ZSCAN18 Promoter is Hypermethylated and Its Restored Expression Inhibits Proliferation in Gastric Cancer

Baoqing Ren

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Bin Li (✉ binli@tmu.edu.com)

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

<https://orcid.org/0000-0003-0516-0997>

Gang Ma

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Fenglin Cai

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Pengliang Wang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Yinping Dong

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Yi Zeng

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Yong Liu

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Li Zhang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Yang Yang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Han Liang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Nannan Zhang

Air Force Medical University Xijing Hospital: Xijing Hospital

Yongzhan Nie

Air Force Medical University Xijing Hospital: Xijing Hospital

Jingyu Deng

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Keywords: ZSCAN18, methylation, gastric cancer, carcinogenesis, proliferation

Posted Date: December 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1120023/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Zinc finger and scan domain containing 18 (ZSCAN18) belongs to the zinc finger transcription factor superfamily, which consists of hundreds of members that play critical roles in all steps of tumorigenesis. Although hypermethylation of the ZSCAN18 promoter and its consequent deficiency in a variety of malignancies have been reported recently, its functions and mechanisms in the initiation and progression of gastric cancer (GC) are not clear. This study aims to investigate the roles of ZSCAN18 in gastric carcinogenesis.

Methods: Methylation of ZSCAN18 promoter in GC cell lines was analyzed *via* MassARRAY and treatment with 5-aza-2'-deoxycytidine. Bioinformatics analysis, quantitative real-time polymerase chain reaction (qRT-PCR), western blot, immunohistochemistry, Cell Counting Kit 8, colony formation, and an *in vivo* xenograft tumor model were used to examine the expression and function of ZSCAN18. Survival analysis was performed to determine the correlation between ZSCAN18 expression and the prognosis of patients with GC. Genes modulated by ZSCAN18 in NCI-N87 cells were identified through RNA next-generation sequencing and verified by qRT-PCR in AGS and NCI-N87 cells.

Results: ZSCAN18 expression was markedly reduced in GC tissues compared with adjacent normal tissues as a result of hypermethylation in GC. Likewise, ZSCAN18 expression was significantly reduced in a panel of GC cell lines as a result of the densely methylated ZSCAN18 promoter. Forced expression of ZSCAN18 inhibited proliferation in AGS and NCI-N87 cells *in vitro*. Additionally, ZSCAN18 impaired the growth of NCI-N87 cells in immunodeficient mice. Survival analysis showed that ZSCAN18 expression was positively associated with favorable outcomes in patients with GC. RNA next-generation sequencing showed three representative genes (FGF20, CEACAM5, and TP53INP2) involved in downstream signaling pathways modulated by ZSCAN18.

Conclusions: Collectively, this study unveils the anti-cancer role of ZSCAN18 in GC and provides a promising diagnostic and therapeutic target.

1 Background

Gastric cancer (GC), as a complex heterogeneous disease, is the fifth most common cancer and the third most common cause of cancer deaths globally [1–3]. Although diagnostic and therapeutic strategies for advanced GC have improved significantly in recent years, the treatment remains unsatisfactory, and the prognosis remains poor. Therefore, the molecular mechanisms of GC pathogenesis should be elucidated to explore potential therapeutic targets for clinical use.

It has already been confirmed that nonmutation events, such as epigenetic dysregulation, play substantial roles in carcinogenesis [4–6]. Aberrant promoter DNA hypermethylation is a well-characterized epigenetic hallmark and leads to a series of malignant tumors. Hypermethylation of CpG islands in the promoter region usually results in inactivation of tumor-related genes, affecting human cells via subsequent dysfunctional transcription. Different genes are frequently methylated in GC, but few

of them may be used as cancer biomarkers to detect disease or predict therapeutic efficacy [7–10]. In this regard, the identification of novel genes silenced by promoter methylation may provide new insights to determine potential diagnostic markers and therapeutic targets for GC.

Zinc finger (ZNF) transcription factors (TF) represent the largest TF superfamily, and its members play critical roles in all aspects of cellular processes. Zinc finger and scan domain containing 18 (ZSCAN18) is a ZSCAN TF (a subfamily of ZNF TFs). ZSCAN18 is also known as ZNF447 and is located in chromosome 19q13.43. Recently, hypermethylation in the promoter of ZSCAN18 has been identified in various types of cancers, such as esophageal cancer [11], renal cell carcinoma [12], cholangiocarcinoma [13, 14], colorectal cancer [15, 16], gastric cancer [16–18], hepatocellular carcinoma [18], and pancreatic cancer [16–18]. Low protein expression of ZSCAN18 is reported in these malignancies, especially in gastrointestinal cancers. These findings strongly suggest that ZSCAN18 exerts anti-tumor effects. However, the mechanisms and functions of hypermethylated ZSCAN18 in GC remain poorly understood.

In this study, we analyzed the epigenetic regulation and functions of ZSCAN18 and revealed that promoter hypermethylation contributes to low ZSCAN18 expression in GC. We also confirmed that ZSCAN18 depletion inhibited the proliferation of GC cells, both *in vitro* and *in vivo*. Most importantly, we showed that prognostic outcomes correlated with ZSCAN18 hypermethylation in GC.

2 Materials And Methods

2.1 Data availability

The mRNA expression data and methylation data were obtained from the Gene Expression Omnibus (GEO) database with the login numbers GSE33335 and GSE30601.

2.2 Tissue sample collection and follow-up

The tissues used for immunohistochemistry (IHC) were composed of 83 pairs of tumor and adjacent non-tumor tissues that were collected from patients with GC who underwent curative gastrectomy at Tianjin Medical University Cancer Hospital (Tianjin, China) between January 2004 and August 2007. All patients underwent standard follow-up after curative surgery. The median follow-up time was 26 months (range: 3–70 months). The tissue used for the MassARRAY methylation test consisted of 97 cancerous tissues and 6 paracancer tissues from patients undergoing radical gastrectomy between January 2003 and July 2007 at Tianjin Medical University Cancer Hospital (Tianjin, China), of which 97 cancerous tissues were closely observed. The median follow-up time was 18 months (range: 2–85 months). The clinical data included the patient's age, sex, endoscopy result, chest X-ray, B-mode ultrasound, and surgical methods. Between August and November 2019, we collected 30 pairs of GC and adjacent non-tumor tissues for RNA extraction. The study was conducted with patient consent. The experimental study protocol and the use of clinical data were approved by the Ethics Committee of the Cancer Institute of Tianjin Medical University and the Cancer Hospital of Tianjin Medical University (Tianjin).

2.3 Cell culture and transfection

The cell lines of MKN45, NCI-N87, BGC-823, MGC-803, SGC-7901, SNU-1, KATO-III, HGC-27, and GES-1 were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% antibiotics (penicillin/streptomycin) (Gibco). AGS cells were cultured in F12k (Gibco) with 10% FBS and 1% antibiotics. The HEK293T cell line was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) with 10% FBS and 1% antibiotics. Cells were maintained at 37°C with a 5% CO₂ atmosphere. The expression level of ZSCAN18 was upregulated by a plasmid (pLVX-IRES-Puro-ZSCAN18), and an empty vector was transfected into the cells (pLVX-IRES-Puro-vector). Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) were used to verify the transfection efficiency.

2.4 Immunohistochemistry

We prepared 83 paraffin-embedded primary gastric tumor tissues and adjacent non-tumor tissues. The ZSCAN18 antibody concentration was 1:150. The staining intensity of ZSCAN18 was estimated by three independent pathologists without knowledge of the clinical data. The staining intensity was divided into 0 (negative), 1 (weak), 2 (medium), and 3 (strong) ranks. Zero and 1 were considered negative; 2 and 3, positive.

2.5 Western blot

The western blot experiment was conducted in accordance with standard procedures. The primary antibodies used for this research were the ZSCAN18 antibody (TA505326, OriGene), VINCULIN (ab219649, Abcam), AURORA (ab52973, Abcam), GEMINI (ab195047, Abcam), and LAMIN-B1 (660951-1-AP, Proteintech).

2.6 Cell viability assay

Cell viability was detected using a Cell Counting Kit 8 (CCK-8) reagent. After the cell count, cells were placed in a 96-well culture plate; the AGS cell line had 1×10^3 cells per well, and the NCI-N87 cell line had 3×10^3 cells per well. Testing started 2 h after the addition of 10 µl of the CCK-8 reagent. AGS was tested once a day, and NCI-N87 was tested once every other day.

2.7 Colony formation assay

The colony-forming method was used to detect the proliferation of GC cells. We conducted experiments on AGS and NCI-N87 cell lines using 1000 AGS cells per well and 8000 NCI-N87 cells per well. After the cell count, the cells were inoculated into 6-well plates and incubated at 37°C for 12 to 14 days. Cell fluid was changed periodically until a visible clone formed.

2.8 Animal experiment

Ten 4-week-old female BALB/C nude mice (Vital River) were purchased to construct a subcutaneous tumor xenograft. A total of 3×10^6 overexpressed ZSCAN18 and control NCI-N87 cells were injected into the lateral dorsal subcutaneous flank of nude mice. Xenograft tumor formation was measured every 4 days. Three weeks after injection, the nude mice were euthanized by cervical-dislocation sacrifice after

intraperitoneal injection of 2% pentobarbital sodium (0.5 mL), and the tumor volume (V) was measured by the formula: $V = 1/2 \times \text{length} \times (\text{width})^2$. Tumor tissues were excised for immunohistochemistry.

2.9 5-Aza-2'-deoxycytidine treatment

AGS and NCI-N87 cells were laid 12 h in advance, and the cell density was approximately 30% on the next day. Cells were incubated with a concentration of 2 μM of 5-aza-2'-deoxycytidine (DAC, Sigma, St. Louis, MO). The liquid was changed every 24 h. The treatment lasted for 96 h.

2.10 RNA sequencing and analysis

NCI-N87 cells were stably transfected with PLVX-IRES-Puro-ZSCAN18 or empty vector (PLVX-IRES-Puro). RNA from total samples was isolated and purified using TRIzol (Invitrogen, CA, USA). Then, NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA) was used to control the quantity and purity of total RNA. The captured mRNA was fragmented at 94°C for 5 to 7 min using a magnesium ion fragmentation kit (NEBNext® RNA Fragmentation Module, article no. E6150S, USA). cDNA was synthesized from the segmented RNA using Invitrogen SuperScript™ II Reverse Transcriptase (article no. 1896649, CA, USA). Illumina Novaseq™ 6000 (LC Bio Technology Co, Ltd, Hangzhou, China) was used for double-terminal sequencing according to the standard operation, and the sequencing mode was PE150. Cutadapt (<https://cutadapt.readthedocs.io/en/stable>, version for cutadapt 1.9) was used to remove the joint plane raw data processing software. A significant difference was analyzed between samples, and the multiple of the difference (fold change [FC] > 2 or [FC] < 0.5 with a p value < 0.05) defined differentially expressed genes (DEGs), which were analyzed for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment using R packs.

2.11 MassARRAY analysis of the methylation level of ZSCAN18

Instructions to extract cells or tissues using the DNA extraction kit (BioTeKe Corporation) were observed. The DNA samples to be tested were processed using a commercial NaHSO₃ kit (ZYMO). The gene fragments to be detected were enriched and amplified by PCR reaction, and the product length was 200 to 700 bp. The PCR product was treated with shrimp alkaline phosphatase to remove free deoxyribonucleoside-5'-triphosphates (dNTPs) in the system. A transcriptase digestion reaction was followed by resin purification. The purified products of the resin were transported to a 384-well SpectroCHIP® bioarray (Agena, Inc) using an Agena NanodispenserRS1000 dot sampling instrument (Agena, Inc). EpiTYPER™ software provided advanced and convenient quantitative analysis of DNA methylation.

2.12 Statistical analysis

IBM SPSS Statistics (version 24.0; Armonk, NY, USA) and GraphPad Prism 8 software (GraphPad Software Inc, La Jolla, CA, USA) were used to analyze the data for this study. The t-test was used to compare statistical differences between the two groups. The chi-squared test and Fisher's exact test were used to analyze the relationship between methylation and various clinicopathological parameters. Overall

survival was determined by log-rank test and the Kaplan-Meier method. The Spearman correlation test was used to analyze the correlation between gene expression and the methylation level. A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of methylation sites in cancerous and adjacent tissues. In univariate analysis, survival differences were estimated using the Kaplan-Meier method (log-rank test), and independent prognostic factors were subsequently identified in Cox proportional risk regression models for multivariate analysis. $p < 0.05$ was considered statistically significant.

3 Results

3.1 Downregulation of ZSCAN18 in primary GC is associated with poor survival

Initially, bioinformatics analysis of the data from The Cancer Genome Atlas (TCGA) datasets showed that expression of ZSCAN18 was depressed in a variety of cancers (Fig. 1A), and analysis from the public datasets (GSE33335) showed that ZSCAN18 is downregulated in GC samples (Fig. 1B). Subsequently, we compared the mRNA expression of ZSCAN18 in 30 GC tissue samples and matched adjacent non-tumor tissue samples by qRT-PCR. Accordingly, ZSCAN18 was downregulated significantly in primary tumor compared with in the adjacent non-tumor tissues (Fig. 1C). Next, using IHC analysis, we semi-quantified ZSCAN18 protein levels of 83 fresh gastric adenocarcinoma tissues and matched adjacent non-tumor tissues. IHC demonstrated that ZSCAN18 was positively stained in the nuclei of gastric mucosal cells and that its protein levels were considerably lower in GC tissues than in matched adjacent non-tumor tissues (Fig. 1D, E). Kaplan-Meier analysis showed that the overall survival of patients with negative expressions of ZSCAN18 was significantly shorter than survival of patients with positive expressions (Fig. 1F). Tumor size, depth of tumor invasion (pT_{3-4} vs pT_2), lymph node metastasis (pN_{1-3} vs pN_0), and low expression of ZSCAN18 predicted an increased risk of cancer-related death in univariate analysis (Table 1). Multivariate analysis revealed that depth of tumor invasion (pT_{3-4} vs pT_2), lymph node metastasis (pN_{1-3} vs pN_0), and expression of ZSCAN18 were independent prognostic factors (Fig. 1G). These results demonstrate that ZSCAN18 is downregulated in primary GC and is associated with poor survival.

Table 1
Univariate Cox proportional hazard models for overall survival of gastric cancer patients

Predictor	Univariate analysis	
	HR(95% CI)	p
Gender		
Female vs Male	0.880(0.516-1.499)	0.637
Age (year)		
<65 vs ≥65	0.755(0.458-1.242)	0.268
Tumor size (cm)		
>4 vs ≤4	3.561(2.045-6.201)	<0.001 ^{***}
Lauren type		
Intestinal vs diffuse	1.234(0.687-2.214)	0.482
Tumor location		
Middle 1/3 vs up 1/3	0.663(0.274-1.605)	0.363
Low 1/3 vs up 1/3	1.228(0.586-2.571)	0.587
>2/3 stomach vs up 1/3	1.262(0.585-2.720)	0.553
Tumor invasion		
pT ₃₋₄ vs pT ₂	4.976(2.473-10.014)	<0.001 ^{***}
Lymph node metastasis		
pN ₁₋₃ vs pN ₀	2.714(1.488-4.949)	0.001 ^{**}
Expression level of ZSCAN18		
High vs low	0.258(0.141-0.471)	<0.001 ^{***}
** $p < 0.01$; *** $p < 0.001$		

3.2 ZSCAN18 is frequently hypermethylated in primary GC and is associated with poor survival

To explore the regulation mechanism of ZSCAN18 in GC, we focused on epigenetic alterations, especially DNA promoter methylation. Bioinformatics analysis of the data from the public dataset (GSE30601) confirmed a high methylation level of ZSCAN18 in GC (Fig. 2A). The MassARRAY analysis spanned the promoter region from -209 to 280, including 50 CpG islands (of which 41 were detectable), and was used

to evaluate the methylation level (Fig. 2B). The level of ZSCAN18 methylation was investigated in 97 primary GC tissues and 6 adjacent non-tumor tissues by MassARRAY analysis, which identified significantly higher methylation of ZSCAN18 in GC (Fig. 2C). Aberrant ZSCAN18 promoter hypermethylation in various CpG islands (CpG11.19.39, CpG23.37.44, CpG26.27, and CpG45.46) was detected more often in GC tissues than in adjacent non-tumor tissues (Fig. 2D). The ROC curve suggested that the methylation level of CpG islands (CpG11.19.39, CpG23.37.44, CpG26.27, and CpG45.46) could distinguish patients with or without GC (Fig. 2E). As shown in the Kaplan-Meier survival curves, patients with GC and high ZSCAN18 methylation had significantly shorter survival times compared with those with GC who had low ZSCAN18 methylation (Fig. 2F). The association between clinicopathologic features and ZSCAN18 methylation in GC tissues is listed in Table 2; the expression of ZSCAN18 correlated with tumor size, which suggests that hypermethylation of ZSCAN18 may be involved in GC proliferation. Tumor size, depth of tumor invasion (pT_{3-4} vs pT_2), lymph node metastasis (pN_{1-3} vs pN_0), and hypermethylation in CpG45.46 predicted an increased risk of cancer-related death in univariate analysis (Table 3). Additional multivariate analysis showed that ZSCAN18 promoter hypermethylation in CpG45.46, depth of tumor invasion (pT_{3-4} vs pT_2), and lymph node metastasis (pN_{1-3} vs pN_0) were independent prognostic factors (Fig. 2G). Collectively, these results reveal that ZSCAN18 is frequently hypermethylated in primary GC and that hypermethylation contributes to poor survival in patients with GC; also, the methylation level of CpG45.46 may be an important marker of prognosis.

3.3 Promoter hypermethylation inhibits ZSCAN18 expression in GC cells

We examined the mRNA expression of ZSCAN18 in 9 GC cell lines by qRT-PCR. Reduced expression of ZSCAN18 was found in 8 GC cell lines (AGS, MGC-803, MKN45, SNU-1, BGC-823, NCI-N87, KATO-III, and SGC-7901); in contrast, ZSCAN18 was overexpressed in only 1 cell line (HGC-27) compared with the human gastric epithelial cell line (GES-1) (Fig. 3A). We next explored the role of promoter methylation in the regulation of ZSCAN18 by MassARRAY analysis. Consistent with the reverse tendency of ZSCAN18 expression, hypermethylation was detected in 8 of 9 GC cell lines (AGS, MGC-803, MKN45, SNU-1, BGC-823, NCI-N87, KATO-III, and SGC-7901), and hypomethylation (equal to the level in GES-1 cells) was found only in HGC-27 cells (Fig. 3B). A significant negative correlation was observed between mRNA expression and the methylation level of ZSCAN18 in all cell lines (GES-1, HGC-27, AGS, MGC-803, MKN45, SNU-1, BGC-823, NCI-N87, KATO-III, and SGC-7901) (Fig. 3C). To confirm whether promoter methylation mediated ZSCAN18 silencing, 2 methylated cell lines (AGS and NCI-N87) that showed silencing of ZSCAN18 were treated with the DNA methyltransferase inhibitor DAC, and the mRNA expression of ZSCAN18 was restored in both lines (Fig. 3D). The change in the ZSCAN18 methylation level was synchronously validated by MassARRAY analysis in AGS cells (Fig. 3E). Furthermore, the decreased methylation level of ZSCAN18 was confirmed in a variety of CpG islands (Fig. 3F). These results indicate that promoter hypermethylation is associated with the transcriptional silence of ZSCAN18 in GC cells; moreover, additional demethylation restores the expression of ZSCAN18.

3.4 Overexpression of ZSCAN18 inhibits GC cell proliferation *in vitro* and *in vivo*

To elucidate the functional significance of ZSCAN18 in GC, we first upregulated ZSCAN18 in AGS and NCI-N87 cells, and overexpression of ZSCAN18 was identified by qRT-PCR and western blot (Fig. 4A). The CCK-8 assay showed that ZSCAN18 overexpression could inhibit the proliferation of both AGS and NCI-N87 cells (Fig. 4B). This inhibitory effect on cell growth was also confirmed with a colony formation assay in both AGS and NCI-N87 cells (Fig. 4C). Western blot showed that ZSCAN18 overexpression led to a decrease in the level of GEMINI and AURORA in both AGS and NCI-N87 cells (Fig. 4D), suggesting that ZSCAN18 overexpression in the nucleus might disrupt circadian rhythms in GC cells.

Subsequently, NCI-N87 cell lines transfected with ZSCAN18 overexpression or empty vector control cells were used to establish xenograft tumors in mice to examine whether ZSCAN18 could suppress the growth of GC cells *in vivo*. The tumor volumes were $52.67 \pm 13.575 \text{ mm}^3$ in xenografted mice with ZSCAN18-overexpressed NCI-N87 cells and $167.69 \pm 10.893 \text{ mm}^3$ in xenografted mice with empty vector control NCI-N87 cells (Fig. 4E, F). IHC analysis of NCI-N87/ZSCAN18 tissues and NCI-N87/control tissues taken from nude mice revealed remarkable overexpression of ZSCAN18 in NCI-N87/ZSCAN18 tumor tissues (Fig. 4G). The tumor growth was significantly lower in ZSCAN18-transfected nude mice than in the vector control mice, suggesting that ZSCAN18 does function as a tumor suppressor in gastric carcinogenesis.

3.5 Identification of genes modulated by ZSCAN18 in GC cell lines

As IHC results showed, ZSCAN18 localized mainly to the nucleus; thus, it is reasonable to speculate that ZSCAN18 might be implicated in the regulation of gene expression. To gain insights into the molecular mechanisms underlying the tumor inhibition of ZSCAN18, we analyzed the downstream signaling pathways modulated by ZSCAN18 through RNA next-generation sequencing in ZSCAN18-overexpressed and control NCI-N87 cells. GO enrichment revealed that gene functions focused on cell division and telomere alteration (Fig. 5A). These DEGs were classified by GO into different KEGG enrichment pathways, including the Wnt signaling pathway, the MAPK signaling pathway, and the nuclear factor kappa B signaling pathway—all of which are well established as playing important roles in carcinogenesis (Fig. 5B). We found that the expressions of 510 genes (DEGs) were significantly changed ($FC > 2$, $p < 0.05$) in NCI-N87 cells (Fig. 5C); among them, 289 genes were upregulated, whereas 221 were downregulated. Among these DEGs, three representatives (FGF20, CEACAM5, and TP53INP2) and their function profiles were summarized (Fig. 5D). Expression levels of these three genes were also verified by qRT-PCR in AGS and NCI-N87 cells (Fig. 5E).

Table 2 Association of ZSCAN18 methylation with clinicopathologic features in gastric tumor

Clinical factor	ZSCAN18 methylation status		<i>p</i>
	Low-methylated	High-methylated	
Gender			0.635
Male	37	22	
Female	22	16	
Age (year)			0.975
≤60	39	25	
>60	20	13	
Tumor size (cm)			0.020*
≤4	16	3	
>4	43	35	
Tumor location			0.065
Up 1/3	9	12	
Middle 1/3	15	12	
Low 1/3	29	10	
>2/3 stomach	6	4	
Lauren type			0.953
Intestinal	15	10	
Diffuse	40	26	
Mixed	4	2	
Tumor invasion			0.740
pT ₂	9	5	
pT ₃	31	23	
pT ₄	19	10	
Lymph node metastasis			0.065
pN ₀	19	6	
pN ₁	9	4	
pN ₂	14	7	

pN ₃	17	21	
Recurrence			0.578
No	42	29	
Yes	17	9	

Chi-square test; * $p < 0.05$

Table 3 Univariate Cox proportional hazard models for overall survival of gastric cancer patients

Predictor	Univariate analysis	
	HR(95% CI)	<i>p</i>
Gender		
Female vs Male	0.916(0.599-1.401)	0.685
Age (year)		
≥60 vs <60	0.986(0.653-1.487)	0.946
Tumor size (cm)		
>4 vs ≤4	1.402(0.834-2.357)	0.203
Lauren type		
Diffuse vs Intestinal	1.079(0.677-1.721)	0.749
Mix vs Intestinal	1.231(0.498-3.044)	0.653
Tumor location		
Middle 1/3 vs up 1/3	0.942(0.531-1.673)	0.839
Low 1/3 vs up 1/3	0.704(0.406-1.221)	0.211
>2/3 stomach vs up 1/3	0.880(0.413-1.873)	0.740
Tumor invasion		
pT ₃₋₄ vs pT ₂	2.388(1.293-4.410)	0.005**
Lymph node metastasis		
pN ₁₋₃ vs N ₀	4.758(2.603-8.698)	<0.001***
Methylation High vs low		
CpG11	1.134(0.722-1.783)	0.585
CpG23	0.852(0.425-1.708)	0.652
CpG26.27	1.221(0.532-2.803)	0.637
CpG45.46	2.060(1.301-3.261)	0.002**

p* < 0.01; *p* < 0.001.

4 Discussion

In this study, we provide substantial evidence of a suppressive effect of ZSCAN18 on GC. We found that ZSCAN18 is widely downregulated in GC tissues and in most GC cell lines, suggesting a suppressive

effect on carcinogenesis. As confirmed by demethylation treatment and MassARRAY analyses, these decreased expression are mostly attributed to ZSCAN18 promoter hypermethylation in various CpG islands. However, ZSCAN18 hypermethylation was not detected in normal gastric mucosal tissues or GES-1 cells, suggesting that the hypermethylation of the ZSCAN18 promoter appears to follow a chronological sequence during carcinogenesis and is involved in the early stage of the multistep process of gastric carcinogenesis [16, 19, 20]. Strikingly, no prominent downregulation of ZSCAN18 was noted, with hypomethylation in only HGC-27 cells, indicating that the carcinogenicity of ZSCAN18 may be influenced by other transcription regulating mechanisms [21]. This study revealed that promoter hypermethylation is a unique mechanism of ZSCAN18 inactivation in GC. In addition, we confirmed for the first time, to our knowledge, that downregulation of ZSCAN18 with hypermethylation in CpG44.45 is associated with poor survival in primary GC; this finding suggests that ZSCAN18 may serve as a biomarker of poor prognosis [9, 22, 23]. Furthermore, the restorability of CpG44.45 after treatment with DAC (also known as decitabine, a chemotherapy agent) indicates that ZSCAN18 may be a potential target for an anti-tumor drug.

Given that ZSCAN18 reduction leads to poor survival, it is reasonable to postulate that ZSCAN18 could affect the progression and development of GC. Furthermore, the tumor-suppressive function of ZSCAN18 in GC was investigated both *in vitro* and *in vivo*. First, gain-of-function experiments using ZSCAN18 were performed in AGS and NCI-N87 cells, and the effect of ZSCAN18 on the proliferation of these cells was examined using CCK-8 and colony formation assays. The results showed that ZSCAN18 overexpression inhibited GC cell proliferation. In addition, decreased tumor growth resulting from ZSCAN18 overexpression was observed in nude mice and supports the inhibitory role of ZSCAN18 in gastric carcinogenesis. However, dysfunction of circadian rhythm was associated with the development and progression of tumors [24–27]. Our results revealed that ZSCAN18 overexpression might influence the circadian rhythm in GC cells by reducing the levels of GEMINI and AURORA. Taken together, these results confirm the crucial role of ZSCAN18 as a functional anti-tumor gene through suppression of cell proliferation and disruption of the circadian rhythm in GC.

To extend our investigation into the molecular mechanisms, we analyzed the downstream signaling pathways modulated by ZSCAN18. We elucidated, to our knowledge for the first time, the molecular mechanism of the tumor suppressive function of ZSCAN18: regulation of the oncogenic Wnt signaling pathway, the MAPK signaling pathway, and the nuclear factor kappa B signaling pathway. Abnormal overexpression of ZSCAN18 caused by the downregulation of FGF20 and CEACAM5 and the upregulation of TP53INP2 plays an extremely important role in the suppression of GC. As a member of the fibroblast growth factors, FGF20 plays important roles in morphogenesis, angiogenesis, tissue remodeling, and carcinogenesis through the Wnt/ β -catenin signaling pathway [28, 29]. CEACAM5 may also promote tumor invasion and migration and may be a promising biomarker for GC in pre-diagnostic and prognostic evaluations [30, 31]. Moreover, TP53INP2 is a key positive regulator of autophagy and may act as a novel autophagic adaptor through recruitment of ubiquitinated substrates to autophagosomes for degradation [32–35]. These alternative downstream genes may induce carcinogenesis when ZSCAN18 is aberrantly

expressed through dysregulation of a pathway component. Thus, a search for therapeutically relevant compounds targeting ZSCAN18 is needed.

5 Conclusions

In conclusion, this study found that frequent hypermethylation of the ZSCAN18 promoter leads to gene silencing and involvement in gastric carcinogenesis. ZSCAN18 contributes to the suppression of gastric carcinogenesis by decreasing cell proliferation, inducing disruption of the circadian rhythm, and regulating the oncogenic pathways (Wnt signaling pathway, MAPK signaling pathway, and nuclear factor kappa B signaling pathway). ZSCAN18 also may serve as a potential epigenetic biomarker of prognosis in GC and has potential as a target for drug development.

Abbreviations

ZSCAN18

Zinc finger and scan domain containing 18

GC

gastric cancer

qRT-PCR

quantitative real-time polymerase chain reaction

ZNF

Zinc finger

TF

transcription factors

GEO

Gene Expression Omnibus

IHC

immunohistochemistry

FBS

fetal bovine serum

DMEM

Dulbecco's modified Eagle medium

CCK-8

Cell Counting Kit 8

DAC

5-aza-2'-deoxycytidine

DEGs

differentially expressed genes

GO

Gene Ontology

KEGG
Kyoto Encyclopedia of Genes and Genomes
dNTPs
deoxyribonucleoside-5'-triphosphates
ROC
receiver operating characteristic
TCGA
The Cancer Genome Atlas
GES-1
human gastric epithelial cell line.

Declarations

7.1 Ethics approval and consent to participate

This research was approved by the Ethics Committee of Tianjin Medical University Cancer Hospital, and oral and written informed consents were obtained from all patients before enrolling in the research program. The *in vivo* assay using nude mice was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University Cancer Hospital.

7.2 Consent for publication

Not applicable.

7.3 Availability of data and materials

The data used to support the findings of this study are available from the corresponding author on reasonable request.

7.4 Competing interests

The authors declare that they have no competing interests

7.5 Funding

This work was supported by the National Key R&D Program of China (Grant No.2016YFC1303200) and the National Natural Science Foundation of China (Grant No. 81974373).

7.6 Authors' contributions

BQR, BL, GM, HL and JYD made the final approval of the version to be published. BQR, BL, GM and JYD conceived and designed the experiments. BQR, BL, FLC, YPD, YZ, YL, LZ and YY conducted the experiments. BQR, BL, GM and PLW analyzed the data. BQR, BL, GM, PLW, NNZ, YZN and JYD contributed reagents/materials/analysis tools. BQR and BL wrote the paper. All authors read and approved the final manuscript.

7.7 Acknowledgements

Not applicable.

References

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. *CA Cancer J Clin.* 2021;71(1):7–33.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2021;71(3):209–49.
3. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric cancer. *Lancet.* 2020;396(10251):635–48.
4. Bennett RL, Licht JD. Targeting epigenetics in cancer. *Annu Rev Pharmacol Toxicol.* 2018;58:187–207.
5. Hogg SJ, Beavis PA, Dawson MA, Johnstone RW. Targeting the epigenetic regulation of antitumour immunity. *Nat Rev Drug Discov.* 2020;19(11):776–800.
6. Flavahan WA, Gaskell E, Bernstein BE. Epigenetic plasticity and the hallmarks of cancer. *Science.* 2017;357(6348):eaal2380.
7. Du W, Wang S, Zhou Q, Li X, Chu J, Chang Z, et al. ADAMTS9 is a functional tumor suppressor through inhibiting AKT/mTOR pathway and associated with poor survival in gastric cancer. *Oncogene.* 2013;32(28):3319–28.
8. Yu J, Cheng YY, Tao Q, Cheung KF, Lam CN, Geng H, et al. Methylation of protocadherin 10, a novel tumor suppressor, is associated with poor prognosis in patients with gastric cancer. *Gastroenterology.* 2009;136(2):640–51.e1.
9. Tahara T, Arisawa T. DNA methylation as a molecular biomarker in gastric cancer. *Epigenomics.* 2015;7(3):475–86.
10. Ebrahimi V, Soleimanian A, Ebrahimi T, Azargun R, Yazdani P, Eyvazi S, et al. Epigenetic modifications in gastric cancer: focus on DNA methylation. *Gene.* 2020;742:144577.
11. Oka D, Yamashita S, Tomioka T, Nakanishi Y, Kato H, Kaminishi M, et al. The presence of aberrant DNA methylation in noncancerous esophageal mucosae in association with smoking history: a

- target for risk diagnosis and prevention of esophageal cancers. *Cancer*. 2009;115(15):3412–26.
12. Morris MR, Ricketts CJ, Gentle D, McDonald F, Carli N, Khalili H, et al. Genome-wide methylation analysis identifies epigenetically inactivated candidate tumour suppressor genes in renal cell carcinoma. *Oncogene*. 2011;30(12):1390–401.
 13. Andresen K, Boberg KM, Vedeld HM, Honne H, Hektoen M, Wadsworth CA, et al. Novel target genes and a valid biomarker panel identified for cholangiocarcinoma. *Epigenetics*. 2012;7(11):1249–57.
 14. Andresen K, Boberg KM, Vedeld HM, Honne H, Jebsen P, Hektoen M, et al. Four DNA methylation biomarkers in biliary brush samples accurately identify the presence of cholangiocarcinoma. *Hepatology*. 2015;61(5):1651–9.
 15. Mitchell SM, Ross JP, Drew HR, Ho T, Brown GS, Saunders NF, et al. A panel of genes methylated with high frequency in colorectal cancer. *BMC Cancer*. 2014;14:54.
 16. Vedeld HM, Andresen K, Eilertsen IA, Nesbakken A, Seruca R, Gladhaug IP, et al. The novel colorectal cancer biomarkers CD01, ZSCAN18 and ZNF331 are frequently methylated across gastrointestinal cancers. *Int J Cancer*. 2015;136(4):844–53.
 17. Jiang H, Gu J, Du J, Qi X, Qian C, Fei B. A 21-gene support vector machine classifier and a 10-gene risk score system constructed for patients with gastric cancer. *Mol Med Rep*. 2020;21(1):347–59.
 18. Yu CB, Zhu LY, Wang YG, Li F, Zhang XY, Dai WJ. Systemic transcriptome analysis of hepatocellular carcinoma. *Tumour Biol*. 2016;37(10):13323–31.
 19. Leung WK, Yu J, Ng EK, To KF, Ma PK, Lee TL, et al. Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. *Cancer*. 2001;91(12):2294–301.
 20. Qu Y, Dang S, Hou P. Gene methylation in gastric cancer. *Clin Chim Acta*. 2013;424:53–65.
 21. Goodman JI, Watson RE. Altered DNA methylation: a secondary mechanism involved in carcinogenesis. *Annu Rev Pharmacol Toxicol*. 2002;42:501–25.
 22. Matsuoka T, Yashiro M. Biomarkers of gastric cancer: current topics and future perspective. *World J Gastroenterol*. 2018;24(26):2818–32.
 23. Abbas M, Faggian A, Sintali DN, Khan GJ, Naeem S, Shi M, et al. Current and future biomarkers in gastric cancer. *Biomed Pharmacother*. 2018;103:1688–700.
 24. Kettner NM, Katchy CA, Fu L. Circadian gene variants in cancer. *Ann Med*. 2014;46(4):208–20.
 25. Williams GH, Stoeber K. The cell cycle and cancer. *J Pathol*. 2012;226(2):352–64.
 26. Soták M, Sumová A, Pácha J. Cross-talk between the circadian clock and the cell cycle in cancer. *Ann Med*. 2014;46(4):221–32.
 27. Fu L, Kettner NM. The circadian clock in cancer development and therapy. *Prog Mol Biol Transl Sci*. 2013;119:221–82.
 28. Kirikoshi H, Sagara N, Saitoh T, Tanaka K, Sekihara H, Shiokawa K, et al. Molecular cloning and characterization of human FGF-20 on chromosome 8p21.3-p22. *Biochem Biophys Res Commun*. 2000;274(2):337–43.

29. Katoh M. Multi-layered prevention and treatment of chronic inflammation, organ fibrosis and cancer associated with canonical WNT/ β -catenin signaling activation (Review). *Int J Mol Med*. 2018;42(2):713–25.
30. Zhou J, Fan X, Chen N, Zhou F, Dong J, Nie Y, et al. Identification of CEACAM5 as a biomarker for prewarning and prognosis in gastric cancer. *J Histochem Cytochem*. 2015;63(12):922–30.
31. Liu JN, Wang HB, Zhou CC, Hu SY. CEACAM5 has different expression patterns in gastric non-neoplastic and neoplastic lesions and cytoplasmic staining is a marker for evaluation of tumor progression in gastric adenocarcinoma. *Pathol Res Pract*. 2014;210(10):686–93.
32. Zhang W, Li P, Wang S, Cheng G, Wang L, Mi X, et al. TP53INP2 promotes bovine adipocytes differentiation through autophagy activation. *Animals (Basel)*. 2019;9(12):1060.
33. Xu Y, Wan W. TP53INP2 mediates autophagic degradation of ubiquitinated proteins through its ubiquitin-interacting motif. *FEBS Lett*. 2019;593(15):1974–82.
34. Xu Y, Wan W. The bifunctional role of TP53INP2 in transcription and autophagy. *Autophagy*. 2020;16(7):1341–3.
35. Ivanova S, Polajnar M, Narbona-Perez AJ, Hernandez-Alvarez MI, Frager P, Slobodnyuk K, et al. Regulation of death receptor signaling by the autophagy protein TP53INP2. *EMBO J*. 2019;38(10):e99300.

Figures

Figure 1

Deregulation of ZSCAN18 expression in GC is correlated with poor prognosis. (A) Bioinformatics analysis of The Cancer Genome Atlas database (<http://ualcan.path.uab.edu>) between tumor tissues (red) and normal tissues (blue) in various cancers. (B) Bioinformatics analysis from the public datasets (GSE33335) in adjacent non-tumor tissues and tumor tissues. (C) mRNA expression levels of ZSCAN18 in GC tissue samples and matched adjacent non-tumor tissue samples ($n = 30$) are determined by quantitative real-time polymerase chain reaction (qRT-PCR). (D) ZSCAN18 expression score analysis of immunohistochemistry (IHC) staining in GC tissues and adjacent non-tumor tissues ($n = 83$). A score of (–) or (+) is classified as negative, whereas a score of (++) or (+++) is classified as positive. (E) Representative IHC results show the expression levels of ZSCAN18 in GC tissues and adjacent non-tumor tissues (left bar: 200 μ m; right bar: 30 μ m). (F) Kaplan-Meier curves show the association of overall survival rate in patients with GC and ZSCAN18 expression. Green: patients with negative expression ($n = 49$, median survival, 22 months); orange: patients with positive expression ($n = 34$, median survival, 43 months, $p < 0.001$, log-rank test). (G) Multivariate analysis shows independent prognostic factors. Data are presented as mean $\pm$ standard deviation (SD). ** $p < 0.01$, *** $p < 0.001$.

Figure 2

ZSCAN18 is frequently hypermethylated in primary GC and is associated with poor survival. (A) Bioinformatics analysis from the public datasets (GSE30601) in adjacent non-tumor tissues and tumor tissues. (B) Methylation status of the ZSCAN18 promoter was confirmed by MassARRAY. (C) Methylation levels of ZSCAN18 in GC tissue ($n = 97$) and adjacent non-tumor tissue ($n = 6$) samples are determined by MassARRAY. (D) Methylation levels of ZSCAN18 in GC tissue and adjacent non-tumor tissue samples are detected by MassARRAY analysis in CpG11.19.39, CpG23.37.44, CpG26.27, and CpG45.46. (E) Receiver operating characteristic (ROC) curve evaluation showed that the methylation level of CpG islands (CpG11.19.39, CpG23.37.44, CpG26.27, and CpG45.46) could distinguish between patients with or without GC. (F) Kaplan-Meier curves show the association of overall survival rate of patients with GC (ZSCAN18 high-methylation: 13.5 months; low-methylation: 20.0 months). (G) Multivariate analysis shows independent prognostic factors. Data are presented as mean $\pm$ standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3

The expression and methylation status of ZSCAN18 in GC cells. (A) ZSCAN18 mRNA expression was reduced in all GC cell lines except HGC-27. GES-1, normal gastric epithelial cell line. (B) Hypermethylation of ZSCAN18 was detected in all GC cell lines except HGC-27. GES-1, normal gastric epithelial cell line. (C) Correlation between mRNA expression and methylation level of ZSCAN18 in GC cell lines. (D) The mRNA expression of ZSCAN18 in AGS and NCI-N87 cells was restored after treatment with the demethylation agent 5-aza-2'-deoxycytidine. (E) Demethylation of ZSCAN18 was observed in AGS cells after treatment with the demethylation agent 5-aza-2'-deoxycytidine, as examined by MassARRAY analysis. (F) The methylation level of ZSCAN18 was detected in a variety of CpG islands by MassARRAY analysis. Data are presented as mean $\pm$ standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4

The effects of aberrant ZSCAN18 expression on GC cell proliferation *in vitro* and *in vivo*. (A) Overexpression of ZSCAN18 in AGS and NCI-N87 cells was evidenced by qRT-PCR (above) and western blot (below). (B) Growth curves represent cell viability that was inhibited by ZSCAN18 in both AGS and NCI-N87 cells. (C) Overexpression of ZSCAN18 suppressed colony formation in both AGS and NCI-N87 cells. (D) ZSCAN18 suppressed the expression of GEMINI and AURORA in both AGS and NCI-N87 cells. (E, F) Tumor growth was inhibited in nude mice subcutaneously inoculated with NCI-N87 cells that overexpressed ZSCAN18 compared with control inoculations. (G) IHC results show the expression levels

of ZSCAN18 in ZSCAN18/NCI-N87 tumors and ZSCAN18/control tumors from nude mice. Data are presented as mean $\pm$ standard deviation (SD). ** $p < 0.01$, *** $p < 0.001$.

Figure 5

Downstream molecular mechanisms of ZSCAN18 in GC were identified by RNA next-generation sequence screening. (A, B) GO and KEGG enrichment plots of DEGs were generated from mRNA sequencing analysis of ZSCAN18-overexpressed and control NCI-N87 cells. (C) The volcano plots of DEGs were generated from mRNA sequencing analysis of ZSCAN18-overexpressed and control NCI-N87 cells. (D) Downstream potent molecular events were identified. (E) Three representative downstream DEGs were validated by qRT-PCR in AGS and NCI-N87 cells. Data are presented as mean $\pm$ standard deviation (SD). *** $p < 0.001$, **** $p < 0.0001$.