	12	0.244	18	0.414	21	0.550
		<62[a]	60	58		54		52		52	
Gender		Male	80	63	0.053	61	0.133	69	0.152	66	0.164
		Female	8	5		4		5		5	
Smoking		Consumers	74	57	<0.05	58	<0.05	61	0.199	62	0.240
		Non-consumers	14	11		7		13		9	
Alcohol use		Consumers	71	56	<0.05	56	<0.05	61	0.665	60	0.060
		Non-consumers	17	12		9		13		11	
Betel quid Chewing		Consumers	68	51	<0.05	52	<0.05	61	<0.005	57	<0.05
		Non-consumers	20	17		13		13		14	
HPV-L1		HPV positive	8	6	<0.005	6	0.061	8	<0.005	7	<0.005
		HPV negative	80	62		54		66		64	
Tumor grade	OC	Grade 3, 4	19	14	<0.05	13	<0.05	15	0.311	16	0.289
		Grade 1, 2	31	25		24		28		27	
	OPC	Grade 3, 4	8	6	<0.05	5	<0.05	4	<0.05	2	0.146
		Grade 1, 2	22	17		17		21		21	
Tumor stage	OC	Stage (III, IV)	23	18	<0.005	17	<0.05	17	0.233	19	0.712
		Stage (I, II)	8	7		6		7		6	
	OPC	Stage (III, IV)	13	8	0.525	10	0.930	10	0.076	11	0.964
		Stage (I, II)	2	2		2		2		1	

[a]Mean age; [b]Mann-Whitney U-test; N:Total number of patients in each category; Meth: Number of patients with methylated gene promoter regions.

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Table 5. CombiROC curve analyses and validation tests of the quadruple-methylation marker panel.

	AUC	ACC	Error rate (1-ACC)	SE %	SP %	PPV %	NPV %
(a) Performance in discriminating OC from healthy controls							
Whole-cohort	0.92	0.92	0.08	91.7	92.3	95.7	85.7
10-fold CV	0.85	0.87	0.14	83.3	92.3		
Permutated models	0.71	0.71	0.29	69.7	76.0		
(b) Performance in discriminating OPC from healthy controls							
Whole-cohort	0.97	0.96	0.04	99.8	92.1	93.7	98.6
10-fold CV	0.82	0.89	0.11	86.7	92.3		
Permutated models	0.60	0.66	0.33	65.1	68.7		
(c) Performance in discriminating OC from OPC							
Whole-cohort	0.65	0.72	0.28	91.7	40.0	61.2	53.3
10-fold CV	0.60	0.56	0.44	33.3	93.3		
Permutated models	0.70	0.70	0.30	68.8	73.9		

Clinical performance of the quadruple-methylation marker panel in discriminating (a) OC from healthy controls, (b) OPC from healthy controls and (c) OC from OPC was determined with respect to all key parameters. AUC: Area under the curve; ACC: Accuracy; SE: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

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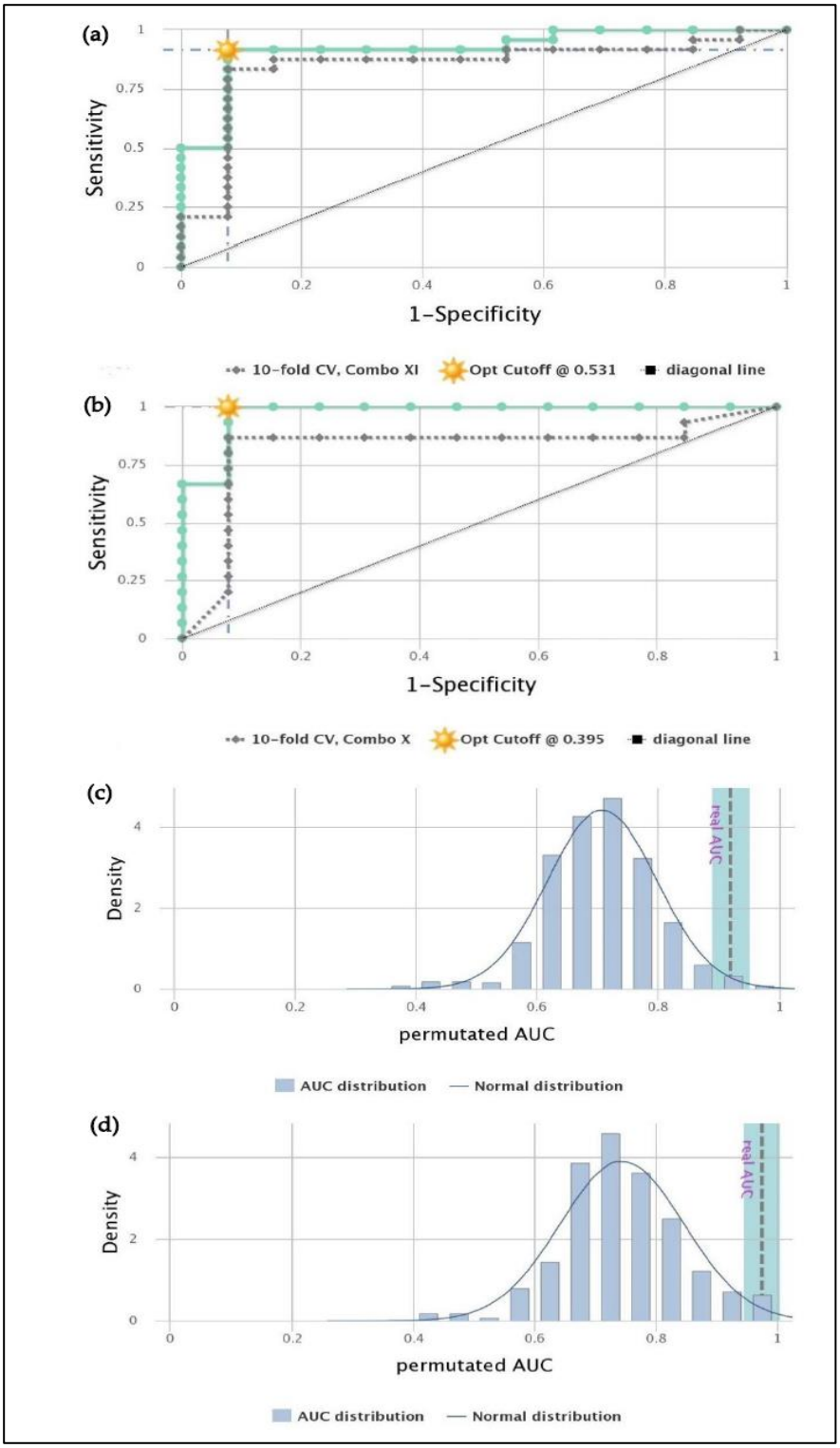

Figure 4. Clinical performance of the quadruple-methylation marker panel. CombiROC curve analysis of the marker panel in discriminating (a) OC patients and (b) OPC patients from normal healthy controls. Green bar-ROC curve of the marker panel for the whole-cohort. Grey dotted bar-10-fold CV test of the marker panel. Diagonal line-Reference line with zero discriminating power (0.5 sensitivity and specificity). Density distribution of permutated AUC values compared to the normal distribution, illustrating the significance of real AUC value generated for discriminating (c) OC subjects and (d) OPC subjects from healthy controls.

4. Discussion

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The present study evaluated a population of OC and OPC patients and matched healthy controls to determine the ability of MS-PCR approach combined with a densitometry analysis in measuring promoter methylation levels of *p16^{INK4a}*, *RASSF1A*, *TIMP3* and *PCQAP/MED15* TSGs in DNA derived from saliva that could distinguish these groups. Conferring to the high diagnostic accuracy detected, the strong potential of this quadruple-methylation marker panel in discriminating OC and OPC patients from healthy controls was clearly evident.

TSGs analyzed in our study were found to be associated with common oncogenic transformation pathways and cellular functions that are frequently dysregulated in many cancers including OC and OPC. *p16^{INK4a}*encodes for a cyclin-dependent kinase 4 (CDK 4) inhibitor which regulates the retinoblastoma (Rb) pathway and arrest the cell cycle[41]. Hence, its Inactivation may lead to disrupt cell cycle control, playing a key role in tumorigenesis in various cancers including OC [42-46]. However, no significant *p16^{INK4a}* promoter methylation was observed in both OC and OPC salivary rinses, although this gene was reported to be highly methylated in previous HNSCC studies [15, 26, 44, 47, 48].This contradiction of detecting different methylation frequencies can be explained by the differences in sampling methods, sensitivity of the detection method, location and length of investigated CpG repeats, and cohort composition.

RASSF1A, acts as a down-stream negative effector of Ras protein, which induces growth arrest of cells (apoptosis) [49]. It is well documented that *RASSF1A* TSG is frequently inactivated in primary oral tumors by *de novo* methylation of promoter CpG islands, subsequently triggering OC initiation [26, 39, 50]. Significant *RASSF1A* promoter hypermethylation detected by our study in both OC and OPC salivary rinses, suggests its strong association with OC and OPC risk.

Tissue inhibitor of metalloproteinases (TIMPs) are known as inhibitors of cellular invasion, metastasis and angiogenesis[51]. Loss of TIMPs expression by *TIMP3* promoter hypermethylation has been speculated specifically with HNSCC neoplastic evolution [38, 52].Our study reiterates previous reports that evidence the positive correlation between *TIPM3*promoter hypermethylation in oral malignancies [12, 38, 53, 54].

Similarly, we observed a substantial *PCQAP* hypermethylation in both OC and OPC, which is in line with previous studies on HNSCC [11, 39] . Although, *PCQAP* methylation in HNSCC was identified and validated recently, it has been frequently implicated in prostate and endometrial cancer etiologies [11, 42]. PCQAP is identified as a transcriptional co-activator mediator, responsible for the transcriptional regulation of ligand-activated proteins which plays a pivotal role in cellular regulation, proliferation and differentiation[11, 55].

Age and sex-specific differences in promoter hypermethylation of *p16^{INK4a}* and *RASSF1A* TSGs were previously reported [56]. Conversely, no direct association was observed between TSG promoter methylation with age and sex in both cancer types, in agreement with a few previous reports [39]. It is well known that *p16^{INK4a}* and *RASSF1A* promoter methylation status is strongly correlated with the cancer stage [57, 58]. By confirming these findings, our study established a significant *p16^{INK4a}* and *RASSF1A* promoter hypermethylation in advanced stage (3 and 4) of OC subjects, suggesting that these alterations may occur late in the carcinogenesis of the oral cavity. Also, we found a significant promoter hypermethylation in advanced tumor grades of OC for *p16^{INK4a}* and *RASSF1A*and of OPC for *p16^{INK4a}*, *RASSF1A and TIMP3*, suggesting the correlation between promoter hypermethylation of tumor related genes and poor prognosis of OC and OPC[26, 59, 60].

Our results strengthened the notion that smoking and alcohol consumption are strong predictors of *p16^{INK4a}* and *RASSF1A*promoter hypermethylation [61-63]. Carcinogens in tobacco smoke could drive genetic and epigenetic mutations in frequently exposed tissues [64]. Tobacco smoking also induces DNA methyltransferases (DNMTs) activity, thereby causing *de novo* methylation on disposed loci in a gene-specific basis [65, 66]. Significant promoter hypermethylation observed in OPC subjects, compared to OC subjects, might be attributable to the higher independent risk of smoking, seen in OPC subjects. In addition, it is well established that betel nut as well as betel quid, with or without tobacco, are carcinogenic to HNSCC [67, 68]. Betel quid chewing is critically associated with high-risk pre-cancerous oral lesions[15, 69]. Furthermore, recent studies report that silencing of *RASSF1A* and *p16^{INK4a}* gene expression by promoter hypermethylation may play a

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critical role in betel-associated oral carcinogenesis[15, 70]. In agreement with the above findings, we describe a strong association between betel quid chewing and promoter hypermethylation of all four TSGs studied.

HPV is recognized as an additional independent risk factor for the development of OSCC, particularly OPSCC, confirmed by our risk analysis [71-73]. In addition, we reported that, *p16^{INK4a}*, *TIMP3* and *PCQAP* promoter regions were hypomethylated in HPV-positive cases compared to HPV-negative cases. This result seems to be paradoxical since HPV infection appears to have a greater association with promoter hypermethylation of TSGs, due to the over expression and increased DNMTs activities induced by HPV oncoproteins (E7)[74-76]. This contradiction may be caused by differences in sampling methods, ethnic origin of the subjects and by the HPV genotype.

It follows that lifestyle factors contribute to tumorigenesis through revocable epigenetic dysregulations, hence holding a great promise in disease prevention, and treatment [77]. In addition, methylation profiles of TSGs in combination with clinicopathological characters would be useful in predicting the behavior of OC and OPC. The significance of the association between clinicopathological characteristics and promoter methylation of TSGs evaluated by the current study, led us to hypothesize that the degree of effect of risk factors may have affected the performance of the quadruple-methylation marker panel. For instance, the higher AUC and sensitivity in detecting OPC patients compared to OC patients may be attributed to the substantial effect of risk factors on the development of OPC compared to OC.

Early detection of cancer, at a stage where it is localized and treatable will subsidize substantially to reduce mortality, owing to the disease. For this reason, diagnostic tumor biomarkers has become an imperative field pursued in biomedical engineering[78]. It is now well established that individual biomarkers may not be sufficiently accurate in diagnosing tumors as heterogeneity of individual tumors are triggered by specific molecular alternations in different tumor related genes [79]. Thus, combined biomarker panels are developed to attain significant specificity and sensitivity values for effective translation into diagnostic settings [22, 52]. Our results suggest that *p16^{INK4a}* methylation alone should not be considered as a tumor marker in OC or OPC, since no significant methylation was found in normal and tumor samples. However, hyper-methylation of *p16* has been found to be a promising diagnostic and prognostic bio-marker for recurrence-free survival of OC and OPC [80-82]. Hence, CombiROC analysis has allowed us to develop a better performing combination of independent methylation markers, by lowering the risk of missing p16 marker. We defined the high performance of this quadruple-methylation marker panel which outperformed the previously published individual markers and other marker combinations substantiated by previous studies in different ethnic groups [26, 39, 52, 79, 83]. We have further strengthened our study by analyzing the performance, robustness and consistency of the marker panel, through cross-validation and permutation tests, as such features of this panel would be decidedly useful as a robust diagnostic and screening tool.

High performance of this marker panel prompts one to study the prognostic potential of it that can be used during surveillance after cancer treatment or with high-risk cohorts, such as smokers, alcohol users. Notably, evaluating the accuracy of genetic screening tests for oral HPV infections has become a promising area of research. However, no genetic screening method for oral HPV infections is still approved by the Food and Drug Administration **(**FDA), hence is rarely practiced by developing countries. A previous study reported the high accuracy of a methylation marker panel in discriminating HPV positive HNSCC cases from HPV negative counterparts [39]. Given the low percentage of HPV positive OC/OPC cases in our study, the current marker panel was not assessed for its performance in discriminating HPV positive cancer cases from their HPV negative counterparts. The low prevalence of HPV in our analysis (9.1 %) is probably explicated by the abundance of squamous cell carcinomas of the oral cavity (61.3 %) in our samples, in agreement with previous reports [84, 85].

Since its introduction, the MS-PCR method has progressively gained favor as a preferred tool for detecting the DNA methylation status of CpG islands. Nevertheless, some studies showed that it is prone to overestimate methylation prevalence leading to a high number of false positives (i.e. low specificity), due to incomplete bisulfite conversion [86, 87]. Also it cannot distinguish the

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methylation status between normal epithelium and tumor cells. This explains the different methylation levels observed in the current study compared to the literature. Previous study, validating CCNA1, DAPK, DCC and TIMP3 methylation markers for diagnosing OSCC, showed that quantitative methylation-specific PCR analyses (Q-MSP) may be a choice in improving the level of detection. However, recently pyrosequencing has emerged as a robust and versatile platform in global DNA methylation quantification, which may overcome the limitations of MSP based methods [88, 89]. Several studies have evaluated both the accuracy and precision of pyrosequencing for the analysis of DNA methylation in heterogeneous mixtures of tumor DNA samples [90, 91].

Our work clearly emphasizes the need of assessing other promising genes in saliva in order to improve the sensitivity and specificity of this current marker panel. Optimized marker panel will finally be evaluated for performance in prospective trials to depict its clinical efficacy. Validation of this panel is expected to involve screening of high-risk populations followed by clinical assessment using biopsy tests for individuals whose methylation profile expresses the highest risk for OC or OPC. Our findings highlight the potential of this marker panel comprising epigenetically silenced TSGs in saliva which can be used for the earliest possible identification of OC/OPC incidence, permitting careful monitoring to guide immediate intervention and further evaluation.

5. Conclusions

We report that frequent exposure to established risk factors such as smoking, alcohol use, and betel quid chewing may drive oral, and oropharyngeal carcinogenesis through promoter hypermethylation of TSGs. Importantly, we established the exceptional diagnostic accuracy of the methylation marker panel consisting of *p16^{INK4a}*, *RASSF1A*, *TIMP3* and *PCQAP/MED15* TSGs in the early diagnosis of OC and OPC.

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Conflicts of Interest: None

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