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Hypermethylated *PAX1* and *ZNF582* genes in the tissue sample are associated with aggressive progression of oral squamous cell carcinoma

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Abstract

Background: DNA methylation of *paired box gene 1 (PAX1)* and *zinc finger 582 (ZNF582)* is promising cancer biomarkers for oral squamous cell carcinoma detection. This study aims to investigate the correlation between *PAX1* or *ZNF582* methylation and the progression of oral squamous cell carcinoma (OSCC).

Materials and Methods: A total of 135 OSCC cases from Peking University School and Hospital of Stomatology were enrolled in this study. Tissue specimens were collected from the lesion site and corresponding adjacent normal site. The methylation level of these two genes was evaluated in primary and recurrent OSCC group.

Results: Hypermethylation of *PAX1* or *ZNF582* was observed in lesion sites among primary and recurrent OSCC cases. In the lesion site of primary cases, promoter methylation was observed in T3/T4 (*PAX1*: $P = .02$; *ZNF582*: $P = .01$), stage III/IV (*PAX1*: $P = .03$; *ZNF582*: $P = .01$), and bone invasion cases (*PAX1*: $P = .02$; *ZNF582*: $P = .047$). In the subgroup analysis, the correlation between hypermethylation and OSCC severity remains significant with exposure to smoking/alcohol consumption.

Conclusions: Hypermethylated *PAX1* and *ZNF582* can sufficiently act as biomarkers to reflect the severity or progression of OSCC.

KEYWORDS

DNA methylation, oral squamous cell carcinoma, paired box1, progression, Zinc finger protein 582

1 | INTRODUCTION

Oral cancer account for approximately 40% of head and neck cancer, which is the fifth most common cancer in the world.[1] Oral squamous cell carcinoma (OSCC) is the most prevalent oral cancer.[2] According to the World Health Organization's report, the incidence rate of OSCC is higher in Asian countries than in western countries.[3] In addition, the 5-year relative and period survival rates of OSCC remained about 50% in the past decades.[4] Although oral cancer is not primary cancer in China, yet, its incidence persists, and most of the diagnosed patients were found to be in the advanced stage.[5] In general, the rising trend of incidence and the severity were accompanied by poor prognosis in OSCC in China.[3,5] It is crucial to find a marker to predict the progression of OSCC.

DNA methylation is a widely studied epigenetic modification, which associates with carcinogenesis by altering gene expression

without affecting the DNA sequences.[6] DNA methylation often occurs in cytosine-guanine dinucleotide (CpG-rich) promoter region of the genes. The high density of methylcytosine accumulation in the CpG region leads to chromatin remodeling and subsequent gene silencing. As a result, loss of functions of tumor suppressor genes (TSGs) might trigger carcinogenesis.[7] Several studies suggested that OSCC arises as a result of the accumulation of genetic and epigenetic alterations, which lead to the activation of proto-oncogenes and inactivation of multiple TSGs.[7,8] DNA methylation changes targeting tumor suppressor genes were identified as the first epigenetic alterations in cancer and have been related to the early stages of carcinogenesis.[9]

Paired-box1 (PAX1), located on chromosome 20p11.2, is a member of the paired box family of transcription factors that play a vital role in the development of the skeletal system, thymus, and parathyroid.[10] *Zinc finger protein 582 (ZNF582)*, located at chromosome 19q13.43, is a zinc finger protein containing one KRAB-AB domain and nine zinc finger motifs,[11] which affect cell differentiation, cell proliferation, apoptosis, and neoplastic transformation.[12] *PAX1* and *ZNF582* hypermethylation was found in oral cancer and cervical cancer.[13,14] In particular, several studies in Taiwan suggested that methylation of *PAX1* and *ZNF582* is potential biomarkers for OSCC detection.[13] Hypermethylation of *PAX1 and ZNF582* genes in oral scrapings collected from cancer-adjacent normal oral mucosal sites was associated with aggressive progression and poor prognosis.[15-17] Whether hypermethylated *PAX1 and ZNF582* collected from tumor tissue was associated with progression were still uncertain. This study was aimed at determining whether hypermethylated *PAX1* and *ZNF582* genes in tissue specimens from the lesion and adjacent normal mucosal sites are associated with OSCC progression.

2 | METHODS AND MATERIALS

2.1 | Study population and sample preparation

The study was conducted at Peking University School and Hospital of Stomatology, and approved by the ethics committee for human experiments at the Peking University School and Hospital of Stomatology. The full study protocol can be accessed in the Chinese Clinical Trial Registry, and the registration number of this study is ChiCTR1800015542.

Patients with OSCC diagnosis from April 2018 to August 2019 were enrolled at the Department of Oral and Maxillofacial Surgery. Patients who are older than 18 years old and had signed informed consent forms were recruited into the study. Primary OSCC patients without a history of previous tumor-specific treatment or recurrent OSCC were enrolled in this study. All OSCC patients received lesion en bloc resection after recruitment. Formalin-fixed paraffin-embedded (FFPE) and fresh-frozen tissue specimens were prepared from the center of lesions, and 1.5-2.0 cm adjacent area away from the lesion. The tissue specimens proceeded for hematoxylin and eosin staining and methylation assay. The presence of tumor phenotypes was confirmed by two board-certified oral pathologists (Figure 1). The differentiation level of OSCC is classified according to WHO guidelines.[18,19] Tumor stages are determined according to the p-TNM classification of tumors.[20]

2.2 | Genomic DNA extraction, bisulfite conversion, and methylation determination

Genomic DNA (gDNA) obtained from cases was extracted from tissue specimens using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of genomic DNA was determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). Methylation status was determined using the bisulfite-converting method in this study. Briefly, a total of 500 ng gDNA were bisulfite-converted using the "EpiGene" Bisulfite Conversion Kit (iStat Biomedical Co., Ltd.). Bisulfite-converted genomic DNA was subjected to quantitative methylation-specific PCR (Q-MSP). Q-MSP reactions of *PAX1 and ZNF582* were performed using TaqMan technologies accompanied by 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 minutes, followed by 50 cycles of 95°C for 10 seconds, and annealing at 60°C for 40 seconds, and a final extension at 40°C for 40 seconds. According to previous report,[21] *COL2A1* is a fully unmethylated gene in which the CpG island around the transcription start site of the *COL2A1* promoter was completely unmethylated. It was therefore used as an unmethylated internal control to ensure the quality of bisulfite conversion and Q-MSP processing for DNA quantity normalization. Several ATCC cancer cell lines were used as DNA methylation controls. C33A and CaSki are human cervical epidermoid carcinoma derived from the cervix and small intestine, respectively, whereas A375 is human malignant melanoma derived from skin. Since the hypermethylation of *PAX1* and *ZNF582* expression in these cell lines has been demonstrated in the previous study.[22] gDNA samples from CaSki cells were used as positive (methylation) control for both genes, while genomic DNA samples from C33A cells and A375 cells were used as negative (non-methylation) control for *PAX1* and *ZNF582*, respectively (Figure 2). As shown in Figure S1, standard curves were created using a mixture of fully methylated and unmethylated DNA by serial dilution. Positive signals from the methylated gene and negative signal from the unmethylated gene were considered as internal controls for PCR reaction in order to exclude the presence of PCR inhibitors. In addition to the two controls, the reaction was considered invalid when the crossing point (Cp) value of *COL2A1* > 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 index (M-index), which was calculated with the formula $(2^{-\triangle Cp}) * 100$.

2.3 | Data analysis

The Student *t* test was used for M-index comparison by SPSS 24.0, while Spearman rank-order correlation coefficient (one-tailed) was

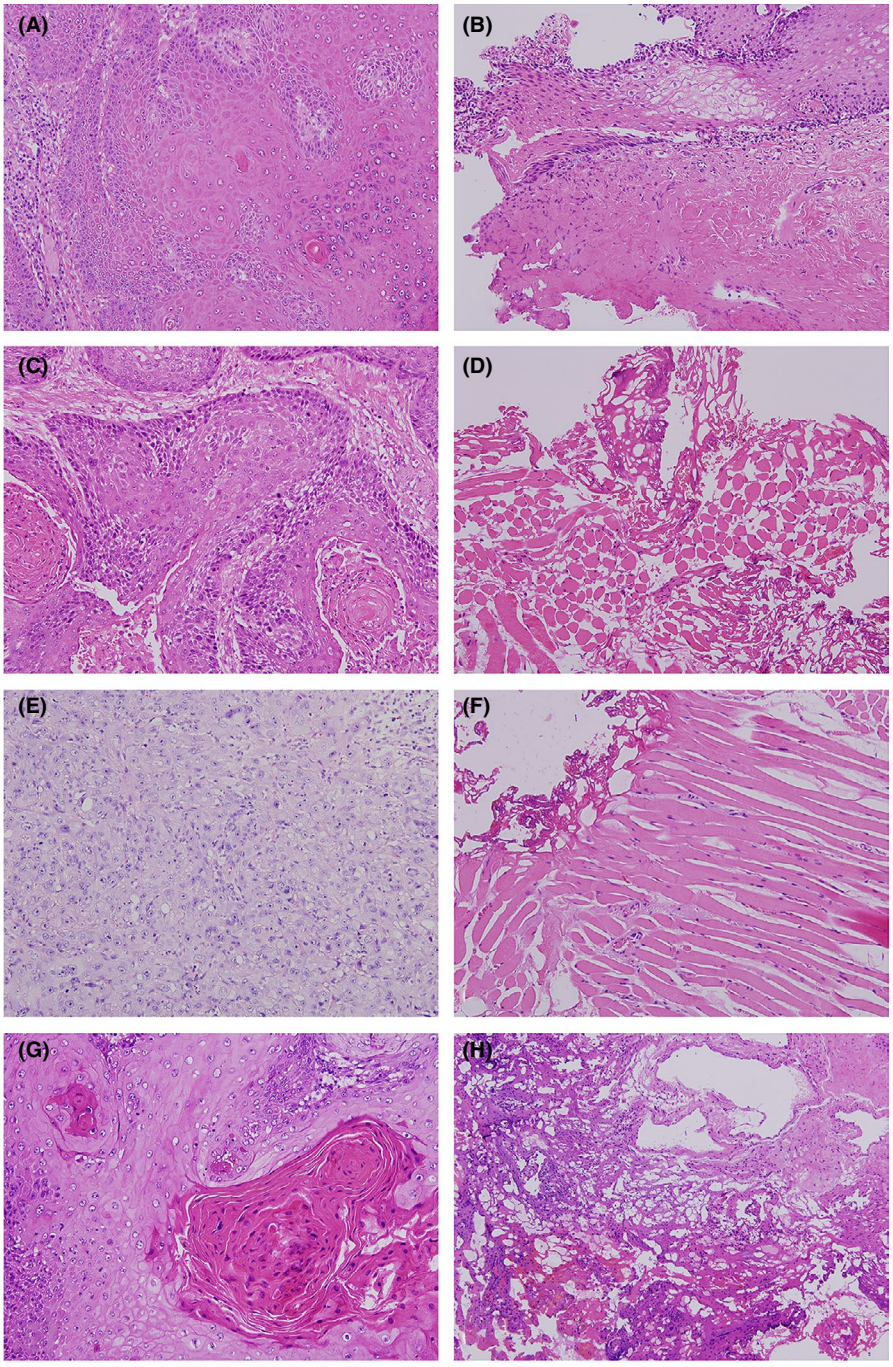

FIGURE 1 Histopathological manifestations of the primary OSCC cases (A)-(F) and recurrent OSCC (G)-(H): A, High-differentiated primary OSCC; B, Corresponding adjacent normal tissue of high-differentiated OSCC; C, Moderately differentiated primary OSCC; D, Corresponding adjacent normal tissue of moderately differentiated OSCC; E, Low-differentiated primary OSCC; F, Corresponding adjacent normal tissue of low-differentiated OSCC; G, Recurrent OSCC; and H, Corresponding adjacent normal tissue of recurrent OSCC (stain, H&E; magnification 200X)

used to measure how highly correlated between specific variables and M-index of genes, and a *P*-value of <0.05 was considered statistically significant.

3 | RESULT

3.1 | Patient characteristics

A total of 135 OSCC patients were enrolled at Peking University School and Hospital of Stomatology from April 2018 to August 2019. Among OSCC patients, there were 108 primary cases and 27 recurrent cases. Overall, there were 82 males and 53 females (60.7% and 39.3%, respectively) with a mean age of 57.9 ± 12.6. Approximately 38.5% of lesions were located in the tongue, 23.7% in the buccal site, and 37.8% in other sites (gum 17.1%, floor of mouth 9.6%, palate 8.2%, lip 1.5%, retromolar area 1.5%). Regarding the potential risk factors, nearly half of the patients who ever have smoking (49.6%) and alcohol consumption (45.9%) habits. Only 5.1% of OSCC patients have areca-nut chewing habits in this study. Based on similar exposure to smoking or alcohol consumption, there were slightly more patients with larger T4 (38.0%), stage IV (45.4%), and moderate differentiation (57.4%) among primary OSCC in this study. However, the proportion between high and moderate differentiation was similar among recurrent OSCC patients (Table 1).

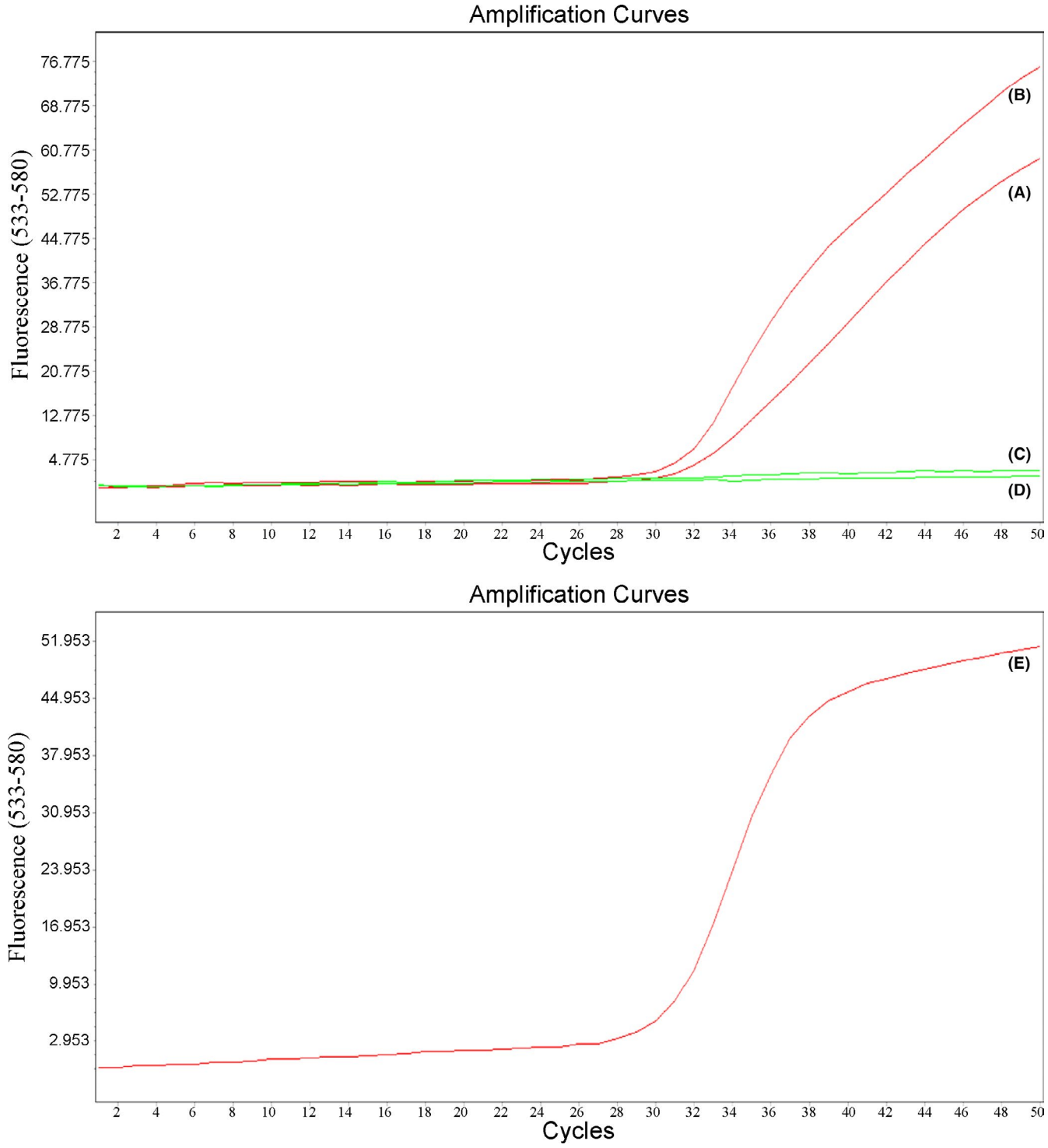

FIGURE 2 The methylation-specific-QPCR results of *PAX1* and *ZNF582*. A, Full-methylation *PAX1* of CaSki cell; B, Full-methylation *ZNF582* of CaSki cell; C, Unmethylated *PAX1* of C33A; D, Unmethylated *ZNF582* of A375; and E, *COL2A1* (reference gene)

3.2 | Methylation level of *PAX1* and *ZNF582* of primary OSCC

To evaluate the gene methylation level of *PAX1* or *ZNF582* in tissue, we collected paired tissue specimen from the lesion and adjacent normal mucosal tissue of each OSCC patients. As for the lesion site of primary OSCC, no significant difference was found between the group of age, sex, and risk factors. However, the significantly higher M-index of *PAX1* or *ZNF582* was observed among cases of T3/4, stage III/IV, and the lesions invaded the bone (Table 2). Using Spearman rank-order analysis, there exist slightly positive correlations between the M-index and tumor stages, tumor sizes in the lesion sites (Table S1). Hypermethylation of *PAX1* or *ZNF582* was observed in some adjacent normal sites of primary cases; however, no

TABLE 1 Clinicopathological characteristics of 135 OSCC patients

	Total		Primary cases		Recurrent cases	
	n = 135		n = 108		n = 27	
	Case number	%	Case number	%	Case number	%
Gender						
Male	82	60.7	69	63.9	13	48.1
Female	53	39.3	39	36.1	14	51.9
Age						
<60	75	55.6	62	57.4	13	48.1
≥60	60	44.4	46	42.6	14	51.9
Location						
Tongue	52	38.5	43	39.8	9	33.3
Cheek	32	23.7	24	22.2	8	29.6
Gum	23	17.0	19	17.6	4	14.8
Other	28	20.7	22	20.4	6	22.2
Risk factor (Ever)						
Smoking	67	49.6	57	52.8	10	37.0
Alcohol consumption	62	45.9	53	49.1	9	33.3
Areca-nut chewing	7	5.1	7	6.5	0	0
Stage						
0			5	4.6%		
I			12	11.1%		
II			24	22.2%		
III			18	16.7%		
IV			49	45.3%		
T						
Tis			5	4.6%		
T1			15	13.9%		
T2			28	25.9%		
T3			19	17.6%		
T4			41	38.0%		
N						
pN0			64	59.3%		
pN1			16	14.8%		
pN2			15	13.9%		
pN3			3	2.8%		
Missing			10	9.3%		
Tumor differentiation						
High	53	39.3	41	38.0	12	44.4
Moderate	76	56.3	62	57.4	14	51.9
Low	6	4.4	5	4.6	1	3.7

significant difference between groups divided by age, sex, and risk factors or severity (Table S2).

Regarding smoking and alcohol consumption, which were considered as potential risk factors of OSCC, we performed a subgroup analysis to evaluate the correlation between methylation level and OSCC severity. In general, the hypermethylation of *PAX1* or *ZNF582* was detectable in the lesion site among primary OSCC patients (Never vs. Ever, Tables S3 and S4). Among patients with smoking

history (Table S3), the methylation level of *PAX1* was significantly higher in the cases of T3/T4 (P = .02) and stage III/IV (P = .01). As for patients with a history of alcohol consumption (Table S4), the methylation level of *PAX1* or *ZNF582* was significantly higher in the cases of T3/T4 and stage III/IV. Among patients without a history of smoking or alcohol consumption, non-statistical higher methylation level was also observed among patients with large tumor size or later stage. These results suggested that the correlation between hypermethylation and OSCC severity remains significant with exposure to smoking/alcohol consumption.

3.3 | Methylation level of *PAX1* and *ZNF582* of recurrent OSCC

In the recurrent OSCC cases, a similar pattern with tissue specimen of primary OSCC was observed (Table 3). In general, the methylation level of lesion site obtained from recurrent OSCC was similar with those from primary OSCC (*PAX1*: 336.04 ± 579.91 vs 706.78 ± 1035.34, P = .08; *ZNF582*: 428.41 ± 868.36 vs 434.10 ± 764.26, P = .98). Notable hypermethylation was also found among patients with bone invasion. However, no statistical

TABLE 2 PAX1 and ZNF582 methylation of tissue specimen at lesion site in primary OSCC

		PAX1		*ZNF582*	
	N	M-index (Mean ± SD)	*P*	M-index (Mean ± SD)	*P*
Age					
<60	62	333.27 ± 622.29	.95	461.53 ± 1048.66	.65
≥60	46	339.76 ± 524.09		383.77 ± 546.54	
Sex					
Male	69	352.83 ± 655.57	.69	485.15 ± 865.11	.37
Female	39	306.33 ± 420.04		328.02 ± 876.23	
Risk factor					
Smoking					
Never	51	371.98 ± 592.24	.54	399.62 ± 1040.59	.75
Ever	57	303.88 ± 571.98		454.17 ± 687.40	
Alcohol consumption					
Never	55	328.88 ± 563.13	.90	277.14 ± 757.12	.07
Ever	53	343.46 ± 602.13		585.39 ± 952.36	
Areca-nut chewing					
Never	101	322.94 ± 554.69	.38	435.14 ± 889.81	.76
Ever	7	524.97 ± 908.06		331.35 ± 489.21	
T					
Tis + T1+T2	48	202.94 ± 409.59	.02*	214.05 ± 377.31	.01*
T3 + T4	60	442.51 ± 671.05		599.91 ± 1089.40	
N					
pN0	64	316.74 ± 484.02	.44	415.96 ± 797.36	.66
pN1-3	34	412.90 ± 746.22		501.05 ± 1061.95	
Stage					
0 + I+II	41	194.24 ± 429.33	.03*	193.91 ± 356.98	.01*
III + IV	67	422.91 ± 642.92		571.91 ± 1044.11	
Differentiation					
High	41	369.35 ± 605.60	.64	313.52 ± 572.25	.28
Low or moderate	67	315.65 ± 567.29		498.72 ± 1005.37	
Bone invasion					
No	65	218.14 ± 450.59	.02*	271.21 ± 491.50	.047*
Yes	43	514.25 ± 702.38		666.05 ± 1207.00	

*P < .05.

correlation was observed between methylation level and recurrent frequency. Relative lower but still significant hypermethylation was found among OSCC patients ever receiving radiation or chemotherapy than patients. Taken together, hypermethylation of *PAX1* or *ZNF582* remains detectable among recurrent OSCC.

3.4 | Overall survival

In this cross-sectional study, primary OSCC and recurrent patients were enrolled separately from April 2018 through August 2019. A preliminary follow-up by phone call for the overall survival of all

TABLE 3 PAX1 and ZNF582 methylation of tissue specimen at lesion site in recurrent cases

		PAX1		*ZNF582*	
	N	M-index (Mean ± SD)	*P*	M-index (Mean ± SD)	*P*
Age					
<60	13	610.05 ± 1132.94	.65	592.17 ± 947.58	.31
≥60	14	796.60 ± 970.06		287.32 ± 539.60	
Sex					
Male	13	717.03 ± 951.12	.96	434.28 ± 573.04	.34
Female	14	697.26 ± 1143.95		433.94 ± 930.09	
Risk factor					
Smoking					
Never	17	623.72 ± 839.81	.60	415.33 ± 579.56	.87
Ever	10	847.98 ± 1344.50		466.01 ± 1043.28	
Alcohol consumption					
Never	18	687.55 ± 1072.31	.89	414.11 ± 859.76	.85
Ever	9	745.24 ± 1018.74		474.07 ± 569.93	
Betel chewing					
Never	27	706.78 ± 1035.34	N/A	434.10 ± 764.26	N/A
Ever	0	0			
Differentiation					
High	12	1116.02 ± 1343.45	.10	627.40 ± 1057.66	.30
Low or moderate	15	379.38 ± 560.57		279.46 ± 384.91	
Recurrent times					
1	20	678.74 ± 1002.80	.82	482.77 ± 795.07	.59
≥2	7	786.87 ± 1204.37		295.05 ± 706.29	
Lymph node metastasis					
No	24	774.72 ± 1079.84	.34	446.44 ± 808.49	.82
Yes	3	163.27 ± 164.02		335.38 ± 244.34	
History of radiation therapy					
No	21	808.88 ± 1135.90	.35	440.95 ± 839.04	.93
Yes	6	349.42 ± 464.71		410.13 ± 469.49	
History of chemotherapy therapy					
No	23	768.65 ± 1097.60	.47	482.61 ± 819.41	.44
Yes	4	350.99 ± 507.28		155.18 ± 128.56	
Bone invasion					
No	14	474.27 ± 855.61	.23	343.04 ± 557.72	.53
Yes	13	957.17 ± 1182.57		532.16 ± 953.13	

participants was performed at the end of March 2020. Among the 135 patients, ten were lost of contact. A total of 125 were follow-up at a duration varied from 1 month to 23 months, with a mean of 13.9 ± 4.56 months. Among them, three patients passed away because of post-operative hemorrhage (n = 1, with 1 month of follow-up duration) and systemic failure (n = 2, with 5 and 9 months of follow-up duration, respectively). Interestingly, two of three patients had hypermethylation at their adjacent normal site. A further follow-up study is needed in the future to fulfill the limited information and time duration collected from phone calls.

4 | DISCUSSION

Previous studies suggested that methylation of *PAX1* and *ZNF582* is potential biomarkers for OSCC detection.[23] In this study, hypermethylation of *PAX1* or *ZNF582* in tissue specimens was detectable among primary and recurrent OSCC patients, which were not influenced by age, gender, and habit of or alcoholic consumption. Besides, the positive correlations were observed between DNA methylation level and severity, including tumor size, stage, and bone invasion among primary OSCC, as well as bone invasion among recurrent OSCC patients. Besides, the methylation level seems higher among recurrent OSCC than primary OSCC patients. Taken together, our study demonstrated that the hypermethylation of *PAX1* and *ZNF582* was correlated with severity among primary and recurrent OSCC patients.

Previous studies demonstrated that hypermethylation of *PAX1* and *ZNF582* was associated with OSCC occurrence. Studies using 43 tumor tissues and 42 non-cancerous matched tissues reported that high methylation levels of both genes in tumors were more commonly observed in poor prognosis patients.[13] However, the correlation between hypermethylation and severity among OSCC patients has not been demonstrated. Tumor size, stage, and bone invasion often considered as severity indicators of OSCC. However, the correlation between bone invasion and OSCC prognosis is still controversial.[24,25] They were using paired biopsy-confirmed tissue specimens of lesion site from 108 incident and 27 recurrent OSCC patients, the statistical correlation between methylation level and severity of OSCC, reflecting the fact hypermethylation related to the progression of OSCC. Thus, our study demonstrated that hypermethylation of the target gene at the lesion site is useful biomarkers to predict or detect the OSCC incidence and recurrence.

Many studies have reported several possible OSCC prognostic factors, including clinical or pathologic characteristics, as well as tumor molecular factors. Tumor characteristics, including stage, tumor size, resection margin free of disease, and dissemination, are considered as factors with significant influence on the prognosis. Regarding tumor biomarkers, there has been an increasing interest in the study evaluating gene or protein expression of tumor biomarkers. Some biomarkers in tissue, blood, or saliva samples have been reported strongly correlated with the different outcomes, including survival, tumor recurrence, advanced grading, and lymph node metastasis. However, clinical trials for proving clinical importance of the validated predictors for survival, tumor recurrence, lymph node metastasis, and therapy resistance were still needed.[26] DNA methylation has been shown to enable early diagnosis, prognosis prediction, and screening for cancers. In particular, several studies have shown the potential of DNA methylation as a prognostic marker. For instance, methylation of *PAX1*, *ZNF582*, and *p16* derived from different specimen sources such as serum or oral scrapings was shown promising as a prognostic marker for colorectal cancer or OSCC.[15,27] Furthermore, a longitudinal study has demonstrated that hypermethylated *PAX1* or *ZNF582* genes in oral scrapings collected from cancer-adjacent normal[15] oral mucosal sites are associated with aggressive progression and poor prognosis of oral cancer. In this present study, we demonstrated that statistical correlations between methylation level of *PAX1* or *ZNF582* in tissue specimens and indicators of OSCC severity, including tumor size, stage, and bone invasion. Indeed, hypermethylation of *PAX1* or *ZNF582* was observed in adjacent normal tissue among some cases; the prognosis outcomes need to be further followed and evaluated. Taken together, these results suggest that gene methylation levels in adjacent normal tissue might serve as potential biomarkers for prognosis, and further evaluation will still be needed in the future.

Smoking, alcoholic consumption, and areca-nut chewing are well-known risk factors of OSCC.[1] The relationships between gene DNA methylation and habits of smoking or alcohol consumption are still uncertain.[28-30] Additionally, the impact of risk factors on the correlation between hypermethylation of *PAX1* and *ZNF582* was still limited. Thus, subgroups analysis by risk factors was used for evaluating the correlation between methylation level and OSCC severity. In general, no significant differences could be found between cases with or without smoking or alcohol consumption history (ever versus never) in lesion sites of primary OSCC cases. Furthermore, the correlation between hypermethylation and OSCC severity remains significant, although the only statistical difference was observed among patients ever exposed to smoking or alcohol consumption. Interestingly, we found significantly higher methylation in lesion sites of recurrent OSCC than primary OSCC. Our results gave a preliminary concept from limited cases of recurrent OSCC; however, further evidence is still needed in the future.

Currently, histopathology is the gold standard for diagnosis of OSCC, including identifying primary OSCC and monitoring OSCC recurrence. In this study, our results revealed the positive correlation between hypermethylation of *PAX1* or *ZNF582* and pathological characteristics of tumor in the tissue, with exposure to smoking/alcohol consumption. Besides, the level of methylation remained detectable in recurrent OSCC cases and was significantly higher than that of the primary OSCC cases. Methylation-specific real-time PCR was used to verify the methylation status of the promoter by targeting limited CpGs status. Thus, we were yet to understand the entire methylation status of the tumor. Next-generation sequencing (NGS) would be an ideal approach further to explore the complete spectrum of epigenetic modification in tumors. This technology grants the next possible study to identify potential markers and their association with

OSCC. Currently, this cross-sectional study revealed the association between methylation level and disease severity. A preliminary overall survival among study cohort was performed, and further follow-up study for the methylation level at baseline and disease progression will be conducted in the future. To our knowledge, this is the first study that demonstrates a correlation between hypermethylation and severity using paired biopsy-confirmed tissue specimens of the lesion site from primary and recurrent OSCC patients. A further study is recommended with an increase of recurrent OSCC case numbers for comprehensive understanding. In conclusion, hypermethylated *PAX1* and *ZNF582* can sufficiently act as effective biomarkers to reflect the severity or progression of OSCC.

CONFLICT OF INTEREST

Dr YC Juan and Dr YF Su are employees of iStat Biomedical Co., Ltd., Taiwan. The other authors declare that they have no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Rui Sun: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, validation, visualization, writing-original draft, writing-review and editing. **Yi-chen Juan:** Conceptualization, data curation, formal analysis, investigation, methodology, resources, software, validation, writing-original draft, writing-review and editing. **Yee-fun Su:** Formal analysis, investigation, ethodology, resources, software, writing-original draft, writing-review and editing. **Wen-bo Zhang:** Conceptualization, investigation, methodology, project administration, resources, software. **Yao Yu:** Formal analysis, investigation, methodology, resources, software. **Hongyu Yang:** Conceptualization, investigation, project administration, resources, supervision, validation. **Guang-Yan Yu:** Conceptualization, data curation, funding acquisition, project administration, resources, supervision. **Xin Peng:** Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing-review and editing.

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REFERENCES

1. Mignogna MD, Fedele S, Lo RL. The World Cancer Report and the burden of oral cancer. *Eur J Cancer Prev*. 2004;13(2):139-142.
2. Muller S. Update from the 4th Edition of the World Health Organization of Head and Neck Tumours: Tumours of the Oral Cavity and Mobile Tongue. *Head Neck Pathol*. 2017;11(1):33-40.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424.
4. Noone AMHN, Krapcho M, Miller D. In: *SEER Cancer Statistics Review, 1975–2015*. Bethesda, MD: National Cancer Institute; 2018.
5. Zheng CM, Ge MH, Zhang SS, et al. Oral cavity cancer incidence and mortality in China, 2010. *J Cancer Res Ther*. 2015;11(6):149-154.
6. Jones LK, Vaskar S. Chromatin modification, leukaemia and implications for therapy. *Br J Haematol*. 2002;118(3):714-727.
7. Williams HK. Molecular pathogenesis of oral squamous carcinoma. *Mol Pathol*. 2000;53(4):165-172.
8. Asokan GS, Jeelani S, Gnanasundaram N. Promoter hypermethylation profile of tumour suppressor genes in oral leukoplakia and oral squamous cell carcinoma. *J Clin Diagn Res*. 2014;8(10):ZC09-12.
9. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. 2010;31(1):27-36.
10. Lang D, Powell SK, Plummer RS, Young KP, Ruggeri BA. PAX genes: roles in development, pathophysiology, and cancer. *Biochem Pharmacol*. 2007;73(1):1-14.
11. Huntley S, Baggott DM, Hamilton AT, et al. A comprehensive catalog of human KRAB-associated zinc finger genes: insights into the evolutionary history of a large family of transcriptional repressors. *Genome Res*. 2006;16(5):669-677.
12. Urrutia R. KRAB-containing zinc-finger repressor proteins. *Genome Biol*. 2003;4(10):231.
13. Yang C-C, Wu C-H, Chang C-F, et al. DNA methylation confers clinical potential to predict the oral cancer prognosis. *Clin Res Trials*. 2018;4(4):1-8.
14. Lai H-C, Lin Y-W, Huang THM, et al. Identification of novel DNA methylation markers in cervical cancer. *Int J Cancer*. 2010;123(1):161-167.
15. Cheng S-J, Chang C-F, Ko H-H, et al. Hypermethylated ZNF582 and PAX1 genes in oral scrapings collected from cancer-adjacent normal oral mucosal sites are associated with aggressive progression and poor prognosis of oral cancer. *Oral Oncol*. 2017;75:169-177.
16. Guerrero-Preston R, Michailidi C, Marchionni L, et al. Key tumor suppressor genes inactivated by "greater promoter" methylation and somatic mutations in head and neck cancer. *Epigenetics*. 2014;9(7):1031-1046.
17. Morandi L, Gissi D, Tarsitano A, et al. CpG location and methylation level are crucial factors for the early detection of oral squamous cell carcinoma in brushing samples using bisulfite sequencing of a 13-gene panel. *Clin Epigenetics*. 2017;9:85.
18. Thompson L. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. *Ear Nose Throat J*. 2006;85(2):74.
19. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ. *WHO Classification of Head and Neck Tumours*. 4th ed. Lyon: IARC; 2017.
20. Amin MB, Edge S, Greene F, et al. *AJCC Cancer Staging Manual* (8th ed.). Chicago, IL: Springer International Publishing; 2017.
21. Imagawa K, de Andrés MC, Hashimoto KO, et al. Association of reduced type IX collagen gene expression in human osteoarthritic chondrocytes with epigenetic silencing by DNA hypermethylation. *Arthritis Rheumatol*. 2014;66(11):3040-3051.
22. Huang J, Wang G, Tang J, et al. Methylation status of PAX1 and ZNF582 in esophageal squamous cell carcinoma. *Int J Environ Res Public Health*. 2017;14(2):216.
23. Cheng S-J, Chang C-F, Lee J-J, et al. Hypermethylated ZNF582 and PAX1 are effective biomarkers for detection of oral dysplasia and oral cancer. *Oral Oncol*. 2016;62:34-43.
24. Matsushita Y, Yanamoto S, Yamada S-I, et al. Correlation between degree of bone invasion and prognosis in carcinoma of the mandibular gingiva: Soft tissue classification based on UICC classification. *J Oral Maxillofac Surg Med Pathol*. 2015;27(5):631-636.
25. Fives C, Nae A, Roche P, et al. Impact of mandibular invasion on prognosis in oral squamous cell carcinoma four centimeters or less in size. *Laryngoscope*. 2017;127(4):849-854.
26. Blatt S, Krüger M, Ziebart T, et al. Biomarkers in diagnosis and therapy of oral squamous cell carcinoma: a review of the literature. *J Craniomaxillofac Surg*. 2017;45(5):722-730.
27. Nakahara Y, Shintani S, Mihara M, Hino S, Hamakawa H. Detection of p16 promoter methylation in the serum of oral cancer patients. *Int J Oral Maxillofac Surg*. 2006;35(4):362-365.

28. Cheng SJ, Chang CF, Ko HH, et al. Hypermethylated ZNF582 and PAX1 genes in mouth rinse samples as biomarkers for oral dysplasia and oral cancer detection. *Head Neck*. 2018;40(2):355-368.
29. Strzelczyk JK, Krakowczyk L, Owczarek AJ. Aberrant DNA methylation of the p16, APC, MGMT, TIMP3 and CDH1 gene promoters in tumours and the surgical margins of patients with oral cavity cancer. *J Cancer*. 2018;9(11):1896-1904.
30. Strzelczyk JK, Krakowczyk L, Owczarek AJ. Methylation status of SFRP1, SFRP2, RASSF1A, RARbeta and DAPK1 genes in patients with oral squamous cell carcinoma. *Arch Oral Biol*. 2019;98:265-272.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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