**ZNF132 promoter hypermethylation as a potential biomarker for esophageal squamous cell carcinoma (ESCC)**

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**Abstract**

**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 andmortality produced by the International Agency for Research on Cancer (IARC), 455,800 new esophagealcancer cases and400,200 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. The hypermethylatedstatus of promoters 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 pMD2G and 1.2 μg pCD513B-ZNF132expression plasmid. one day before infection, CaEs-17 or Ec-109 cells were seeded in 6-well plate with the density of 2×105cells 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 extraction, bisulfite conversion and targeted bisulfite sequencing**

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 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, sequenced with the Illumina Hiseq 2000 platform according to the manufacturer's protocols. BSseeker2 software was utilized for reads mapping and methylation calling. Samples and CpG sites with high missing rates (>30%) were removed.

**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 Table 2. 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 1000 cells/well. Then 10 µL CCK8 (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 /ml, 200μL 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 500μL 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 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/PI. 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[[20](#_ENREF_20" \o "Zhang, 2016 #247)]. The fragment in ZNF132 promoter region (chr19:58951628-58951928), was cloned to pGL3-Basic vector (Promega, Madison, WI, USA) 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-promoter or 200 ng methylated pGL3-ZNF132-promoter 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 0,500ng Sp1 expression vector and 200 ng pGL3-ZNF132-promoter or 200 ng methylated pGL3-ZNF132-promoter 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.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed as previously described[[21](#_ENREF_21" \o "Milne, 2002 #248), [22](#_ENREF_22" \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 ZNF132promoter region encompassing the putative Sp1-binding site. Speciﬁc ChIP primers used for PCR were:5’-C AGCCGAGGAGACAGGCACTT-3’ (forward) and 5’-CCCAGGGA GCCTCCAAGATT-3’ (reverse).

**DNA Pull-down assay**

The DNA pull-down assay was performed according to a previous report[[23-25](#_ENREF_23" \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, 5’-CACTTCCGGGCGGAGTGTAAGA-3’, reverse primer, 5’-TTCCGTCCCTCGCCTGACAAC-3’. The methylated biotinylated dsDNA were methylated in vitro using 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 mMTris-HCl(pH 7.5), 1 mM EDTA and 100 mMNaCl. 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 mMTris, 150 mMNaCl), 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.

**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 of 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[[26](#_ENREF_26" \o "Wang, 2011 #254)].The tumors were collected and weighed 30 days.

**Statistical analysis**

We tested the differential methylation of the CpG sites between cancer and normal tissues using Wilcoxon rank-sum test. Methylation and gene expression correlation in cancer samples were applied linear regression after log-transform to relative gene expression. 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**

Targeted bisulfite sequencing method was used to determine methylation status of ZNF132 in ESCC tissues. Fifteen CpG sites in the promoter region of ZNF132 were sequenced in 94 paired tumor and adjacent normal tissues (Table 1).21 CpG sites located at the promoter of LINE-1 gene (Hypermethylation 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 Table 2. As shown in Figure 1, the methylation percentage of ZNF132 were significantly higher in ESCC tumors than that in adjacent control tissues on 14 tested CpG sites in the paired tissues of 94 ESCC patients (Figure 1A). The results are also summarized in Table 3 showing a significant association of between ZNF132 methylation and ESCC (P=8.71×10-14). In consistence with results reported[[27](#_ENREF_27" \o "Chalitchagorn, 2004 #261)], 13 sites of LINE-1, as a positive control, also showed significantly higher methylation percentage in tumor tissues than that in adjacent normal tissues, while 11 sites of ChrM , a negative control, show no difference (Figure 1B, 1C, and Table 3), demonstrating the reliability and robustness of our targeted bisulfite sequencing method. The results of 14 CpG sites in 94 paired tissues were individually shown in Figure1D indicating a significant higher average methylation level in ESCC tissues. The results described above shows clearly the association of hyper-methylation of ZNF132 promoter and ESCC.

To examine the clinical diagnostic value of hyper-methylation of ZNF132, the methylation status was evaluated in ESCC cancer and adjacent normal samples. We found the methylation was significantly increased in ESCC cancer samples compared with corresponding normal control (P-value =8.71×10-14). To evaluate the prediction ability, sensitivity, specificity and area under curve (AUC) were calculated with a logistic regression prediction model. As shown in Table 3 and Figure 1E, sensitivity, specificity and area under curve (AUC) are 77.53%, 80.43% and 0.83 respectively without adjustment for the risk factors of age, gender, alcohol, smoking and stage illustrating strong prediction ability.

**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 94 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.The results show gene expression of ZNF132 was significantly negative correlated with DNA methylation level in cancer clinical samples (P-value=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, measured by qRT-PCR, 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. Ec-109 cells and Caes-17 cells are transfected by the plasmid pCD513B-ZNF132 or pCD513B. 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 pCD-513B cells. Starting at day 3 the growth rates between experiment and control groups became significant (Figure 3A). 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 3B). The abilities of the cells in migration were also negatively affected by the presence of pCD513B-ZNF132(Figure 3C,3D). 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 andCaes-17 cells significantly increased by high expression of ZNF132 (Figure 3E,3F). 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 a xenograft model. Ec-109 cells transfected with either the plasmid pCD513B-ZNF132or pCD513B were inoculated in to BALB/c nude mice. On day 30, 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). 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**

As CpG was in silico predicted to be harbored in transcriptional activator Sp1-binding site at ZNF132 promoter, 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 (Figure5C). 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).

Combined with our results described above, the results revealed, at least in part, the mechanism underlying the association of hypermethylation of ZNF132 promoter region and ESCC. Methylation of Sp1-bining site prevents the ranscriptional activator Sp1 from binding to ZNF132 promoter, silencing ZNF132 tumor suppressor gene.

**Discussion**

ESCC is one of the most aggressive forms of cancer. Consumption of tobacco and alcohol are two major risk factors in ESCC carcinogenesis[[28](#_ENREF_28" \o "Nasrollahzadeh, 2008 #190)]. Tobacco smoking is associated with promoter region hyper-methylation in group tumor suppressor genes of human ESCC[[29](#_ENREF_29" \o "Oka, 2009 #191)]. Alcohol consumption has a stronger association with ESCC than other human cancers, and is also associated with hypermethylation of tumor suppressor gene promoter region in human esophageal cancer[[30](#_ENREF_30" \o "Islami, 2011 #257), [31](#_ENREF_31" \o "Toh, 2010 #193)]. Increasing, but still 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)]. However, 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 hypermethylated 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. Significant association between overall survival and methylation status was observed. 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 without adjustment for age, sex, smoking, alcohol consumption and stage, indicate moderate prediction ability of the test. Taken together, ZNF132 hypermethylation is an independent factor to predict overall survival regardless of other risk factors, such as age, sex, or stage etc.

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. Overexpression 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 overexpressied 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 transcriptional activator. 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 mechanisms 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.

**Table 1. Clinical Characteristics of the Study Population**

|  |  |  |  |
| --- | --- | --- | --- |
| characteristics |  | Patient distribution | |
|  | N=94 | | % |
| Age(years) |  | |  |
| <60 | 31 | | 33.0 |
| ≥60 | 63 | | 67.0 |
| Age(mean ±SD) | 63.31 ±8.55 | |  |
| Gender(n) |  | |  |
| Male | 69 | | 73.4 |
| Female | 25 | | 26.6 |
| Drinking |  | |  |
| Yes | 35 | | 37.2 |
| No | 57 | | 60.6 |
| Unknown | 2 | | 2.2 |
| Smoking |  | |  |
| Yes | 58 | | 61.7 |
| No | 36 | | 38.3 |
| Stage |  | |  |
| 0-II | 46 | | 48.9 |
| III-IV | 46 | | 48.9 |
| Unknown | 2 | | 2.2 |

**Table 2：Sequences of primers used in this study**

|  |  |  |  |
| --- | --- | --- | --- |
| Primer | Target | Sequence, 5’-3’ | Use |
| ZNF132 F | Coding region | GTCATTGAGAGGCGGGACT | qPCR |
| ZNF132 R | Coding region | TCGGGAACACCTTGGCTCAT | qPCR |
| ZNF132 Xba I | CDS sequence | GCTCTAGAATGGCCCTGCCCAGC | PCR |
| ZNF132 Not I | CDS sequence | ATAAGAATGCGGCCGCTCAGGTATGAATCTT | PCR |
| GAPDH F | Coding region | GAAGGTGAAGGTCGGAGTC | qPCR |
| GAPDH R | Coding region | GAAGATGGTGATGGGATTTC | qPCR |
| ZNF132 F | Promoter region | GGTGTTTTAGGGTTGGTTATTGG | BSP |
| ZNF132 R | Promoter region | TACCTTCCTCRCTCCTATTTCCATAA | BSP |
| LINE-1 F | Positive control | AGTAGGGYGAGGTATTGTTTTATTTG | BSP |
| LINE-1 R | Positive control | AAACTACTATACTAACAATCAACARAATTCC | BSP |
| ChrM F | Negtive control | TGTGTGGAAAGTGGTTGTGTAGATATT | BSP |
| ChrM R | Negtive control | AATCACAAATCTATCACCCTATTAACCA | BSP |

**Table 3 The methylation of ZNF132 gene and control gene in ESCC**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Gene name | Mean（Case） | Mean（Control） | P valuea | OR（95%CI） | P valueb | Sensitivity | Specificity | Area Under Curve（AUC） |
| ZNF132 | 0.40 | 0.16 | 8.71×10-14 | 3.53（2.51,4.74） | 2.36×10-9 | 77.53% | 80.43% | 0.83 |
| LINE-1 | 0.55 | 0.73 | 2.16×10-8 |  |  |  |  |  |
| ChrM | 0.03 | 0.02 | 2.59×10-1 |  |  |  |  |  |

**Figure and Table Legends**

**Figure 1. Methylation status of ZNF132 in ESCC and adjacent control tissues**

**A.**Median % methylation in ESCC and adjacent control tissues of 15 CpG sites of ZNF132 promoter region. **B.** Median % methylation values of 13 CpG site of LINE-1. **C.** Median % methylation of 11 CpG sites of ChrM.

**Figure 2. The methylation of ZNF132 in the tissues of the ESCC detected by Methyl target and ROC curves. A.** The methylation of ZNF132 in the 94 cases of ESCC tissues and adjacent tissues (each point represents the absolute ratio of methylation in each tissue) **B.** 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 without the adjustment for gender, age, smoking status and alcohol status.

**Figure 3.Methylation status and gene expression of ZNF132 in ESCC patients and esophageal cancer cell lines.**

**A.** Expression of ZNF132 measured by q-PCR in ESCC tissues was significantly lower than that in adjacent tissues. **B.** 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. **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.

**Figure 4. The effects of high expression ZNF132 on characteristics of esophageal cancer cell lines in vitro.**

**A.** Effect of ZNF132 gene on the proliferation of esophageal cancer cells. The results of cell proliferation analysis showed that the up-regulation of ZNF132 gene could inhibit the proliferation of CaEs-17 and Ec-109 cells. **B.**In Vitro Scratch Healing Experiment showed that high expression of ZNF132 in Ec-109 cells and CaEs-17 cells significantly inhibits cells healing ability.**C.** The up-regulation of ZNF132 gene in Ec-109 cells and CaEs-17 cells reduced cell migration ability in a transwell assay.**D.**Flow cytometry demonstrates that upregulation of ZNF132 in CaEs-17 and Ec-109 cells could significantly increase the cell apoptosis rate.

**Figure 5. 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. **C.** Wet tumor weight of experiment group was significantly lighter than control group. **D.** There was no significant difference in body weight between the experimental group and the control group during the whole experiment.

**Figure 6.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. **B.**Trascriptional activity was significantly reduced by methylation of Sp1 site of ZNF132 promoter. **C.**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 showed that the methylated Sp1-bining site probe had weaker binding ability with speciﬁc proteins compared with the unmethylated Sp1-bining site probe.