Identification of a DNA methylaion-based biomarkers panel for esophageal squamous cell carcinoma(ESCC) in Chinese Han population

Abstract

Background: DNA methylation-based biomarkers was suggested to be promising for early cancer diagnosis. Currently, limited suitable DNA methylation-based biomarkers have been identified in esophageal squamous cell carcinoma(ESCC), especially in Chinese Han population.

Methods: candidate genes were selected through literature search and two high-throughput DNA methylation microarray datasets were collected for initial confirmation. targeted bisulfite sequencing was applied in an independent cohort of 94 pairs of ESCC and normal tissues from Chinese Han population for further validation.

Results: In our study, four candidate genes (*ADHFE1, EOMES, SALL1* and *TFPI2*) were selected based on the literature search. Eight CpG sites located at these four genes were taken as the candidate differentially methylated CpG sites between ESCC and adjacent normal tissues in two high-throughput datasets. In the validation stage, four genomic regions covering the eight CpG sites were designed and the methylation status of these candidate CpG sites were validated (cg08090772, p= 2.90×10-2; cg20295442, p= 5.10×10-3; cg20912169, p= 2.10×10-3; cg16971668, p= 1.60×10-9; cg22383888, p= 3.30×10-9; cg04550052, p= 2.50×10-4; cg04698114, p= 1.10×10-6; cg12973591, p = 3.30×10-5). Moreover, through the targeted bisulfite sequencing method, we could also test the CpG sites located at the targeted regions. The mean methylation percent of each region was calculated and took as the representative for each gene. The diagnostic model based on the combination of these four genomic regions yielded a robust performance (Sensitivity = 0.66, Specificity = 0.87, AUC = 0.81). Nine statistical models following with fivefold cross validation were applied for further evalation. In addition, subgroup analyses revealed a significant difference in diagnostic performance between the alcohol use and non-alcohol use subgroups.

Conclusion: In summary, based on literature reports and high-throughput DNA methylation microarray dataset, we identified four genes as diagnosis biomarkers. Methylation profiles of *ADHFE1*, *EOMES*, *SALL1* and *TFPI2* could be an effective methylation-based assay for ESCC diagnosis.

Keywords: Esophageal squamous cell carcinoma, DNA methylation, Biomarker, Diagnosis, Methyltarget

**Background**

Esophageal cancer is one of the most aggressive malignant tumors with high prevalence and poor prognosis worldwide [1]. Esophageal cancer usually occurs as two subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), which differed significantly in pathogenesis, pathology, epidemiology and geographical distribution [2]. The regions of the highest occurrence of esophageal cancer streching from northern China to northwestern Iran, including Japan and India are localized in the so-called Asian Esophageal Cancer Belt [3, 4]. Moreover, the prevalence of ESCC and EAC in these regions are significantly unbalanced with 90% of esophageal cancer patients are ESCCs [5]. In addition, the clinical outcomes of ESCC patients depend largely on its diagnosed stage [2]. The majority of ESCCs are diagnosed at advanced stages and the overall 5-year survival rate is relatively poor, while the 5-year survival rate for early-stage diagnosed ESCC patients is significantly higher [6]. Therefore, it is imperative to identify biomarkers for early diagnosis of Chinese as well as Asian ESCC patients.

DNA methylation, which usually occurs in CpG dinucleotides, functioning as an epigenetic modification in mammalian genome and is involved in gene and microRNA expression regulation and alternative splicing. [7-9]. Global hypomethylation as well as the hypermethylation of CpG islands in the tumor suppressor genes have been identified in the the tumorigenesis [10, 11]. Comparing with the biomarkers based on SNP/mutation, copy number variations (CNV) and gene/microRNA expression, DNA methylation is advantageous due to its stability, easy detection as well as accuracy [12-14]. Numerous studies have suggested that DNA methylation could be one of the most promising early-detection biomarkers for several types of cancers [15-19]. For example, multiple tumor-related genes have been reported to be hypermethylated in ESCC, including *APC, MGMT, CDH1, RASSF1* [20-23]. However, due to the heterogeneity of ESCC, a single biomarker could only achieve relatively limited prediction ability, which calling for the comprehensive combinations of these candidate biomarkers.

In the present study, we conducted a literature search then manually selected four of the candidate hypermethylated genes (*ADHFE1, EOMES, SALL1, TFPI2*) previously reported as potential tumor suppressor genes in several types of cancers, and validated these biomarkers with high-throughput datasets from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. Moreover, the methylation profiles of these four genes were further validated with targeted bisulfite sequencing method in 94 pairs of ESCC tumor and adjacent normal tissues from Chinese Han population.

**Patients and Methods**

**Biomarker selection based on publications and public datasets**

After careful publication search and manual selection, we identified four candidate tumor-associated genes (*ADHFE1, EOMES, SALL1* and *TFPI2*) which have been reported in several kinds of cancers. In order to test the methylation status of these four genes in ESCC patients, we searched high-throughput microarray databases in TCGA and GEO database to collect the DNA methylation profiles of the ESCC samples. After stringent quality control, we found that TCGA project has quantified the methylation profiles of 84 ESCC and 3 normal tissues, as well as 78 EAC and 13 normal tissues. In addition, a dataset in GEO database named GSE52826 was also retrieved, including 4 ESCC and 8 normal tissues. Due to the similarities between the adjacent normal tissues of EAC and ESCC, which was shown by the principle component analysis (Supplementary Figure 1), we included the 13 normal tissues of EAC as controls and obtained 84 ESCC and 16 normal tissues in total from TCGA for further study.

Wilcoxon rank-sum test was conducted to rank differentially methylated CpGsites located in these four genes in the datasets from TCGA (Supplementary Table 1). We firstly filtered out the CpG sites according to the methylation percent (β ≥ 0.25 in ESCC patients and β < 0.25 in controls). Moreover, the regions covering multiple CpG sites which had a similar methylaiton pattern were preferred. It is widely acknowledged that the methylation status of CpG sites was largely variable in different cell types. As a result, we must ensure that the methylation percent should be lower for the candidate biomarker in the peripheral blood so as to be used for non-invasive cancer diagnosis in the future. As a result, we then filtered the candidate CpG sites with high methylation percentage in the peripheral blood mononuclear cells (PBMC, N = 111) and peripheral blood leucocytes (PBL, N = 527) of the healthy normal samples from the GEO database. The PBMC dataset came from the GSE53045 dataset, and the PBL dataset was the combination of GSE36054 and GSE42861 dataset. Finally, we obtained four candidate regions, which covered 1-3 CpG sites each, as the representatives for four candidate genes (Table 2). We then constructed the multiplex PCR reaction system for the representative regions for each candidate gene for further validation.

**Patients, samples and DNA**

ESCC samples and their paired adjacent normal tissues for validation study were obtained from the First Affiliated Hospital of Soochow University and Fourth Military Medical University between the years of 2011 and 2015. All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. The studies were approved by the institutional review boards of Soochow University at Jiangsu Province and Fudan University, Shanghai, China. Written informed consent was obtained from each study subject. In addition, all of the subjects were re-examined and confirmed by professional pathologists for histopathological diagnosis. All tissues were immediately frozen at -80oC after surgical resection. Face-to-face interviews were conducted by professional investigators with a comprehensive questionnaire, including clinical information on tobacco smoking, alcohol consumption and family history.

**DNA extraction, bisulfite conversion and targeted bisulfite sequencing**

Genomic DNA from ESCC tumor tissue and adjacent normal tissue samples were extracted by AIIperp DNA/RNA Mini Kit (Qiagen, Duesseldorf, Germany) according to manufacturer’s protocols. For methylation analysis, 500ng genomic DNA was subjected to bisulfite conversion using the EpiTect Fast DNA Bisulfite Kit (Qiagen, Duesseldorf, Germany). All unmethylated cytosines were converted to uracil, then to thymidine during the subsequent PCR step. A multiplex PCR was performed first with optimized primer sets combination. PCR amplicons were diluted and amplified using indexed primers and the products (170bp - 270bp) were separated by agarose electrophoresis and purified by QIAquick Gel Extraction kit (Qiagen, Duesseldorf, Germany). Libraries from different samples were quantified and pooled together, sequenced with the Illumina Hiseq 2000 platform according to manufacturer's protocols. FastQC was applied for the quality control of the sequenced reads. Filtered reads were mapped to genome using Blast. In addition, one positive and one negative control were also included for internal control. The methylation profiles in chrM (chrM: 1 - 200) is a part of chromsome from mitochondria, which has been shown to have no DNA methylation appearance and was taken as our negative control. Meanwhile, the methylation status of APC was taken as a positive control since it has been reported hypermethylated in ESCC tumor tisses.

**Statistical analysis and machine learning**

In the first and second stage, we tested the differential methylation of the CpG sites between cancer and normal tissues using Wilcoxon rank-sum test. False discovery rate (FDR) correction was conducted for multiple test correction. In order to discriminate the ESCC tumor and nomal tissues, we utilized serveral machine learning methods, including logistic regression (Package stats), support vector machine (SVM, Package e1071), random forest (Package randomForest), naïve bayes (Package e1071), neural network (Package nnet), linear discriminant analysis (LDA, Package mda), mixture discriminant analysis (MDA, Package mda) as well as the flexible discriminant analysis (FDA, Package mda) followed with five-fold cross-validation. All statistical analyses were all conducted using R 3.2.1 [24].

**Results**

**Public datasets collection and CpGsites validation**

In order to quantify the methylation status of these four candidate genes, public DNA methylation microarray datasets of ESCC were carefully searched. In total, 84 ESCC tumor tissues as well as 16 adjacent normal tissues were employed for the discovery stage analysis. In addition, the GSE52826 dataset from GEO database, incorporating 4 ESCC tumors and 8 adjacent normal tissues was also utilized as the validation dataset [25]. Based on the CpG sites selection criteria which was described in Methods, eight CpG sites (cg08090772, cg20295442, cg20912169, cg16971668, cg22383888, cg04550052, cg04698114, cg12973591) located at the four candidate genes were selected for validation (Table 2). Four of the eight CpG sites were significantly hypermethylated in ESCC tissues compared to the adjacent normal tissue, while the other four CpG sites were also shown to be hypermethylated in ESCC tissues but did not reach the significance threshold (Table 2). Moreover, a similar methylation pattern of these 8 CpG sites were also validated in an independent GEO dataset (Supplementary Table 1). In addition, all 8 CpG sites showed a hypo-methylated state in the PBMC and PBL from healthy samples (Supplementary Table 1).Integratively, we believed that though some of the eight CpG sites did not reach the significance threshold due to the limited sample size, all of these 8 CpG sites may be of potential as the non-invasive potential biomarkers for ESCC and thus were included for validation. To test the prediction ability based on these eight CpG sites, we built a prediction model based on the logistic regression using the methylation status of these 8 CpG sites without adjustment for age, gender and other covariates, which provided a fair good performance to discriminate between ESCC and normal tissues (Sensitivity = 0.94, Specificity = 0.94, AUC = 0.98). To further evaluate and validate the diagnostic ability of these eight CpG sites, we then conducted the validation study in 94 paired ESCC and adjacent normal tissue samples obtained from patients from the Chinese Han population.

**Methylation status validation with targeted bisulfite sequencing**

The characteristics of the ESCC patients are shown in Table 1. In order to give a robust characterization of the methylation status of these 8 CpG sites as well as the four genes, we applied the targeted bisulfite sequencing method, which was based on the next generation sequencing (NGS) platforms. Because the NGS platforms could generate millions of reads with length > 200 bp, we then designed to test four genomic regions, covering all these eight genes for validation (Table 3). In the quality control process, we found that the bisulfite conversion rate of our samples were higher than 98%, and no significant difference was found between the tumor and adjacent normal tissues (Figure 3E). In addition, the samples and the CpG sites with high missing rates and low coverages were also filtered out as described in Materials and Methods. After that, 163 samples remained for further study. Differential methylation analyses were conducted for the four genomic regions, suggesting a major difference between the ESCC and adjacent normal tissue (Figure 2A-E). A logistic regression model was then applied, and showed significant hypermethylation status of the eight selected CpG sites in the ESCC tissues (Table 2, cg08090772, p= 2.90×10-2; cg20295442, p= 5.10×10-3; cg20912169, p= 2.10×10-3; cg16971668, p= 1.60×10-9; cg22383888, p= 3.30×10-9; cg04550052, p= 2.50×10-4; cg04698114, p= 1.10×10-6; cg12973591, p = 3.30×10-5). To better characterize the methylation status of the four genomic regions as well as the four candidate genes, we averaged the methylation status of all the CpG sites in each genomic region as representatives of the regions for further analysis (Figure 3A-D). Based on the mean methylation status of the four genomic regions, the prediction ability of each region separately was evaluated through logistic regression without adjustment for age, gender and other covariates. The sensitivity of each region ranges from 0.29 to 0.69, while the specificity ranges from 0.77 to 0.94, and the AUC ranges from 0.64 to 0.78 (Table 3). Moreover, in the logistic model taking all of the four regions as predictors, we obtained the sensitivity of 0.66 and specificity of 0.87, as well as the AUC of 0.81 (Figure 3F).

**The prediction ability of the diagnosis panel with different statistical models**

Several machine learning methods, including logistic regression model, random forest, support vector machine (SVM), neural network (NN), Naïve Bayes (NB), linear discriminant analysis (LDA), mixture discriminant analysis (MDA), flexible discriminant analysis (FDA) and gradient boosting machine (GBM) following with fivefold cross validation were utilized for ESCC diagnosis based on the targeted bisulfite sequencing results. It turned out that the GBM model achieved the highest classification accuracy aomong all machine learning methods in train stage, whose sensitivity, specificity and accuracy were 0.826, 0.856, 0.840. The naive bayes model achieved the best specificity (0.916) in the train stage. In the test stage, the random forest and naive bayes performed with the best sensitivity (0.728) and specificity (0.910), respectively. However, the linear discriminant analysis and flexible discriminant analysis model both achieved the best accuracy (0.735). Interestingly, in both of the train and test stage, we found that the specificity of all the models were much higher than the sensitivity, indicating that the diagnosis panel consisting of these four genes could detect the ESCC with relatively low false positive rate.

**The diagnositic ability in the ESCC subgroups**

Previous studies have found several risk factors for the incidence of ESCC, including age, gender, smoking status, and alcohol status [26-29]. In order to explore the effects of these risk factors on the ESCC diagnosis, we conducted the subgroup analyses. Similarly, the mean methylation percentage of each genomic region was utilized. To explore the diagnostic ability in the young/old samples, we firstly divided the samples according to the median age of our patients. No significant difference between the sensitivity, specificity and the AUC between the two subgroups (Supplementary Table 3). The overall AUCs using all the variables in the two subgroups was 0.82 and 0.80 for the young and old subgroups, respectively (Supplementary Figure 3A-B). When it comes to the gender, still no significant difference was found between the two subgroups. (AUC: 0.79 vs. 0.82 for male and female subgroups, Supplementary Table 3). Similarly, no significant difference of the diagnostic performances was found between smoker/non-smoker subgroup analysis (Supplementary Table 4 and Supplementary Figure 3E-F). However, when concentrating on the effect of alcohol use, we found that the non-alcohol use subgroup showed significantly higher AUC than that of the alcohol use subgroup (0.84 vs. 0.77 respectively, Supplementary Table 5). The significant difference in the diagnostic performance between the alcohol use and non-alcohol use subgroup indicates that alcohol use may contribute to the epigenetic changes in ESCC as well as to the pathogenesis of ESCC [27].

**Disscussion**

Althought epigenetic aberrations were reported having critical role in cancer progression and prognosis in many studies, limited DNA methylation based biomarkers have been utilized for the diagnosis and prognosis of ESCC. In this study, we retrieved four tumorosis-associated genes from literature screening and tested their methylation status from the TCGA and GEO dataset. Specifically, eight candidate CpG sites, located at the four candidate genes (*ADHFE1, EOMES, SALL1, TFPI2*) were found to be hypermethylated in ESCC tissues and hypomethylated in the adjacent normal tissues as well as the peripheral blood samples. In order to further confirm its methylation status and quantify its potential to be the ESCC diagnositic biomarkers, we validated these eight CpG sites in an independent 94 pairs of ESCC and adjacent normal tissues from Chinese Han population. Targeted bisulfite sequencing method was conducted to detect the methylation status of the candidate genes. Because that the targeted bisulfite sequencing is based on the NGS technology, we could not only detect the methylation status of these eight CpG sites but also their genomic regions as well. To give a robust estimation of the methylation status of the candidate regions, we averaged the methylation percent of all the CpG sites of each region, and built several machine learning methods to assess the diagnostic ability of these DNA methylation based biomarkers. In addition, the subgroup analyses were also conducted and the alcohol use was found to be associated with the dignostic ability, indicating the importance of taking the epidmiological information into account when performing the ESCC diagnosis.

All four candidate genes have been reported to be associated with the tumorigenesis in several tumor types. Alcohol dehydrogenase, iron containing 1(*ADHFE1*) encodes hydroxyacid-oxoacid transhydrogenase, which is responsible for the oxidation of 4-hydroxybutyrate in mammalian tissues [30]. *ADHFE1* promoter hypermethylation was found in [colorectal cancer](https://www.ncbi.nlm.nih.gov/pubmed/24886599/) (CRC) and the alcohol could downregulated the expression of *ADHFE1* through hypermethylation and further induce the proliferation of CRC cells [31, 32]. *EOMES* belongs to the TBR1 (T-box brain protein 1) sub-family of T-box genes, enncoding a transcription factor which is necessary for the embryonic development. It has been reported that *EOMES* promoter methylation could serve as a promising biomarker for the prediction of occurrence, recurrence and prognosis of bladder cancer [33-35]. In addition, EOMES has also been confirmed to have potential anti-cancer functions through siRNA experiments, and was regarded as candidate tumor suppressor gene for human hepatocellular carcinoma [36]. Spalt like transcription factor 1(*SALL1*) encodes a zinc finger transcriptional repressor, which has recently been identified as a tumor suppressor gene, whose expression was in positive correlation with *CDH1* and associated with the survival of patients in breast cancer [37]. In addition, *SALL1* hypermethylation has already been confirmed as the diagnositic biomarker for breast cancer and other epithelial cancers, especially for the colorectal cancer [38]. Tissue factor pathway inhibitor 2 (*TFPI2*) encodes a member of the Kunitz-type serine proteinase inhibitor family, and was found to be a tumor suppressor gene in several types of cancer [39-42]. Moreover, numerous studies have suggested that the *TFPI2* promoter methylation could be of potential as a biomarker for cancer progression and prognosis in hepatocellular carcinoma, pancreatic adenocarcinoma, epithelial ovarian cancer and melanomas [43-46].

The accurate early diagnosis of cancer is a great challenge due to the cancer heterogeneity. In our study, we selected four candidate tumorigenesis genes and applied the targeted bisulfite sequencing method to explore the methylation status of our candidate CpG sites as well as their adjacent genomic regions, thus yielding a robust estimation of the methylation status of the candidate genes. With the fast development of next generation sequencing (NGS), the targeted bisulfite sequencing method is becoming more popular for methylation detection because of high accuracy, high-throughput and cost-effective. According to the our results, the panel consisting of these four candidate genes could distinguish the ESCC tumors with high specificity and relatively lower sensitivity. In order to achieve a better panel of DNA methylation-based biomarkers for diagnosis of ESCC, we should then search for the candidate biomarkers with high sensitivity through the integration of literature information and public datasets, and combined with the biomarkers from our current panel. In summary, a panel with four genes was identified and achieved a fair accuracy in classfying ESCC from normal tissues. However, diagnostic sensitivity of this panel still need to be improved more to reach the needs for clinical use. Multi-omics datasets, including genomics, epigenomics and proteomics, which could provide biomarkers in different biological layers, could contribute to the accurate non-invasive diagnosis of esophageal squamous cell carcinoma in the future.

**Conclusion**

Integrated analysis of public literatures and multiple-platform high-throughput DNA methylation microarray datasets were conducted and discovered eight candidate CpG sites located at four genes (*ADHFE1, EOMES, SALL1, TFPI2*) as the candidate biomarkers for ESCC diagnosis. All four genes were then successfully validated in an independent cohorts including 94 pairs of ESCC and adjacent normal tissues using the targeted bisulfite sequencing method. Methylation profiles of *ADHFE1, EOMES, SALL1, TFPI2* could be an effective methylation-based assay for the ESCC diagnosis with high specificity.

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