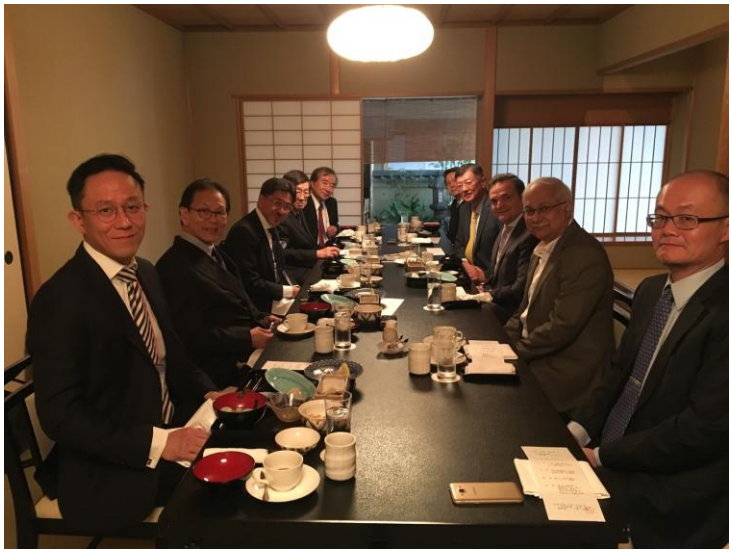
2019 年 10 月 25-26 日 高壽延副院長受邀出席

第 68 屆日本口腔顎面外科學會議

高副院長出席第 68 屆日本口腔顎面外科學會議，這是日本最高的口腔顎面外科會議，本次大會邀請發表論文，題目為 DNA methylation confers clinical potential to predict theoral cancer prognosis，希望與國際口腔顎面外科相關專業人士討論此議題，並促進國際口腔醫學學術經驗分享與交流，以及提昇本院之專業素養、擴展國際視野與經驗，同時也是本院專科醫療行銷推廣的一種途徑。



此外，高副院長身為亞洲口外學會顧問，參與本次日本口外 JSOMS、台灣口外 TAOMS、亞洲口外 Asian AOMS、國際口外 IAOMS 四方的理監事交流會面，旨在分享台灣成功舉辦 2018 亞洲口腔顎面外科學大會之經驗，獲得各方好評後，欲利用此行表達申辦世界會議之決定，會中獲得日方強力支持協助，深感意義非凡，這同時也是口外學會很關鍵會晤。

DNA methylation confers clinical potential to predict theoral cancer prognosis

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Key words: Oral Squamous Cell Carcinoma (OSCC), methylation, PAX1, ZNF582

Abstract

Oral squamous cell carcinoma (OSCC) has high incidence worldwide and poor prognosis for the past few decades. The common diagnostic procedure is to perform a visual oral examination (VOE)followed by a biopsy examination; however, these procedures have limitations in effectively detecting lesions with diffuse patterns or without clear pathological phenotypes. Thus, effective biomarkers for accurate diagnostic assessments are urgently needed to ensure optimal clinical management. In this current study, 43 tumor tissues and 42 non-cancerous matched tissues (NCMT) were harvested to determine new biomarkers for this purpose. Te methylation levels of *ZNF582*^{m} and *PAX1*^{m} were determined by quantitative methylation-specific PCR. Clinical information was masked until the completion of methylation tests. ZNF582 and PAX1were highly methylated in tumors of OSCC patients with a true positive rate of 88.4% (*ZNF582*) and 76.7% (*PAX1*). The high methylation levels of both genes in tumors were more commonly observed in poor prognosis patients compared to those with well prognoses. Also, a high positive rate in NCMT was observed in poor prognosis patients. In conclusion, high methylation levels of *ZNF582* and *PAX1* in tumors and NCMT can be potential biomarkers in predicting OSCC prognosis, and can lead to optimal clinical management for OSCC patients.

Abbreviations

OSCC: Oral squamous cell carcinoma; VOE: Visual oral examination; NCMT: Non-cancerous matched tissue; HNSCC: Head and neck squamous cell carcinoma; ZNF582: Zinc finger protein 582; PAX1: Paired box protein 1; QMSP: Quantitative methylation specific PCR; COL2A: Te type II collagen gene; Cp: crossing point; TPR: True positive rate; TNR: True negative rate; AUC: Area under curve; OR: Odds ratio; PCR: Polymerase chain reaction.

Background

Head and neck cancers comprise a group of cancers that occur in the oral cavity, the oropharynx, the nasal cavity, paranasal sinuses, the nasopharynx, the hypopharynx, and the larynx. Most (90%) of these cancers have squamous cell carcinoma histology and are called head and neck squamous cell carcinoma (HNSCC). HNSCC is the seventh most common cancer worldwide, with around 600,000 new diagnoses each year. Oral squamous cell carcinoma (OSCC) is listed as the top common type and has been listed as one of the top 10 cancers that lead to death. To combat this cancer, the government offers biennial visual oral examination (VOE) for people who are over 30 years old and exposed to oral cancer risk factors. Although there were advances in the therapeutic strategies and the implementation of screening programs, the prognosis of OSCC/OSCC remained unchanged for the following reasons: (i) more than half of oral cancer patients are diagnosed at late stages (III or IV and (ii) OSCC/OSCC is subject to field cancerization, leading to having higher risk of developing local recurrences, second primary tumors, or distant metastases.

Most of the current preoperative methods for clinical diagnoses of OSCC are based on the imaging of the neck region through palpation, ultrasound examination, CT scan, MRI examination, and aspiration cytology. These techniques are suboptimal and can misdiagnose the presence or absence of cancer in many patients. To ensure optimal management of patients with OSCC, an effective biomarker for accurate assessment is required. It is especially true for people with special risk habits, such as betel nut chewing, smoking, and alcohol drinking.

The affected sites of OSCC are usually able to be assessed by direct inspection or endoscopic examination followed by biopsy and pathologic checking. It is, however, a great challenge in the cases of clinical lesions manifested with diffuse patterns or those without strong pathologic evidence of malignancy after biopsy. Then, identifying and developing other biomarkers for optimal clinical management becomes critical. Carcinogenesis in OSCC is a complex process which involves aberrant genetic and epigenetic events. Previous studies have shown that aberrant DNA methylation of zinc finger protein 582 (*ZNF582*) and paired box protein 1(*PAX1*) is a hallmark of neoplastic disease. Also, mass studies of these two methylated genes in cervical cancer have shown great clinical potential. Since both genes were also found to be highly associated with squamous cell carcinoma, here we would like to explore the clinical utility of both genes in OSCC prognoses by using paired specimen, including cancerous and non-cancerous matched tissues (NCMTs).

Methods

Patients' recruitment, specimen collection, and DNA preparation

This study was approved by the Institutional Review Board (IRB) of Taipei Veterans General Hospital (VGH, IRB No. 2015-06-009AC). We collected total of 43 tumor tissues and 42 non-cancerous matched tissues (NCMT) as training test to determine the optimum cut-off values of M-index for each gene. These tissues were harvested from OSCC patients in Taipei Veterans General Hospital who had received surgical ablations of their primary tumors. Using the same M-index values generated from the training set, another 65 patients as a validation set were analyzed. Among them, 46 cancer patients were recruited, and two swabs were collected (one from the oral cancer lesion and the other one from the adjacent lesion (visually normal oral lesion), and 19 patients from the training test who regularly came back for follow up examination were also collected the swab from the operative site as the control group. The flow chart of patient enrollment for the tissue collection was illustrated (Figure 1) .

Bisulfte conversion and DNA methylation analysis

All the methylation tests were performed in an ISO17025-certifed laboratory. Briefly, the tissues were ground, and the genomic DNA (gDNA) samples were extracted from the tissues/ swabs and bisulfite converted using the Epigene™ Nucleic Acid Extraction Kit and Bisulfite Conversion Kit (iStat Biomedical Co., Ltd., New Taipei City, Taiwan),respectively

Quantitative methylation-specific PCR (QMSP) was performed to determine the methylation levels of *ZNF582* and *PAX1* (*ZNF582*^{m} and *PAX1*^{m}) by using TaqMan-based technologies with the Light Cycler LC480 system (Roche Applied Science, Penzberg, Germany). The type II Collagen gene (*COL2A*) was used as the internal reference and quality indicator. The PCR reactions consisted of an initial incubation at 95° C for 10 minutes, followed by 50 cycles of denaturation at 95° C for 10 seconds, and annealing and extension at 60° C for 40 seconds. Fluorescence data were collected during the annealing /extension step for determination of the crossing point(Cp). The ΔCp is the difference of crossing-point (Cp) values between the methylated genes and COL2A ($\Delta Cp = Cp_{Gene}{}^{m} - Cp_{COL2A}$). The methylation levels were expressed as the M-Index with the formula of 10,000 X $2^{-\Delta Cp}$. When ΔCp is equal to ten (M-Index=9.77), there are about 0.1% of cancer cells in the specimen calibrated by cancer cell lines, and this defines positive methylation as seen in previous studies.

Statistical analysis

R Project ver. 3.3.1 was used for all statistical analyses. To evaluate the performance of DNA methylation in identifying the SCC lesion, Receiver operating characteristic (ROC) curve analysis was performed by plotting the true positive rate (TPR) and the true negative rate (TNR) at various M-indexes. In the training set, the Wilcoxon signed-rank tests were used to compare the methylation levels in paired tissues (NCMT and Tumor), for tumor location or prognosis analyses, Wilcoxon rank-sum tests were used. In the validation set, the correlation among methylation M-index and clinic-pathological parameters were analyzed by Student's t-test or chi-square test, where appropriate. Statistical significance was declared at a p-value of less than 0.05.

Results

Patient Characteristics

In this study, we collected 85 tissue specimens including 43 tumors and 42 NCMTs as training set and 108 swab specimens including 44 tumors, 45 NCMTs and 19 normal as validation set. The detailed clinical information was indicated in Table 1. 34 males and 9 females with an average age of 53.60 ± 11.37 were included in the training set. In the 43 tumor specimens, 15 were located at buccal and 28 at non buccal locations. As for the tumor development stages, 5 patients were at stage I, 13 patients were at stage II/III, and 25 patients were at stage IV. 56 males and 8 females with an average age of 57.73 ± 10.73 were also included in the validation set. We also distinguished the patients according to the lesion site, 13were located at buccal and 32 at non buccal locations. Te tumor development stages were also shown in the table, 11 patients were at stage I, 15 patients were at stage II/III, and 19 patients were at stage IV.

DNA methylation in cancerous tissue and non-cancerous matched tissue (NCMT)

As mentioned, ROC methods were used to evaluate the performances of methylated genes in identifying the SCC lesions (Figure 2a). Both *ZNF582*^{m} and *PAX1* ^{m} had significantly high area under the curve (AUC)/odds ratio (OR) with values of 92.0%/39.1 and 83.9%/5.0,respectively (all p < 0.001). The methylation levels of the two genes at the tumor sites and NCMT were analyzed by box-and-whisker plots (Figure 2b). As expected, the methylation levels of both *ZNF582*^{m} and *PAX1*^{m} were higher at the tumor site (median M-index: 1695.8 and 824.7, respectively) than those in NCMT (median M-index 0.53 and 0.01, respectively) with p values less than 0.001. At the methylation-positive cut off (M-index=9.77), *ZNF582*^{m} had 88.4% of TPR and 83.7% of TNR compared to those in *PAX1*^{m} with rates of 76.7% TPR and 60.5%TNR, respectively.

The anatomic location of OSCC, the buccal mucosa, has long been correlated to oral habits like chewing tobacco or betel quid. Thus, the association between cancer location and hypermethylation was investigated. Results showed that buccal tumors had significantly higher *ZNF582*^{m} methylation levels than tumors in non-buccal areas (median M-index: 2932 and 1644, respectively, p=0.045) (Supplement Figure S1). Similar methylation trends were observed in *PAX1*^{m}: buccal tumors had higher *PAX1*^{m} methylation levels than those tumors in nonbuccal areas (median M-index: 2059 and 1199, respectively, p=0.09)(Supplement Figure S1). No significant differences in the methylation of both *ZNF582*^{m} and *PAX1* ^{m} were observed in either buccal or nonbuccal areas in NCMT.

Validation of Clinical Cut-off value

To validate the cut-off value generated from the training set, we recruited 65 patients as the validation set. Their tissues harvested by surgical excision were used for the methylation assay. As observed in the training set, the difference methylation level of *ZNF582* and *PAX1* between non-cancer and cancer group were analyzed. The methylation level of *ZNF582*^{m} and *PAX1*^{m} in cancer groups were significantly higher (391.47 ± 1595 vs. 20/363.23 ± 1575.77) than those in non-cancer groups (0.36 ± 519 vs. 90/19.78 ± 7.57).

The sensitivity, specificity and odds ratio were calculated for the detection of cancer. *ZNF582*^{m} showed a moderate sensitivity (72.73%) and high specificity (89.47%) and odds ratio (22.67), while PAX1^{m} had lower sensitivity (68.18%), specificity (78.95%) and odds ratio (8.04) (Supplement Figure S2).

For clinical application as a non-invasive tool for OSCC discrimination, exofoliative cells collected by swabbing with sponge rolls were processed for methylation assays. In this validation set, both *ZNF582*^{m} and PAX1^{m} had the similar results to those in the training set.

The box and whisker plots depict the significantly higher methylation levels of *ZNF582*^{m} and *PAX1*^{m} in tumor sites (median M-index: 1249.42± 1595.20 and 1006.44 ± 1575.77, respectively) than those in adjacent normal sites (median M-index: 501.06 ± 989.49 and 289.90 ± 780.52, respectively) (Figure 3).

In table 2, we further analyzed the difference methylation level of *ZNF582*^{m} and *PAX1*^{m} between adjacent normal and tumor in each group. We grouped those patients by age, gender, location, tumor size, lymph node metastasis and tumor histological differentiation. Both *ZNF582*^{m} and PAX1m had significantly high methylation level of tumor in the male patients and with below 50 years of age. In the analysis of location, *ZNF582*^{m} showed markedly difference methylation level in the buccal site, however,*PAX1*^{m} had the significantly difference in the non-buccal site. We observed both *ZNF582*^{m}and *PAX1*^{m} had distinct different methylation level between adjacent normal and tumor in the T4 group.

The variation of methylation levels in well and poor prognostic conditions

To evaluate whether the methylation level of these two genes could be used as predictors for prognosis, scatter plots of *ZNF582*^{m} and *PAX1* ^{m} were used to analyze the prognoses of the training set in a 3-year follow-up (Figure 4). In the cohort of training set, 10 out of 43 patients had treatment failure (defined as poor prognosis), which included 3 with cancer recurrence, 3 with metastasis, and 4 with the development of second primary cancer.

Methylation levels in NCMTs and tumors are represented as X and Y-coordinates, respectively. Methylation levels of *ZNF582* or *PAX1* in tumor and NCMT from the individual patient were represented in each dot on the diagram. In particular, red dots represented patients with poor prognosis conditions, which were defined as patients developing tumor recurrence, metastasis, or second primary tumors after initial treatment. Results showed that *ZNF582* ^{m} and *PAX1* ^{m} were predominantly methylated in the tumor sites as compared to NCMTs (Figure 4). Methylation index was further analyzed in tumor sites in well and poor prognosis conditions.

Results showed that M-indexes of *ZNF582*^{m} and *PAX1* ^{m}in poor prognoses patients were 2.15 and 1.95-fold higher than in patients having well prognoses. Te median M-index of *ZNF582* ^{m}/*PAX1* ^{m} was 1299/1582 for well prognoses, and 2797/3078 for poor prognoses, respectively. The odds ratios were 17.8 for*ZNF582*^{m} and 22.0 for *PAX1*^{m} (p=0.005 and 0.002, respectively). The threshold of M-index distinguishing well to poor prognosis was also determined by ROC methods, and the prognosis threshold values of *ZNF582*^{m} and *PAX1*^{m} were 2000 and 2500, respectively.

Acknowledgments

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