**Epigenetic silencing of ZNF132 mediated by methylation sensitive Sp1-binding promotes cancer progression in esophageal squamous cell carcinoma (ESCC)**

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**Abstract**

The epigenetic alteration of tumor suppression gene (TSG) is one of the most significant indicators in human esophageal squamous cell carcinoma (ESCC). In this study, we identified a novel ESCC hyper-methylation biomarker ZNF132 by integrative [computational](https://www.google.com/search?q=computational&spell=1&sa=X&ved=0ahUKEwj928jy-IveAhWBzVkKHSrPDRMQkeECCCooAA) analysis to comprehensive genome-wide DNA methylation microarray dataset. We validated the hyper-methylation status of *ZNF132* in 91 Chinese Han ESCC patients and adjacent normal tissues with methylation target bisulfite sequencing (MTBS) assay. Meanwhile, *ZNF132* gene silencing mediated by hypermethylation was confirmed in both solid tissues and cancer cell lines. What’s more, we found *in vitro* over-expression of *ZNF132* in ESCC cells could significantly reduce cell abilities in growth, migration and invasion, and tumorigenicity of cells in a *in vivo* nude mouse model. We identified the Sp1 binding site in *ZNF132* promoter region with Chromatin immunoprecipitation (ChIP) assay and demonstrated hyper-methylation status could reduce the Sp1 transcript factor activity. Our results suggest that *ZNF132* plays an important role in the development of ESCC as a tumor suppressor gene and support the underlying mechanism caused by the DNA hyper-methylation mediated Sp1 binding decay and gene silencing.

**Introduction**

Esophageal cancer (EC) ranks 8th in most common cancers and 6th in cancerrelated mortality worldwide[1](#_ENREF_1" \o "Stoner, 2001 #174),[2](#_ENREF_2" \o "Allum, 2009 #175). For the past several decades, the incidence of and estimated deaths due to esophageal cancers have been increasing continuously. Based on GLOBOCAN worldwide estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC), 455,800 new esophageal cancer cases and 400,200 deaths occurred in 2012 worldwide[3](#_ENREF_3" \o "Torre, 2015 #229),[4](#_ENREF_4" \o "Yan, 2018 #213). The five-year survival rate of esophageal cancer remains poor despite of advances in clinical oncology. Esophageal cancer consists mainly of two subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), each with distinct pathologies and etiologies[5](#_ENREF_5" \o "Fichter, 2014 #178). While EAC predominates in North America[6](#_ENREF_6" \o "Brown, 2002 #179),[7](#_ENREF_7" \o "Drahos, 2017 #233), the majority of esophageal cancer cases worldwide are ESCC, which has a high prevalence in East Asia, Eastern and Southern Africa, and Southern Europe[8](#_ENREF_8" \o "He, 2014 #180),[9](#_ENREF_9" \o "Huang, 2016 #181). ESCC accounts for more than 90% esophageal cancers in China nowadays[10](#_ENREF_10" \o "Zhang, 2013 #182). With the characteristics of highly invasive, metastatic and poor prognosis, there is an urgent need for identifying diagnostic and prognostic biomarkers for ESCC.

DNA methylation is one of the most intensively studied epigenetic modifications. DNA methylation involves in many kinds of biological processes, including development, gene expression regulation and imprinting[11](#_ENREF_11" \o "Bell, 2000 #237). Multiple studies have confirmed that global hypomethylation induces genomic instability leading to cell transformation, and hypermethylation of promoter regions of the tumor suppressor genes facilitates tumorigenesis[12](#_ENREF_12" \o "Asokan, 2014 #184). Previous studies have shown that a broad range of genes are silenced by DNA hypermethylation in different cancer types[13](#_ENREF_13" \o "Nunna, 2014 #185). The study of specific DNA methylation has translational potential in the management of ESCC patients, and hypermethylated promoters may serve as candidate biomarkers. Moreover, DNA methylation is reversible which makes it very interesting for therapy approaches[14](#_ENREF_14" \o "Kulis, 2010 #187).

We have previously screened TCGA database for aberrant epigenetic changes in ESCC. The results were validated with DNA methylation datasets from GEO and peripheral blood mononuclear cells (PBMC) and peripheral blood leucocytes (PBL) of healthy controls. Hypermethylation of several candidate genes were identified. One of them is *ZNF132*, which belongs to C2H2 zinc finger protein family. It is located at chromosome 19q13.4, which is usually deleted in thyroid adenomas[15](#_ENREF_15" \o "Tommerup, 1995 #242). *ZNF132* has 18 C2H2 zinc finger motifs according to its predicted structure. The zinc finger protein family has been shown to participate in biological processes such as development and differentiation. Recent studies have also suggested that zinc finger proteins play a role in cancer progression[16](#_ENREF_16" \o "Hajra, 2002 #243). There are not many studies on the biological function of *ZNF132*, however, decreased expression of *ZNF132* has been reported in prostate cancer, and is associated with aggressive prostate cancers[17](#_ENREF_17" \o "Abildgaard, 2012 #189).

To determine the role of *ZNF132* and its potential value as a biomarker in ESCC, we studied the methylation status of the *ZNF132* promoter and the expression level of *ZNF132* in ESCC tumors and adjacent normal tissues. The correlation between methylation status and expression of *ZNF132* were then measured in vitro in EC cell lines with or without epigenetic drugs. Furthermore, we tested the effect of *ZNF132* expression on proliferation, migration, invasion and apoptosis of ESCC cells in vitro. The effect of *ZNF132* overexpression on the tumerigenecity of ESCC cell line was also tested in a nude mouse model. Finally, the mechanism of association of promoter hypermethylation and expression of *ZNF132* was explored.

**Materials and methods**

**Human tissues, Cell lines, transfection and drug treatment**

ESCC samples and their paired adjacent control tissues were obtained for validation study from the First Affiliated Hospital of Soochow University and Fourth Military Medical University between the years of 2011 and 2015. Ec-109 and CaEs-17 cells were obtained from the Shanghai Institute for Biological Sciences and grown in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (GIBCO®, Invitrogen™, Auckland, NZ), penicillin (100 U/ml) and streptomycin (100μg/ml). HEK293T cells was maintained in DMEM culture medium containing 10% fetal bovine serum (GIBCO®, Invitrogen™, Auckland, NZ) that was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO2 and were passaged using pancreatic enzymes two or three times a week. HEK293T cells were co-transfected with the lentiviral vectors 0.8 μg pSPAX2, 0.4 μg pMD2.G and 1.2 μg pCD513B-*ZNF132* expression plasmid. one day before infection, CaEs-17 or Ec-109 cells were seeded in 6-well plate with the density of 2×105 cells per well, and incubated at 37 °C in a humidified atmosphere of 5% CO2 overnight. 5-aza-2’-deoxycitidine (5-Aza) (Sigma-Aldrich, St. Louis, MO, USA) was used as demethylating agent to treat cells. Drug treatment protocol as previously described[18](#_ENREF_18" \o "Lee, 2017 #245),[19](#_ENREF_19" \o "Oka, 2005 #246).

**DNA methylation evaluated by methylation target bisulfite sequencing (MTBS)**

Methylation targeted bisulfite sequencing method (MTBS) was applied for the methylation profile investigation in this study. Non-CpG containing bisulfite sequencing primer (BSP) was used for non-bias bisulfite converted DNA replication with biocode for the sample identification and then followed by next-generation sequencing. Theoretically, multiplies PCR primers could be arranged in one PCR reaction for the multiple region methylation detection for large number samples, details see our previous study[20](#_ENREF_20" \o "Pu, 2017 #153). Genomic DNA from ESCC tumor tissue and adjacent control tissue samples were extracted by AIIperp DNA/RNA Mini Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer’s protocols. For methylation analysis, 500 ng genomic DNA was subjected to bisulfite conversion using the EpiTect Fast DNA Bisulfite Kit (Qiagen, Duesseldorf, Germany). Multiplex PCR was performed first with optimized non-CpG primer sets combination (LINE-1, *ZNF132* and ChrM). PCR amplicons were diluted and amplified using indexed primers and the products (170-270bp) were separated by agarose electrophoresis and purified by QIAquick Gel Extraction kit (Qiagen, Duesseldorf, Germany). Libraries from different samples were quantified and pooled together equally, and then sequenced with the Illumina Hiseq 2000 platform according to the manufacturer's protocols. BSseeker2 software was utilized for reads mapping and methylation calling. Samples with high missing rates (>30%)and CpG sites with high missing rates (>20%)were removed[21](#_ENREF_21" \o "Guo, 2013 #263). 13 CpG sites located at the promoter of LINE-1 gene (Hypomethylation in esophageal cancer tissues) and 11 CpG sites from mitochondrion DNA (ChrM) were also sequenced as positive and negative controls respectively. Primers used are listed in Supplementary Table 1.

**RNA extraction, and quantitative real-time PCR**

Total RNA was isolated by AIIperp DNA/RNA Mini Kit (Qiagen, Duesseldorf, Germany). First-strand cDNA was synthesized from 1 μg total RNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was carried out with an Applied Biosystems 7900 Prism real-time PCR machine and SYBR Premix Ex Taq (Takara, Dalian, Japan), in accordance with the manufacturer’s instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. Quantitative real-time PCR primers used for *ZNF132* were listed in Supplementary Table 1. The target gene expression in test samples was normalized to the corresponding GAPDH level and was reported as the fold difference relative to the GAPDH gene expression.

**Cell Proliferation Assay**

Ec-109 cells and CaEs-17 cells both treated by the plasmid pCD513B-*ZNF132* and pCD513B for 48 hours, were incubated for 0, 24, 48, 72, 96, and 120 hours in 96-wells plate with 1,000 cells/well. Then 10 µL CCK-8 (Tianjin Biolite Biotechnologies, Tianjin, China) was added to each well for 3 hour, followed by light absorbance measurement at a wavelength of 450 nm.

**Transwell assays for cell migration and invasion**

The suspension of the Ec-109 cells treated by the plasmid pCD513B-*ZNF132* and pCD513B was prepared in a non-serum medium with the a density of 2x105 cells/ml, 200 μL suspension of this kind was plated on the top side of a polycarbonate Transwellfilter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA, USA) and incubated at 37 °C for 24 h, and 500 μL culture medium was added in each well, then remove the cells inside the upper chamber with cotton swabs, invaded cells on the lower membrane surface were fixed in 4% paraformaldehyde, stained with crystal violet, and counted (5 random fields per well at 100× magnification).

**Wound-Healing Assay**

CaEs-17 and Ec-109 cells treated by the plasmid pCD513B-*ZNF132* and pCD513B were seeded into 6-well plates at a density of 2x105 cells/well, When they had nearly reached confluency, a wound was created by manually scraping the cell monolayer with a 10μl pipette tip and then cells were washed twice with 1×PBS. Some cells were harvested here (time, 0 h), while others were maintained for 96 h in culture medium, and pictures were taken under the inverted microscope.

**Assessment of apoptosis**

Ec-109 cells treated by the plasmid pCD513B-*ZNF132* and pCD513B were seeded in 6-well plates with the density of 2×105 cells per well for 48 h, Cells were suspended with trypsin, harvested and stained with Annexin V-PE/7-AAD. Afterward, the cells were analyzed by a flow cytometer (FACS Calibur; Becton–Dickinson, Mountain View, CA, USA).

**Dual-luciferase reporter gene assay**

Dual-luciferase reporter gene assay was performed as previously described[22](#_ENREF_22" \o "Zhang, 2016 #247). The fragment in *ZNF132* promoter region (chr19:58951628-58951928, hg19), was cloned to pGL3-Basic vector (Promega, Madison, WI, USA) or pCpGL-Basic vector (The pCpGL-Basic vector was a kind gift of M. Rehli, University Hospital Regensburg) to make a reporter construct. The construct inserts were veriﬁed by sequencing. To confirm the participation of Sp1, HEK293T cells plated on 24-well plate were transfected with 0-1.6 μg Sp1 expression vector and 200 ng pGL3-*ZNF132* or 200 ng methylated pGL3-*ZNF132* by lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). In each transfection, 5 ng of pRL-SV40 vector (Promega, Madison, WI) was used to correct transfection efficiency. To determine whether methylation of Sp1-bining site alters the transcriptional activity of *ZNF132* promoter by derecruiting Sp1. HEK293T cells plated on 24-well plate were transfected with 0, 300ng Sp1 expression vector and 200 ng pCpGL-*ZNF132* or 200 ng methylated pCpGL-*ZNF132* by lipofectamine 2000. The methylated reporter construct were methylated in vitro using SssI (CpG) Methyltransferase as recommended by the manufacturer’s instructions. In each transfection, 5 ng of pRL-SV40 vector was used to correct transfection efficiency. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Promoter activities were expressed as the ratio of Firefly luciferase to Renilla luciferase activities. Transfection experiments were repeated at least three times.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed as previously described[23](#_ENREF_23" \o "Milne, 2002 #248),[24](#_ENREF_24" \o "Hug, 2004 #249). Brieﬂy, Ec-109 cells were transfected with p3×flag-Sp1or p3×flag-cmv-10 vectors. Transfected for 48 hours, cells were crosslinked with 1% formaldehyde for 10 min at room temperature. Chromatin was sheared using Bioruptor® Plus sonication device (Diagenode, Belgium) to obtain DNA fragments of about 400−600 bp. The anti-FLAG affinity gel (Bimake, Product ID. B23101) was used to pull-down the flag tagged proteins. The chromatin was then de-cross-linked at 65°C overnight with proteinaseK (New England Biolabs, Ipswich, MA). DNA was puriﬁed using MinElute PCR puriﬁcation kit. Puriﬁed ChIP DNA was subjected to PCR, which ampliﬁed *ZNF132* promoter region encompassing the putative Sp1-binding site. Speciﬁc ChIP primers used for PCR were: 5’-CAGCCGAGGAGACAGGCACTT-3’ (forward) and 5’-CCCAGGGA GCCTCCAAGATT-3’ (reverse).

**DNA Pull-down assay**

The DNA pull-down assay was performed according to a previous report[25-27](#_ENREF_25" \o "Audebert, 2004 #250). The promoter region of *ZNF132* gene was amplifed by PCR using 5’-biotin-labeled primer. The primer sequences were as follows: forward primer, CACTTCCGGGCGGAGTGTAAGA, reverse primer, TTCCGTCCCTCGCCTGACAAC. The methylated biotinylated dsDNA were methylated in vitro using M.SssI (CpG) Methyltransferase as recommended by the manufacturer’s instructions (New England Biolabs, Beverly, MA). Cell lysates were extracted from HEK293T cells transiently transfected with p3×flag-Sp1 vector. Cell lysis (400 μg) were incubated biotinylated methylated or unmethylated probes (0.5 pmol) in the presence of streptavidin-agarose beads (Roche, USA) at 4℃ for 2 hours in a microfuge tube on a rotating shaker in the binding buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 100 mM NaCl. Due to the affinity of the streptavidin magnetic beads for biotinylated probe, the DNA-protein complexes were pulled down with streptavidin-agarose beads by centrifugation at 200×g for 60s. The pulled-down complex was washed 3 times with 1 ml ice-cold TBS buffer (20 mM Tris, 150 mM NaCl), separated on an SDS-polyacrylamide gel, and analyzed by western blotting. To quantify the strength of the DNA-protein complex, 20×molar non-biotinylated probe were added to the pull-down mixture as competitors for the biotinylated probe. All western blotting experiments in this study were as described by the DNA Pull-down assay.

**Xenograft tumor mouse model**

All animal experiments were approved by the Soochow University. Four-week-old male BALB/c nude mice (nu/nu; n=7) (Soochow University laboratory animal center, China) were anesthetized with an isoflurane/propylene glycol mixture, and Ec-109 stable cell lines with pCD513B-*ZNF132* or pCD513B were subcutaneously injected into each mouse (2.0×106 cells in 200 μl PBS. The tumor sizes were assessed every three days by measuring two dimensions, and the tumor volumes were calculated as the volume = (tumor length)×(tumor width)2/2[28](#_ENREF_28" \o "Wang, 2011 #254).The tumors were collected and weighed 30 days.

**Statistical analysis**

We tested the differential methylation of the overall CpG sites between cancer and normal tissues using Wilcoxon rank-sum test. We evaluated 15 CpGs located in promoter regions of ZNF132 and only the CpGs in the differential methylation region (DMR) was applied in the further diagnosis or prediction analysis or other statistic analysis. Methylation and gene expression correlation in cancer samples were applied linear regression after log-transform to relative gene expression. Correlation analysis between DNA methylation and age, weight was applied with linear regression while other were applied with one way ANOVA, such as TNM, cancer onset location, gender, smoking as well as drinking. All statistical analyses were conducted using R 3.2.1. GraphPad Prism5 (GraphPad, San Diego, CA, USA) and R scripts were used to make the ﬁgures.

**Results:**

**Hypermethylationof *ZNF132* in esophageal squamous cell carcinoma**

Methylation targeted bisulfite sequencing method (MTBS) was used to determine methylation status in the promoter regions (15 CpG sites) of *ZNF132* in 91 ESCC and adjacent normal tissues from Han Chinese population (Table 1). We found methylation level of LINE-1 was significantly lower in cancer samples compared with normal tissues (overall *P*< 2.16×10-8) while technique negative control (ChrM) was absolutely low average methylation fraction (AMF< 0.03) in our samples (Figure 1A, 1B) which are highly consistent with previous studies[29](#_ENREF_29" \o "Chalitchagorn, 2004 #261),[30](#_ENREF_30" \o "Owa, 2018 #264)demonstrating the reliability and robustness of our targeted bisulfite sequencing method. We found the methylation profile of *ZNF132* were significantly higher in ESCC tumors than that in adjacent control tissues (Figure 1C and 1D). A significant differential methylation region (DMR) including 14 CpG sites was identified in the core promoter region of *ZNF132* (overall *P*-value=2.2×10-16 and Table 2). In addition, we found the promoter methylation were slightly different among different cancer onset location (*P*= 0.07, ANOVA). Samples from up side of esophagus have higher methylation levels (AMF=0.53) compared with middle (AMF=0.39) and down (AMF=0.27). Meanwhile, although TNM is not significant associated with ZNF132 methylation (*P*=0.71), we identified a significant association with number of the number of nearby lymph nodes (“N” value in TNM stage). We did not detect other significant correlation between ZNF132 methylation and age (beta=0.002, *P*=0.395), gender (*P*=0.28), drinking (*P*=0.54), smoking (*P*=0.78) and weight (*P*=0.34). We also examined the prediction performance *ZNF132* hyper-methylation in ESCC diagnosis. We built the most acceptable prediction model with logistic regression and we found the model has quite good sensitivity (sensitivity=70.8%), specificity (specificity=80.6%) and area under curve (AUC=0.82) with the adjustment of ESCC main risk factors such as age, gender, smoking and alcohol consumption, demonstrating hyper-methylation of *ZNF132* could be taken as a strong diagnostic biomarker (Table 2 and Figure 1E).

**Regulation of *ZNF132* expression by methylation of its promoter in ESCC patients and esophagus cancer cell lines**

As methylation of gene promoter regions is a well-known gene expression regulation mechanism, we first examined the expression of *ZNF132* in 91 pairs of tumor and adjacent control tissues from ESCC patients. Quantitative Real-time PCR was used to evaluate the expression level of *ZNF132* in the samples. The results demonstrated a significantly higher level of *ZNF132* expression in adjacent control tissues than that in ESCC tissues (Figure 2A), indicating that the expression profile of *ZNF132* in ESCC tissues was altered as a consequence of its promoter hyper-methylation in ESCC patients. The expression-methylation regression analysis methylation and gene expression correlation in clinical samples show gene expression of *ZNF132* was significantly negative correlated with DNA methylation level in cancer clinical samples (P=0.00284) (Figure 2B).

To confirm the relation between *ZNF132* methylation and its expression observed in ESCC patients tissues, two esophagus cancer cell lines (Ec-109, CaEs-17) were treated with demethylation reagent 5-Aza (5-aza-2’-deoxycytidine). *ZNF132* promoter methylation levels of two lines decreased significantly after treatment (Figure 2C), and at the same time *ZNF132* expression level significantly increased (Figure 2D).

The results clearly established that methylation status of *ZNF132* negatively regulates its expression. The fact that epigenetic treatment modulates ZNF expression shows its potential as a epigenetic cancer therapy.

**Effects of *ZNF132* expression on cancer cell characteristics of esophagus cancer lines in vitro**

The results described above raises the possibility that *ZNF132* functions as a tumor suppressor in ESCC. To determine the effects of *ZNF132* protein on characters of ESCC cells we constructed pCD513B-*ZNF132* plasmid and use empty pCD513B as a control. Expression of *ZNF132* measured by q-PCR and western blotting in Ec-109 cells and CaEs-17 cells (Figure 3A, 3C). The effects of high expression of *ZNF132* were then tested on cells’ ability in growth, migration, invasion and apoptosis. Cell growth was monitored daily. Growth of pCD513B-*ZNF132* cells was slower than pCD513B cells. Starting at day 3 the growth rates between experiment and control groups became significant (Figure 3B, 3D). In Vitro Scratch Healing Experiment showed that high expression of *ZNF132* in Ec-109 cells and CaEs-17 cells significantly inhibits cells healing ability (Figure 3E, 3F). The abilities of the cells in migration were also negatively affected by the presence of pCD513B-*ZNF132* (Figure 3G). These results indicate that *ZNF132* plays an inhibitory role in the growth, migration and invasion of esophageal squamous carcinoma cells. Besides cells abilities of growth, migration and invasion, abnormal pattern of tumor cell apoptosis also plays a role in tumor growth and metastasis. Percentage of apoptotic Ec-109 and CaEs-17 cells significantly increased by high expression of *ZNF132* (Figure 3H). The results show that higher expression *ZNF132* greatly reduced tumorigenecity of ESCC cells in vitro.

**Reduction of tumorigenecity of ESCC cells by enforcing *ZNF132* expression in a in vivo xenograft model**

To investigate whether *ZNF132* gene functions as tumor suppressor also in vivo, we established xenograft model. Ec-109 cells transfected with either the plasmid pCD513B-*ZNF132* or pCD513B were inoculated in to BALB/c nude mice. On day 33, the mice were sacrificed, the volumes and wet weights of the tumors were measured individually.

The tumor sizes of pCD513B-*ZNF132* group were visually smaller than pCD513B group (Figure 4A). There were significant differences in tumor volume and wet weight between experiment and control groups (Figure 4B, 4C) while there was no significant difference in body weight of mice between two groups during the experiment (Figure 4D). We further examined the protein expression level of ZNF132 in tumor tissues by Western blotting, and found that ZNF132 was expressed in a subcutaneous injection of ZNF132 stable cell line(Figure 4E). No abnormal daily food and water consumption, and other adverse effects, such as mental state and hematuria were observed. The xenograft study suggests that *ZNF132* plays a role as tomor suppressor gene in preventing ESCC in vivo.

**Methylation of sp1-binding site inhibits *ZNF132* expression at transcriptional level**

In order to demonstrate the ZNF132 gene regulation mechanism, we tried to investigate the transcriptional factors binding status in the ZNF132 promoter region. We found the 6-base-pair (GGGCGG) SP1-binding motif located in the CpG-island of ZNF132 promoter region and this binding region was supported by ENCODE Transcription Factors ChIP-seq data (Supplementary Figure 1). We then try to determine whether methylation of Sp1-binding site play a role in ZNF132 expression regulation. According to the published sequence of *ZNF132* promoter, luciferase reporter constructs was generated and transiently transfected together with increasing doses of Sp1 expression vector into HEK293T cells. The results showed that the transcriptional activity of *ZNF132* promoter were elevated with increasing doses of Sp1, suggesting that the Sp1 may transcriptionally modulate *ZNF132* expression (Figure 5A).

To determine whether methylation of Sp1-bining site alters the transcriptional activity of *ZNF132* promoter by derecruiting Sp1, we generated unmethylated luciferase reporter constructs containing the unmethylated fragments (Sp1-binding sequence). The methylated reporter construct were methylated in vitro using SssI (CpG) Methyltransferase.The results showed that the methylated Sp1-binding site dramatically led to a reduction of luciferase activity compared with the unmethylated one, suggesting that methylation of Sp1-bining site can inhibit *ZNF132* transcriptional expression by interfering with the recruitment of Sp1 to *ZNF132* promoter region (Figure 5B).

To determine directly whether Sp1 binds to *ZNF132* promoter, ChIP assay was performed. Using ChIP DNA purified from cultured cells transfected with p3×flag-Sp1 vector, the results of PCR, which ampliﬁed *ZNF132* promoter region encompassing the putative Sp1-binding site, showed a clear band while no such band was seen if the cells transfected with p3×flag-cmv-10 vectors were used (Figure 5C). This clearly demonstrates that Sp1 can bind to the *ZNF132* promoter region in live cells cultured in vitro.

The DNA pulldown assays was used to confirm transcriptional activator Sp1 could bind to promoter region of *ZNF132* gene and methylation status of *ZNF132* promoter negatively affects the binding. DNA pull-down assays showed that the methylated Sp1-bining site probe had weaker binding ability with Sp1 proteins compared with the unmethylated Sp1-bining site probe (Figure 5D).

As the DNA methylation abnormal theoretically caused by the harmful environment and genetic variants exposure, such as smoking, drinking as well as risk allele carrying and it might be different environment triggers since they contact different environment. Since DNA methylation can be reversed by the appropriate treatment, DNA methylation is considered to be promising diagnosis and prognosis biomarker. Combined with our results described above, the results revealed, at least in part, the mechanism underlying the association of hyper-methylation of *ZNF132* promoter region and ESCC. Methylation of Sp1-bining site prevents the transcriptional activator Sp1 from binding to *ZNF132* promoter, silencing *ZNF132* tumor suppressor gene.

**Discussion**

ESCC is a complex disease caused by different aberrant changes such as epigenetic, genetic and environmental interactions[31](#_ENREF_31" \o "Talukdar, 2013 #385). Since the worse prognosis, early and accuracy diagnosis provide important approach to decrease the mortality. In the past decades, DNA methylation has been demonstrated to be promising early diagnostic biomarker for ESCC, however only quite limited number of DNA methylation markers for early detection, recurrence and prognosis have been identified in ESCC[32](#_ENREF_32" \o "Abbaszadegan, 2005 #259),[33](#_ENREF_33" \o "Liu, 2011 #260). Further, there are not many studies focusing on mechanisms under which epigenetic changes in tumor suppressor gene promoter regions lead to human ESCC initiation and progression.

In this study we show that *ZNF132* gene is silenced in ESCC tumor tissues, but not in adjacent control tissues in paired tissue samples from ESCC patients. In ESCC tumor tissue, the *ZNF132* gene is hyper-methylated in its promoter region. The epigenetic changes in *ZNF132* in ESCC patients samples have been determined by targeted bisulfite sequencing. Methylation status of *ZNF132* promoter region is significantly higher in ESCC tissue than in adjacent control tissue. *ZNF132* expression at RNA level, consistent with its methylation status, is significantly lower in ESCC cells indicating possible tumor suppressor function of *ZNF132*. These results have led us to further explore the clinical value of hypermethylation of *ZNF132* promoter. Logistic regression analysis revealed hypermethylated *ZNF132* is strongly associated with ESCC after adjustment for age, sex, smoking and alcohol consumption. The logistic regression model was also used to evaluate the prediction ability of hypermethylation status of *ZNF132* promoter. Analysis results, sensitivity, specificity and AUC with adjustment for age, sex, smoking and alcohol consumption, indicate moderate prediction ability of the test. Taken together, *ZNF132* hypermethylation is an independent diagnostic factor to together with other risk factors, such as age, gender, smoking and drinking.

To our knowledge so far, there have never been any studies of ZNF132 in ESCC, actually there is only one report on the role of ZNF132 in human cancer, demonstrating the significant inverse correlation between methylation level of ZNF132 and its protein expression in prostate cancer patients tissue samples [17](#_ENREF_17" \o "Abildgaard, 2012 #189). Consistent with our study in ESCC patients, their results also illustrate that ZNF132 have the potential to be a new candidate methylation marker for prostate cancer. The role of methylation promotor and expression of ZNF132 were analysed in in vitro study with EC cell lines. Two EC cell lines showed significant decreased ZNF132 methylation accompanied by increased expression of ZNF132 after demethylation reagent 5-Aza treatments, demonstrating directly the inverse relationship between promoter methylation status and expression of ZNF132 in ESCC. The results indicate the potential of demethylation drugs as a epigenetic cancer therapy. The function of ZNF132 was then studied in ESCC lines. Over-expression of ZNF132 in ESCC cells greatly reduced the abilities of cells in in growth, migration and invasion, and significantly increased apoptotic cell death illustrating in vitro the tumor suppression function of ZNF132. The effect of ZNF132 overexpression was also studied in vivo with a nude mouse model. The tumorigenicity of EC cells with overexpressed ZNF132 is significantly reduced, therefore confirming the above in vitro results. Our study is the first one to show both in vitro and in vivo the tumor suppression function of ZNF132 indicating the pathological importance of reducing ZNF132 expression by hypermethylation of its promoter region. The underlying mechanism of the effect of methylation status of ZNF132 promoter on its expression was explored. Sp1 is a zinc finger protein that belongs to the SP family of transcription factors. The canonical sequence of the Sp1-binding site is 5'-(G/T)GGGCGG(G/A)(G/A) containing GpC in the promoter region[34](#_ENREF_34" \o "Solomon, 2008 #386). Binding of Sp1 to a target gene can be interrupted by DNA methylation, resulting in silencing of gene expression. Sp1 is a ubiquitous transcriptional activator that is involved in a variety of biological processes, including cell proliferation and progression[35](#_ENREF_35" \o "Wang, 2017 #387). However, the role of Sp1 in human cancer remains elusive. Sp1 is thought to be a promoter or repressor of cell proliferation and progression[36](#_ENREF_36" \o "Xie, 2018 #388),[37](#_ENREF_37" \o "Fauquenoy, 2017 #389). CpG was in silico predicted to be harbored in Sp1-binding site at ZNF132 promoter. It was first in this study demonstrated that Sp1 can bind to promoter region of ZNF132, and then that the methylated site prevent Sp1 from binding to the promoter. The mechanism of promoter methylation effects on gene expression is very complicated. However our results imply that preventing of Sp1 binding to ZNF132 promoter region by hypermethylation may be one of the mechanisms of reduced ZNF132 expression in ESCC.

In conclusion, our study for the first time demonstrated that ZNF132 promoter is hypermethylated in ESCC tissues, but not in adjacent control tissues. The effects of the epigenetic change and expression ZNF132 on tumorigenecity of EC cell lines were investigated both in in vitro and in vivo. Preventing Sp1 from binding to ZNF132 promoter was shown to be at least one of the underlying mechanisms. Most important, the methylation status of ZNF132 promoter in ESCC patients tumor tissues is an independent prognostic factor, and has potential use as a biomarker useful in prognosis of ESCC.

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**Conflict of interest**

The authors declare no potential conflict of interest.

**Figure and Table Legends**

**Figure 1. Hypermethylationof ZNF132 in esophageal squamous cell carcinoma**

**A．**Median % methylation values of 13 CpG site of LINE-1. **B.** Median % methylation of 11 CpG sites of ChrM. **C.** Median % methylation in ESCC and adjacent control tissues of 15 CpG sites of ZNF132 promoter region (except the last CpG which is hypermethylated in both normal and cancer samples). DMR represents differentially methylated regions and CpGI represents CpG island. **D.** The methylation of ZNF132 in the 91 cases of ESCC tissues and adjacent tissues (each point represents the absolute ratio of methylation in each tissue) **E.** Represents the overall ROC (Receiver Operating characterstics) curve, which was calculated through a logistic regression model, incorporating the mean methylation percentage of the five genomic regions as the variables, and with the adjustment for gender, age, smoking and alcohol consumption.

**Figure 2. Methylation status and gene expression of *ZNF132* in ESCC patients and esophageal cancer cell lines. A.** methylation and gene expression correlation in clinical samples. Y-axis is log-transferred relative expression same with 4A; x-axis represents average methylation level. Dot line indicates the linear regression line. **B.** Expression of *ZNF132* measured by q-PCR in ESCC tissues was significantly lower than that in adjacent tissues. **C.** Methylation of *ZNF132* in Ec-109 and CaEs-17 was significantly reduced after 5-Aza treatment. **D.** Expression of *ZNF132* measured by q-PCR significantly increased after 5-Aza treatment. Data are presented as the mean±S.D. of three independent experiments. \*, *P*<0.05, \*\*\**P*<0.001.

**Figure 3. The effects of high expression *ZNF132* on characteristics of esophageal cancer cell lines in vitro. A,C.** **B,D.** by Cell Proliferation Assay. Light absorbance measurement at a wavelength of 450 nm was recorded to show **E,F.** In Vitro Scratch Healing Experiment showed that high expression of *ZNF132* in Ec-109 cells and CaEs-17 cells significantly inhibits cells healing ability. **G.** The up-regulation of *ZNF132* gene in Ec-109 cells and CaEs-17 cells reduced cell migration ability in a transwell assay. **H.** Flow cytometry demonstrates that upregulation of *ZNF132* in CaEs-17 and Ec-109 cells could significantly increase the cell apoptosis rate. Data are presented as the mean±SD. \**P*<0.05, \*\*\**P*<0.01, \*\*\**P*<0.001.

**Figure 4. Over-expression of *ZNF132* inhibits the growth of human esophageal squamous cell carcinoma in vivo in a mouse xenograft model. A.** The tumor volume of pCD513B-*ZNF132* group was visually smaller than that of pCD513B group. **B.** difference in tumor volume between two groups increased with passing days.\*\*\**P*<0.001. **C.** Wet tumor weight of experiment group was significantly lighter than control group. \*, *P*<0.05. **D.** There was no significant difference in body weight between the experimental group and the control group during the whole experiment. E. Western blotting results showed that ZNF132 was expressed in a subcutaneous injection of ZNF132 stable cell line.

**Figure 5. Hypermethylation of trascriptional activator Sp1 binding site in *ZNF132* promoter region leading to *ZNF132* gene silencing in esophageal cell line. A.** Transcriptional activity of *ZNF132* promoter elevates with increasing doses of Sp1. Data are presented as the mean±SD. \*\*\**P*<0.001. **B.** Trascriptional activity was significantly reduced by methylation of Sp1 site of *ZNF132* promoter. Data are presented as the mean±SD. \*, *P*<0.05, \*\*\**P*<0.001. **C.** ChIP assay was repeated three times. ChIP assay showed directly that Sp1 protein can bind to *ZNF132* promoter region containing Sp1 site in in vitro cultured cells cells. **D.** DNA pull-down assay was repeated three times. DNA pull-down assay showed that the methylated Sp1-bining site probe had weaker binding ability with speciﬁc proteins compared with the unmethylated Sp1-bining site probe.

**Supplementary Figure 1**. ENCODE ChIP-seq data to show SP1 bindings in CpG-island of ZNF132 promoter region. Integrated Regulation from ENCODE Tracks based on hg19 was captured from UCSC Genome Browses. Transcription Factor ChIP-seq (161 factors) from ENCODE with Factorbook Motifs  Data version: ENCODE Mar 2012 Freeze was used in this study.