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Evaluation of DNA methylation in matched oral swab and tissue specimens from Chinese patients with oral squamous cell carcinoma

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Abstract. The DNA methylation statuses of the paired box 1 (*PAX1*) and zinc finger protein 582 (*ZNF582*) genes have shown promise in the detection of oral squamous cell carcinoma (OSCC). The aim of this study was to investigate the ability of *PAX1* and *ZNF582* methylation to distinguish OSCC and the adjacent normal tissue among cancer patients. This study included 67 patients with OSCC. The methylation levels of these two genes were analysed in tissue specimens (lesion site and adjacent normal site) and in oral swabs (lesion site and contralateral normal site). Levels of DNA methylation were higher at lesion sites than at the corresponding normal sites. According to receiver operating characteristics curve analysis, the area under the curve for *PAX1* and *ZNF582* methylation ranged from 0.73 to 0.82. No significant difference was observed between tissue specimens and oral swabs (*PAX1*, $P = 0.41$; *ZNF582*, $P = 0.28$). For the oral swab, *PAX1* methylation was more pronounced in bone invasion ($Z = 1.988$, $P = 0.047$), and *ZNF582* methylation was more pronounced in early-stage ($Z = 2.354$, $P = 0.02$) and well-differentiated tumours ($Z = 3.731$, $P = 0.0002$). Hypermethylated *PAX1* and *ZNF582* are effective biomarkers to distinguish lesion sites and corresponding normal sites in tissue specimens and oral swabs from OSCC patients.

Key words: oral squamous cell carcinoma; DNA methylation; oral swab; paired box 1; zinc finger protein 582.

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Cancer of the head and neck is the sixth most common cancer in the world. Approximately 40% of head and neck malignancies are oral cancer[1]. Oral squamous cell carcinoma (OSCC) is the most prevalent oral cancer[2]. According to the latest data from the World Health Organization (WHO), a high incidence rate of OSCC is found in Asian countries, including Sri

Lanka, Pakistan, India, Bangladesh, and Japan[3]. Since the development of medical treatment, the 5-year relative survival and period survival rates for OSCC have been about 50% over the past decades[4]. Although oral cancer is not a major cancer in China, its incidence persists, and most patients are diagnosed at an advanced stage[5]. Also, the incidence of oral cancer in China varies widely among regions. Regions with a high incidence are generally located in central and south China, where areca nut (also known as betel nut) chewing is prevalent. However, the prevalence in densely populated cities in eastern China remains unclear[6,7]. In general, the rising trend in incidence and the severity are accompanied by a poor prognosis for OSCC in China[3,5].

Histopathology is the current gold standard diagnostic for OSCC worldwide[8]. However, some limitations remain. First, the histopathology result may be influenced by several factors including the sampling site, biopsy method, and interpretation of the result by the individual reading it. In addition, surgery may be contraindicated in certain patients with multiple lesions or recurrence, as well as in those pre- or post-radiotherapy or chemotherapy. Thus, an alternative diagnostic tool is still needed to detect and monitor the prognosis of OSCC.

DNA methylation is a widely studied epigenetic modification which associates with carcinogenesis by altering gene expression without affecting the DNA sequences[9–11]. DNA methylation often occurs in the cytosine–guanine dinucleotide promoter region of genes through the addition of a methyl group to the 5′ end of cytosine (C) in a CpG dinucleotide by DNA methyltransferases. A high density of methyl-cytosine accumulation in the CpG region leads to chromatin remodelling, and subsequent gene silencing results in a loss of function of tumour suppressor genes, triggering carcinogenesis[11–15]. Paired box 1 is a member of the paired box family of transcription factors, which play a vital role in the development of the skeletal system, thymus, and parathyroid; the paired box 1 gene (*PAX1*) is located on chromosome 20p11.2[16,17]. The zinc finger protein 582 gene (*ZNF582*), located on chromosome 19q13.43, codes for a zinc finger protein containing one KRAB-AB domain and nine zinc-finger motifs[18], which affects cell differentiation, cell proliferation, apoptosis, and neoplastic transformation[19]. Aberrant DNA methylation correlates to several types of cancer. *PAX1* and *ZNF582* hypermethylation have been found in oral cancer and cervical cancer[20,21], and aberrant methylation of *SEPTIN9* has been reported in colorectal cancer[22]. In particular, several studies performed in Taiwan have suggested that methylation of *PAX1* and *ZNF582* can serve as potential biomarkers for OSCC detection[20].

The selection of the specimen type plays a crucial role in the clinical effectiveness of in vitro cancer diagnosis. Several Taiwan-based reports have indicated the feasibility of detecting DNA methylation in oral epithelial cells obtained by oral swab and mouth rinse samples from patients with OSCC[20,23–26]. Nevertheless, DNA methylation underlying OSCC in the China mainland region remains uncertain. In this study, OSCC tissue biopsy specimens and oral epithelial cell specimens obtained using oral swabs were collected from a Chinese population in the mainland region. *PAX1* and *ZNF582* methylation levels were then evaluated to determine their performance and ability to discriminate lesions from adjacent/contralateral normal tissue.

Materials and methods

Study population and sample preparation

The study was conducted at Peking University School and Hospital of Stomatology and was approved by the Peking University School and Hospital of Stomatology Biomedical Institutional Review Board. The Chinese Clinical Trial Registry ID of this study is ChiCTR1800015542.

Patients with a diagnosis of OSCC (International Classification of Diseases, 10th revision, Clinical Modification; ICD-10-CM) between April 2018 and April 2019 were enrolled in the Department of Oral and Maxillofacial Surgery. Patients who were older than 18 years of age and who signed the necessary informed consent forms were recruited into the study. Incident OSCC patients without a history of previous tumour-specific treatment were enrolled. Pregnant patients were excluded.

Oral swabs (OS) were used to collect oral epithelial cells from each patient: one swab was used to collect cells at the lesion site and another was used to collect cells from the contralateral mucosa showing a normal gross appearance or from mucosa showing a normal gross appearance in an area not in contact with the lesion, in order to eliminate the bias of an erroneous judgement of the tumour-free region. The oral epithelial cells collected were stored in a phosphate-buffered saline solution at 4. All samples were sent to the laboratory for the detection of methylation within a week.

Regarding the tissue samples, all OSCC patients underwent a resection of the lesion rather than biopsy after recruitment. The tissue specimens (TS) were collected from the same patients: one from the centre of the lesion and one from the adjacent area at a distance of 1.5 cm from the lesion. Both TS were subjected to histopathological examination. All final

Table 1. Clinicopathological characteristics of the 67 OSCC patients.

	Number of cases	%
Sex		
Male	44	65.7
Female	23	34.3
Age (years)		
<60	35	52.2
≥60	32	47.8
Location		
Tongue	27	40.3
Cheek	13	19.4
Other	27	40.3
Risk factor (ever)		
Smoking	38	56.7
Alcohol consumption	35	52.2
Areca nut chewing	3	4.5
Tumour differentiation		
Well-differentiated	24	35.8
Moderately differentiated	40	59.7
Poorly differentiated	3	4.5
Tumour stage		
Stage 0/I	10	14.9
Stage II	16	23.9
Stage III	10	14.9
Stage IV	31	46.3

OSCC, oral squamous cell carcinoma.

histopathological diagnoses of the specimens were made by two board-certified oral pathologists.

Genomic DNA extraction, bisulphite conversion, and determination of methylation

Genomic DNA (gDNA) was extracted from the tissue samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The concentration of gDNA was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). In this study, the methylation status was determined using the bisulphite-converting method. Briefly, a total of 500 ng gDNA was bisulphite-converted using the EpiGene Bisulfite Conversion Kit (iStat Biomedical Co., Ltd, New Taipei City, Taiwan). Bisulphite-converted gDNA was subjected to quantitative methylation-specific PCR (Q-MSP). Q-MSP reactions of *PAX1* and *ZNF582* were performed using TaqMan technologies and a LightCycler 480 Instrument II real-time PCR system (Roche Applied Science, Penzberg, Germany). The PCR reaction was conducted with an initial incubation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s and annealing at 60 °C for 40 s, and a final extension at 40 °C for 40 s. To ensure the quality of bisulphite conversion and Q-MSP processing, gDNA samples from CaSki cells were used as positive (methylation) controls for both genes, while gDNA samples from A375 cells and C33A cells were used as negative (non-methylation) controls for *PAX1* and *ZNF582*, respectively. In addition to the two controls, the *COL2A1* gene was used as an internal control for DNA quantity normalization. The reaction was considered invalid if the crossing point (Cp) value of *COL2A1* was >35. Finally, the DNA methylation levels were estimated by ΔCp, where ΔCp was calculated as $Cp_{target\ gene} - Cp_{COL2A1}$. The methylation levels were expressed as the methylation

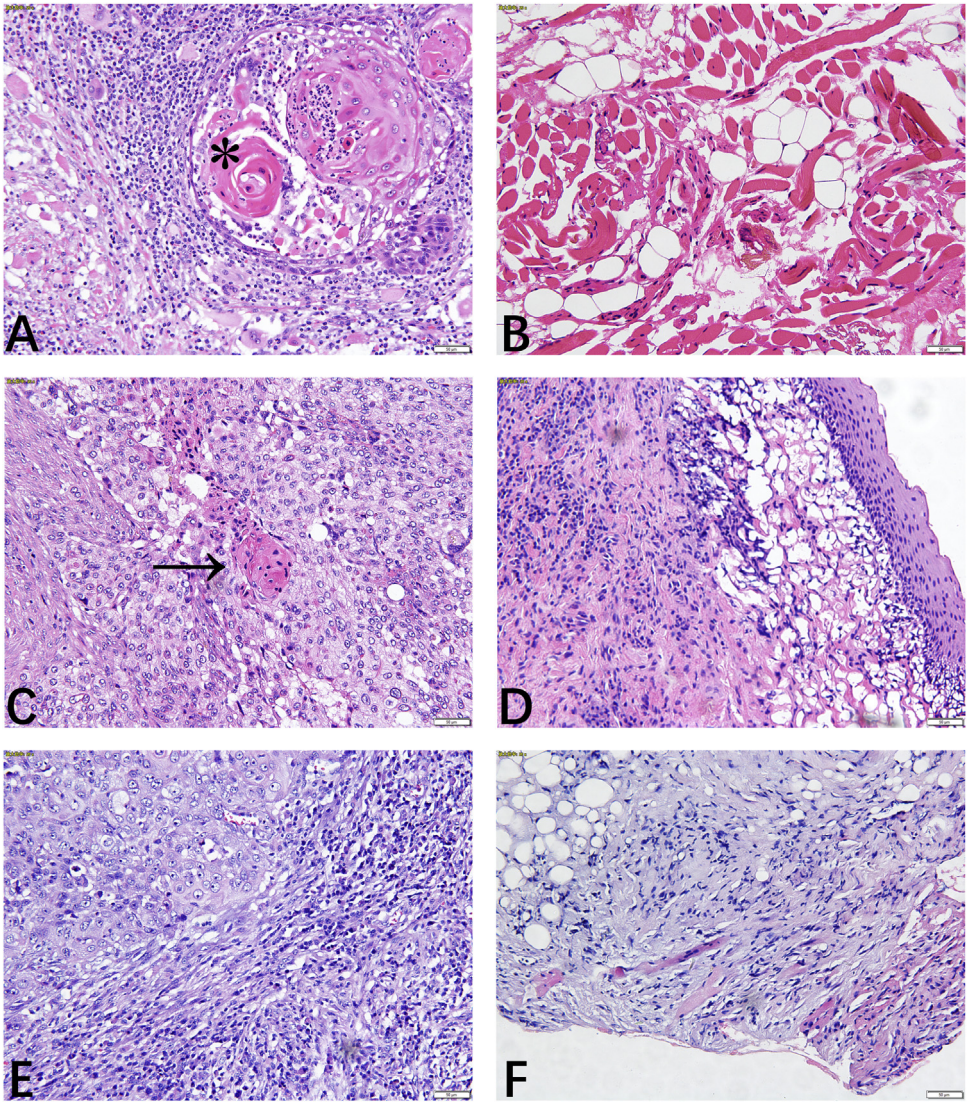

Fig. 1. Lesion sites and adjacent normal sites were confirmed by microscopy. (A) Well-differentiated OSCC; (B) corresponding normal tissue adjacent to the well-differentiated OSCC. (C) Moderately differentiated OSCC; (D) corresponding normal tissue adjacent to the moderately differentiated OSCC. (E) Poorly differentiated OSCC; (F) corresponding normal tissue adjacent to the poorly differentiated OSCC (H&E stain; magnification 20×). The asterisk (*) in Fig. 1a shows keratin pearl formation; the arrow in Fig. 1c shows interconnecting nests of cells with pink cytoplasm.

Table 2. DNA methylation of *PAX1* and *ZNF582* in the OS and TS samples; median (IQR) values.

	OS			TS			OS lesion vs TS lesion
	Lesion	Control	*P*-value	Lesion	Control	*P*-value	*P*-value
PAX1	0.30 (0.05–401.07)	0.02 (0.01–0.05)	<0.0001	120.07 (2.77–382.08)	0.03 (0.00–12.28)	<0.0001	0.16
ZNF582	41.87 (1.68–224.06)	2.11 (0.16–10.47)	<0.0001	20.79 (2.13–225.61)	0.66 (0.11–4.19)	<0.0001	0.83

OS, oral swab; TS, tissue specimen; IQR, interquartile range.

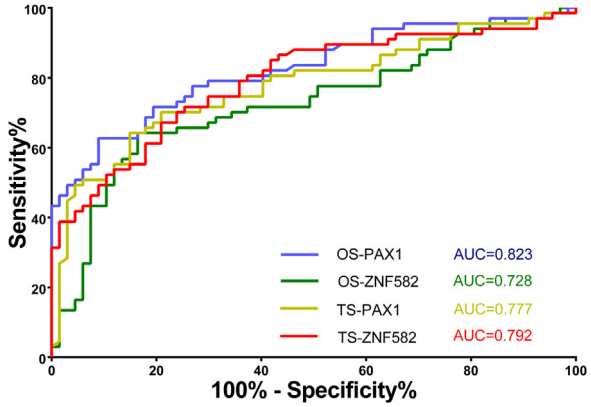

Fig. 2. Receiver operating characteristic (ROC) curves and area under the curve (AUC) for distinguishing lesion and control sites. OS = oral swab, TS = tissue specimen.

index (M-index), which was calculated using the formula $(2^{-\Delta Cp}) \times 10{,}000$.

Data analysis

Receiver operating characteristics (ROC) curve analysis and comparison of the area under the curve (AUC) value by *Z*-test were conducted using MedCalc 19.0 for Windows (MedCalc Software Ltd, Ostend, Belgium). The Mann–Whitney *U*-test was applied for M-index comparison using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA), and a *P*-value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

A total of 67 OSCC patients were enrolled at Peking University School and Hospital of Stomatology between April 2018 and April 2019. The mean age of these OSCC patients was 59.09 ± 11.89 years, and the male to female ratio was 1.9:1. The location of the lesion, risk factors, tumour differentiation, and stage are described in Table 1. Most patients had well-differentiated and moderately differentiated tumours and were at a late stage of OSCC.

Methylation level of *PAX1* and *ZNF582*

To evaluate the gene methylation levels of the lesion and contralateral/adjacent normal tissue, paired samples of TS and OS were collected from each OSCC case. The phenotypes of the TS, including lesion (Fig. 1A, C, E) and adjacent normal tissue (Fig. 1B, D, F), were evaluated by haematoxylin and eosin (H&E) staining and confirmed by the pathologists (Fig. 1). The differentiation of OSCC was classified into well-differentiated, moderately differentiated, and poorly differentiated (Fig. 1A, C, E).

Regarding the methylation levels, the M-index values for *PAX1* and *ZNF582* are reported in Table 2. *PAX1* and *ZNF582* methylation levels were significantly higher in the lesion groups than in the control groups, for both OS and TS. No significant difference in *PAX1* and *ZNF582* methylation levels was found between OS and TS collected from the lesion sites. Overall, these results suggest that the methylation levels of *PAX1* and *ZNF582* in OS could reflect the phenomenon in TS.

Receiver operating characteristics (ROC) curve analysis

ROC curve analysis was performed to evaluate whether *PAX1* and *ZNF582* methylation could be used to differentiate the lesion and normal tissue in OSCC cases. The AUC of OS-*PAX1*, TS-*PAX1*, OS-*ZNF582*, and TS-*ZNF582* for distinguishing lesion and control sites was found to be 0.823, 0.777, 0.728, and 0.792, respectively (Fig. 2). These results suggest that methylation of *PAX1* and *ZNF582* differed significantly between the lesion and the contralateral/adjacent normal tissue in both OS and TS, while there was no significant difference between AUC-OS and AUC-TS (Table 3).

In the subgroup analysis, the results showed no significant difference in AUC according to age, sex, smoking, alcohol consumption, tumour size, and lymphatic metastasis, suggesting that neither *PAX1* nor *ZNF582* methylation is likely to be affected by these factors (Tables 4 and 5; Supplementary Material Tables S1 and S2). However, the AUC of *PAX1* methylation in OS was 0.900 for those with bone invasion, which was significantly different to the AUC for those without bone invasion ($Z = 1.988$, $P = 0.047$) (Table 4). Furthermore, the AUC of ZNF582 methylation in OS was 0.855 in patients

Table 3. Comparison of AUC between the different specimen types.

	Specimen type	Lesion (*n*)	Control (*n*)	AUC	SE of AUC	95% CI of AUC	SE of comparison	95% CI of comparison	*Z*	*P*-value
PAX1	OS	67	67	0.823	0.0362	0.748–0.883	0.0548	−0.0618 to 0.153	0.832	0.41
	TS	67	67	0.777	0.0411	0.697–0.845				
ZNF582	OS	67	67	0.728	0.0447	0.644–0.801	0.0595	−0.0525 to 0.181	1.078	0.28
	TS	67	67	0.792	0.0393	0.713–0.857				

AUC, area under curve; SE, standard error; 95% CI, 95% confidence interval; *Z*, *z*-statistic; *P*, significance level; OS, oral swab; TS, tissue specimen.

Table 4. Comparisons among the different subgroups of the AUC for *PAX1* in oral swabs.

Subgroup		Lesion (*n*)	Control (*n*)	AUC	SE of AUC	95% CI of AUC	SE of comparison	95% CI of comparison	*Z*	*P*-value
Sex	Male	44	44	0.812	0.0475	0.715–0.887	0.0746	−0.120 to 0.173	0.354	0.72
	Female	23	23	0.838	0.0575	0.700–0.930				
Age (years)	<60	35	35	0.826	0.0513	0.717–0.906	0.0730	−0.140 to 0.147	0.0461	0.96
	≥60	32	32	0.823	0.0520	0.707–0.907				
Smoking	Never	29	29	0.827	0.0542	0.705–0.914	0.0740	−0.113 to 0.157	0.161	0.87
	Ever	38	38	0.815	0.0504	0.709–0.895				
Alcohol consumption	Never	32	32	0.850	0.0482	0.739–0.927	0.0744	−0.0823 to 0.209	0.854	0.39
	Ever	35	35	0.787	0.0567	0.672–0.875				
Tumour stage	+	26	26	0.820	0.0594	0.688–0.912	0.0757	−0.145 to 0.152	0.0501	0.96
	+	41	41	0.823	0.0470	0.723–0.899				
T	1 + 2	29	29	0.804	0.0588	0.679–0.896	0.0750	−0.115 to 0.179	0.428	0.67
	3 + 4	38	38	0.836	0.0466	0.733–0.911				
N	0	40	40	0.818	0.0475	0.716–0.896	0.0733	−0.112 to 0.176	0.437	0.66
	1 + 2+3	27	27	0.823	0.0558	0.727–0.933				
Bone invasion	No	40	40	0.764	0.0553	0.656–0.852	0.0682	0.00189 to 0.269	1.988	0.047*
	Yes	27	27	0.900	0.0398	0.787–0.965				
Tumour differentiation	Well-differentiated	24	24	0.859	0.0552	0.729–0.943	0.0735	−0.0849 to 0.203	0.805	0.42
	Moderately or poorly differentiated	43	43	0.800	0.0486	0.700–0.879				

AUC, area under curve; SE, standard error; 95% CI, 95% confidence interval.
* Significant, $P < 0.05$.

with early stage tumours, which was significantly different to those with later stage tumours ($Z = 2.354$, $P = 0.02$), and it was 0.910 in patients with well-differentiated tumours, which was significantly different to those with moderately or poorly differentiated tumours ($Z = 3.731$, $P = 0.0002$) (Table 5).

Discussion

Previous studies have suggested that hypermethylated *PAX1* and *ZNF582* are potential biomarkers for OSCC detection[25]. Since ROC curve analysis has been used widely to analyse the behaviour of diagnostic methods, it was used to test the performance of DNA methylation of lesion specimens and their corresponding control specimens in the same OSCC patients. In this study, ROC curve analysis revealed that the methylation levels of *PAX1* and *ZNF582* differed significantly between the lesion and paired control specimens, for both oral swabs and tissue specimens. In addition, these differences were not influenced by age, sex, smoking habits, or alcohol consumption. Moreover, significant *PAX1* and *ZNF582* hypermethylation in oral swabs was observed among OSCC patients with bone invasion and higher tumour differentiation, respectively. This study is novel in demonstrating that the methylation level in an oral swab from the site of the lesion sufficiently reflects the methylation level of tissue specimens among OSCC patients in China.

A great number of studies have investigated the relationship between DNA methylation levels and malignant tumours[17,27,28]. In addition, many studies have shown the association between DNA methylation and carcinogenesis through several liquid biopsy sources, such as blood and other biofluids. The detection of methylation of the *SEPTIN9* gene in blood samples has been developed as an in vitro diagnostic and has been cleared by the US Food and Drug Administration for market approval[29,30]. Regarding OSCC, several cross-sectional studies have reported a correlation between the methylation of tumour suppressor genes including *PAX1* and the *ZNF* family, *p16*, *APOBEC3A*, and *SALL2* in various specimens (such as tissue specimens, oral scrapings, and serum) and OSCC, suggesting that it might serve as a diagnostic or prognostic tool[23–25,27,31,32].

Previous validation studies have shown that the methylation level of *PAX1* and *ZNF582* in oral epithelial cells obtained using a swab or in mouth rinse samples,

Table 5. Comparisons among the different subgroups of the AUC for *ZNF582* in oral swabs.

Subgroup		Lesion (*n*)	Control (*n*)	AUC	SE of AUC	95% CI of AUC	SE of comparison	95% CI of comparison	*Z*	*P*-value
Sex	Male	44	44	0.712	0.0555	0.606–0.804	0.0956	−0.143 to 0.231	0.462	0.64
	Female	23	23	0.756	0.0778	0.607–0.871				
Age (years)	<60	35	35	0.811	0.0536	0.699–0.894	0.0886	−0.00813 to 0.339	1.868	0.06
	≥60	32	32	0.645	0.0706	0.515–0.761				
Smoking	Never	29	29	0.743	0.068	0.611–0.849	0.0906	−0.153 to 0.202	0.272	0.79
	Ever	38	38	0.718	0.0599	0.604–0.816				
Alcohol consumption	Never	32	32	0.798	0.0596	0.679–0.888	0.0895	−0.0303 to 0.321	1.622	0.10
	Ever	35	35	0.653	0.0669	0.529–0.763				
Tumour stage	+	26	26	0.855	0.0594	0.73–0.937	0.0823	0.0325 to 0.355	2.354	0.02*
	+	41	41	0.661	0.0614	0.548–0.762				
T	1 + 2	29	29	0.812	0.0587	0.688–0.903	0.0861	−0.0329 to 0.305	1.578	0.11
	3 + 4	38	38	0.676	0.063	0.559–0.779				
N	0	40	40	0.772	0.0556	0.664–0.858	0.0942	−0.0699 to 0.299	1.219	0.22
	1 + 2+3	27	27	0.657	0.076	0.515–0.781				
Bone invasion	No	40	40	0.711	0.0593	0.599–0.807	0.0915	−0.142 to 0.217	0.412	0.68
	Yes	27	27	0.749	0.0697	0.612–0.857				
Tumour differentiation	Well-differentiated	24	24	0.910	0.043	0.791–0.973	0.0747	0.132 to 0.425	3.731	0.0002*
	Moderately or poorly differentiated	43	43	0.631	0.0612	0.52–0.732				

AUC, area under curve; SE, standard error; 95% CI, 95% confidence interval.
*Significant, $P < 0.05$.

differed between non-OSCC and OSCC patients[23–25]. Also, a longitudinal study using oral swabs demonstrated that hypermethylation of *PAX1* and *ZNF582* could serve as a prognostic tool for OSCC[24]. In the present study, it was demonstrated that *PAX1* and *ZNF582* methylation differed significantly in the paired samples (control and lesion) among early-stage OSCC patients, suggesting that specific gene methylation can discriminate the lesion and corresponding normal site in the early stage. As a consequence of *PAX1* and *ZNF582* hypermethylation in the oral swab, higher AUCs (>0.9) were observed in the subgroup with bone invasion and in the subgroup with well-differentiated tumours, respectively. Since this study was performed with paired samples (lesion site vs self-control site) from the same patients, these results suggest that DNA methylation information from the different sites could be developed as a detection or prognostic tool in OSCC patients.

According to the WHO[8], histopathology is the current gold standard diagnostic tool for OSCC worldwide. The cost of biopsy examination varies in different countries, depending on the national or private healthcare insurance coverage. In general, a routine biopsy examination takes 4–5 days, and more days will be needed for special staining or second opinions from pathologists for difficult or rare cases. Other methods for OSCC detection have been introduced, such as DNA ploidy analysis[33] and methylene blue staining[34]. However, there are limitations to their clinical application. In contrast, detecting DNA methylation through real-time PCR combined with the oral swab technique is a relatively straightforward, objective, and widely used molecular biology technology. The process, including DNA extraction, bisulphite conversion, and real-time PCR detection, can take no more than 2 days. Compared to biopsy examination, the detection of DNA methylation may be slightly expensive, as it requires specific reagents and instruments to complete the work, but the cost will quickly reduce as the technique becomes popularized and accepted in the healthcare market. Moreover, biopsy examination is well-known for its invasiveness, and most people would prefer not to undergo this procedure unless absolutely necessary. In contrast, DNA methylation coupled with an oral swab technique is relatively non-invasive, which would increase willingness to take the test. Taken together, considering the cost-effectiveness, as well as the risks and benefits of the two approaches, DNA methylation coupled with an oral swab

technique shows enormous potential for development as an auxiliary diagnostic kit for the detection of OSCC, especially for particular populations in whom an operation or biopsy examination is not suitable.

Smoking, alcohol consumption, and areca nut chewing are well-known risk factors for OSCC[1]. The relationships between gene DNA methylation and habits of smoking and alcohol consumption are still uncertain[23,35–37]; however, areca nut chewing is considered to be associated with DNA hypermethylation[23]. Areca nut chewing may not be a cultural habit in northern mainland China, and the number of cases in the current study was limited, so this was not analysed further. Regarding the subgroup analysis of smoking or alcohol consumption habits in this study, similar methylation patterns were demonstrated in both groups, suggesting that these environmental exposures might not influence the detection of DNA methylation. Since environmental exposures (including lifestyle and dietary habits) might be quite different within a geographic area in China, more studies are encouraged in the future.

This study has some limitations. First, there were few OSCC patients with a habit of areca nut chewing, thus a subgroup analysis could not be performed. Second, some exposures, including environmental and dietary habits, as well as the quantity of smoking and alcohol consumption, were not considered in this study. Also, the study population comprised mainly incident OSCC patients who lived in north mainland China. Further studies among different populations are encouraged in the future.

The results of this study demonstrated that *PAX1* and *ZNF582* methylation detected in an oral swab from the lesion site sufficiently reflected the methylation level of the tissue specimen and the actual status of OSCC carcinogenesis. Furthermore, the collection of exfoliated cells with an oral swab is a far less invasive procedure when compared with specimen collection by biopsy, which will likely increase patient willingness to undergo oral cancer diagnostic testing. Besides, DNA methylation shows great potential for development as an alternative method for monitoring the prognosis or therapeutic effect at the lesion site among OSCC patients, especially for patients who are not suitable for biopsy or surgery. Taken together, we suggest that *PAX1* and *ZNF582* methylation detected in oral epithelial cells obtained by oral swab could serve as a non-invasive and alternative or auxiliary diagnostic tool for detecting OSCC.

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Competing interests

The authors declare no competing financial interests.

Ethical approval

Ethical approval was obtained from the Peking University School and Hospital of Stomatology Biomedical Institutional Review Board (PKUSSIRB-201525099a).

Patient consent

All patients received consents.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijom.2020.05.022.

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