KRAB zinc-finger protein 382 regulates epithelial-mesenchymal transition and functions as a tumor suppressor, but is silenced by CpG methylation in gastric cancer

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Abstract. Several studies have recently reported that KRAB zinc finger protein 382 (ZNF382) is downregulated in multiple carcinoma types due to promoter methylation. The exact role of ZNF382 in gastric carcinogenesis, however, remains elusive. In this study, we investigated the alterations and functions of ZNF382 in the pathogenesis of gastric cancer (GC). Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), quantitative (real-time) PCR (qPCR) and immunohistochemistry were carried out to detect the expression patterns of ZNF382 in GC cell lines and gastric tissue samples. Furthermore, its methylation status in GC cell lines, tumor tissues and adjacent non-tumor tissues was detected by methylation-specific PCR (MSP). We observed that ZNF382 was silenced due to promoter methylation in MKN45 and SGC7901 cell lines, and that its silencing could be reversed with 5-aza-2'-deoxycytidine, indicating that its downregulation in GC is due to promoter methylation. In addition, the ectopic expression of ZNF382 significantly inhibited gastric tumor cell clonogenicity, proliferation, migration and epithelial-mesenchymal transition (EMT) through the induction of apoptosis. ZNF382 expression downregulated the expression of SNAIL, Vimentin, Twist, NOTCH1, NOTCH2, NOTCH3, NOTCH4, HES-1, JAG1, matrix metalloproteinase (MMP)2 and MMP11, as well as that of the stem cell markers, NANOG, octamer-binding transcription factor 4 (OCT4) and SOX2. ZNF382 also upregulated the expression of E-cadherin. On the whole, the findings of this study suggest that ZNF382 functions as a tumor suppressor in GC cells, but is frequently methylated in both GC cell lines and primary gastric tumors. ZNF382 can reverse the EMT process in GC cells through NOTCH signaling. Our findings further illustrate the molecular pathogenesis of GC and establish potential biomarkers for this type of cancer.

Introduction

Gastric cancer (GC) is currently the fourth most common malignancy in the worldwide scale. It remains the second leading cause of cancer-related mortality, with a poor prognosis following diagnosis. Regrettably, a large proportion of patients with GC are at a late stage when diagnosed due to a lack of effective screening programs (1-3). Zinc finger proteins (ZFPs) comprise one of the largest transcription factor families and are found exclusively in tetrapod vertebrates (4). The Krüppel-associated box (KRAB) exists in approximately one-third of ZFPs (4). KRAB-ZFPs are considered crucial regulators of diverse cellular progress, such as cell differentiation, proliferation, apoptosis and tumorigenesis (4-6). ZFPs can activate or suppress gene expression by binding to promoters (7,8). The ZNF382 gene, a novel zinc finger transcription factor described previously, is located on chromosome 19q13.12 and contains only one KRAB domain. It has been shown to be a tumor suppressor gene (TSG) and is commonly downregulated due to the hypermethylation of its promoter CpG island in multiple carcinomas, including GC (4,9). Moreover, ZNF382 can inhibit activator protein-1 (AP-1) and nuclear factor (NF)-κB signaling and downregulate multiple oncogenes, including melanogenesis associated transcription factor (MITF), MYC, cyclin dependent kinase (CDK)6 and high mobility group AT-hook 2 (HMGA2), and it can also down-regulate several upstream factors of NF-κB, including signal transducer and activator of transcription (STAT)3, STAT5B and inhibitor of DNA binding 1, HLH protein (ID1) (9).

Epithelial-mesenchymal transition (EMT) is both a physiological and pathological course, regulating cell phenotype...
and function during normal development and tumor development (including GC) (10-12). Previous studies have verified the vital role of SNAIL in suppressing E-cadherin expression; SNAIL downregulates the expression of E-cadherin by binding to the two E-boxes of the E-cadherin promoter (13). Reportedly, various signaling pathways, including NF-κB, Wnt and NOTCH, are involved in this multi-step event (14,15). Notably, NOTCH has been identified as a key factor involved in tumor metastasis (16-18).

As there are limited studies available on ZNF382, at least to the best of our knowledge, its roles during EMT and GC are unclear. Thus, in this study, in order to clarify the role of ZNF382 in GC, the expression level and the methylation status of its promoter in GC cell lines and paired gastric tumor tissues were examined. We further examined its biological function and the potential underlying molecular mechanisms involved in gastric tumorigenesis.

Materials and methods

**Cell culture and tissue samples.** Five GC cell lines (AGS, BGC823, MKN28, MKN45 and SGC7901) were used. The AGS, MKN28 (reported to be a derivative of the MKN74 GC cell line) (19,20) and MKN45 cells were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA) or provided by Professor Qian Tao (the Chinese University of Hong Kong, Hong Kong, China). The BGC823 and SGC7901 cells were purchased from the Cell Resource Center of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were allowed to grow in RPMI-1640 medium ( Gibco-BRL, Karlsruhe, Germany) at 37°C/5% CO₂, supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (FBS; PAALaboratories, Linz, Austria). The MKN45 and SGC7901 cell lines which were transfected with pcDNA3.1-ZNF382-Flag or vector pcDNA3.1 were selected with geneticin (G418). The ectopic expression of ZNF382 was assayed by RT-PCR and western blot analysis prior to the other experimental procedures. A total of 5 normal gastric tissues, 138 primary gastric tumor tissues and 64 matched adjacent non-tumor tissue samples were acquired from 29 matched tumor adjacent tissues. Immunohistochemical staining was then performed as previously described (22,25). The sections were incubated with a rabbit monoclonal anti-SNAIL antibody (SP-9001, 1:100 dilution; ZSGB-BIO, Beijing, China) at 37°C

**DNA and RNA extraction.** The QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) was used for the genomic DNA extraction from the cell lines and tissues in accordance with the manufacturer's instructions. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for the total RNA isolation from the cell lines and tissue samples (-80°C for sample storage).

**Semi-quantitative reverse transcription-PCR (RT-PCR) and quantitative PCR (qPCR).** Briefly, the RNA (1 µg) to 20 µg of cDNA, the Reverse Transcription system (Promega, Madison, WI, USA) was used. RT-PCR was carried out as previously described (21). GAPDH was used as an internal control. RT-PCR was performed (32 cycles for target genes, 23 cycles for GAPDH) with Go-Taq polymerase (Promega). qPCR was carried out in accordance with the instructions of the ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using SYBR®-Green qPCR Master Mix (MBI Fermentas, St. Leon-Rot, Germany). The primers used in this study are listed in Table I.

**DNA bisulfite treatment and methylation-specific PCR (MSP).** DNA bisulfite modifications and MSPs were carried out in accordance with previously described methods (23,24). The primers used for MSP are listed in Table I. AmpliTaq®-Gold DNA Polymerase (Applied Biosystems) was used, and 40 amplification cycles were performed in both the methylated and unmethylated tissue samples. Products were detected on 2% (w/v) agarose gels with 100 bp DNA markers (MBI Fermentas, Vilnius, Lithuania).

**ZNF382 overexpression in GC cell lines.** Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used in accordance with the instructions for the transfection of MKN45 and SGC7901 cells with 4 µg pcDNA3.1- ZNF382-Flag or pcDNA3.1 plasmids (provided by Professor Qian Tao at the Chinese University of Hong Kong) in -10 mmol/l Aza (Sigma-Aldrich, Steinheim, Germany) was used for cell culture. After 3 days, the cells were treated with 100 nmol/l TSA (Sigma-Aldrich) for the following 24 h. Cells were then collected for RNA extraction (22).

**Immunohistochemical staining (IHC) assay.** The gastric cancer tissue samples (n=55) were formalin-fixed and paraffin-embedded. These tissue samples included 55 GC tissues and 29 matched tumor adjacent tissues. Immunohistochemical staining was then performed as previously described (22,25). The sections were incubated with a rabbit monoclonal antibody (HPA049259, anti-ZNF382 antibody, 1:50 dilution; Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight. The following day, samples were rinsed with phosphate-buffered saline (PBS), incubated with rabbit secondary antibody (SP-9001, 1:100 dilution; ZSGB-BIO, Beijing, China) at 37°C for 30 min, stained with diaminobenzidine for 33 sec, and counterstained with hematoxylin for 5 sec to visualize the nuclei. Each section was assessed and scored by two independent pathologists who were blinded to the origin of all tissues. The widely accepted German semi-quantitative scoring criteria was used for scoring, and the staining index was determined...
by multiplying the score for staining intensity with the score for staining extent, as previously described (26). Clinical data included age, sex, histological type, differentiation grade and TNM stage. Clinical follow-up data for 55 patients were censored for the analysis.

**Colony formation assay.** Stable MKN45 and SGC7901 cells were planted in a 6-well plate at 100 cells/well for 14 days, and the medium was refreshed every 2-3 days. The cells were fixed in 4% paraformaldehyde for 30 min, and after staining with Gentian Violet (ICM Pharma, Singapore, Singapore) for 20 min, surviving colonies with >50 cells/colony were counted using ImageJ (V.1.8.0) software (National Institutes of Health, Bethesda, MD, USA) for the analysis. The experiments were repeated 3 times.

**Cell viability.** CCK-8 assay was performed according to the manufacturer's instructions (Beyotime, Shanghai, China). Stable MKN45 and SGC7901 cells were seeded in 96-well plates (2x10^3 cells/well) and cultured for 24, 48 or 72 h, and the medium in each well was then replaced with 100 ml RPMI-1640 (10% FBS) containing 10 ml CCK-8 solution and incubated at 37˚C for 2 h. The absorbance at 450 nm was measured using a microplate reader (Multiskan MK3; Thermo Scientific).

Table I. List of primers used in this study.

| PCR      | Primer | Sequence (5’-3’) | Product size (bp) | PCR cycles | Annealing temperature (˚C) |
|----------|--------|------------------|-------------------|------------|---------------------------|
| RT-PCR   | ZNF382F| CCTTACAGGGATCAGTGTC  | 173               | 32         | 58                        |
|          | ZNF382R| CAACTTGCGGATCATACAG | 281               | 38         | 58                        |
|          | SOX2F  | AGCAACGCGATCAGCA   | 190               | 38         | 58                        |
|          | SOX2R  | TGGAGAAGGGTAACTTGAAC | 303              | 38         | 58                        |
|          | OCT4F  | AAGGGAGAAGTCGGACGAA | 346              | 38         | 58                        |
|          | OCT4R  | GAGGGTTTCTGCTTTGCA  | 206               | 23         | 58                        |
|          | GAPDHF | GAGATGGAATTTTCCATGA | 103              | 60         | 58                        |
|          | GAPDHR | GTGATGGGATTTCCATGA  | 150              | 60         | 58                        |
| RT-qPCR  | E-cadF | TACACTGCCCAGGACGCA  | 103              | 60         |                           |
|          | E-cadR | GGCGAGCTGTGTGAGCATTA | 150          | 60         |                           |
|          | VimentinF| GCACTGCTAAGGACGACA  | 181              | 60         |                           |
|          | VimentinR| GTACATATCTCTCTGCAGGAT | 122          | 60         |                           |
|          | SNAIL1F | CGCCTCTATCTCTGTCAG  | 186              | 60         |                           |
|          | SNAIL1R | TCCGAGTGGCATTGACAGCAG | 150          | 60         |                           |
|          | TwistF  | CCAGTTGGAAGAAGGATCTC | 144          | 60         |                           |
|          | TwistR  | CTATGGTTTTTCGAGGACATG | 130          | 60         |                           |
|          | NOTCH1F | AGGCACTACTCTCCCTTTCG  | 186              | 60         |                           |
|          | NOTCH1R | GGCTCTGGCAAGTCTCTACAA | 150          | 60         |                           |
|          | NOTCH2F | AGGCAGATGGTAGGATGATC  | 150              | 60         |                           |
|          | NOTCH2R | TCCTATGGGAGGCAAAGGAT | 167              | 60         |                           |
|          | NOTCH3R | CAGCAAGGCATGTGGAACATG | 144          | 60         |                           |
|          | NOTCH4F | CTGCGATATAAGGAGGAGGATA | 230          | 40         | 60                        |
|          | NOTCH4R | ACGGAGTAAAGGCAAGGAGGC | 230          | 40         | 60                        |
|          | HES-1F  | AGATAGCTCGCCGCACTCC | 130              | 60         | 58                        |
|          | HES-1R  | GTACCTTCCACGCAACTTTG | 167              | 60         |                           |
|          | JAG1F   | GTGCAGCATCTACAGCTAT | 167              | 60         |                           |
|          | JAG1R   | TGTACTAAGCAGTGCATACCA | 167          | 60         |                           |
| MSP      | ZNF382m1| GGCGATTTACGGCTGTGTTT  | 230              | 40         | 60                        |
|          | ZNF382m2| AAAATTTCACCAACCCGACCTG | 233          | 58         |                           |
|          | ZNF382U1| GTTGGGATTAAATGAGGTGTTT  | 233             | 58         |                           |
|          | ZNF382U2| CAAAATTTCACCAACCCGACTCA | 233          | 58         |                           |

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Fisher Scientific, former Fermentas, Schwerte, Germany) at 24, 48 and 72 h. All experiments were assessed in triplicate.

Flow cytometric analysis. To investigate the cell cycle status, the cells were collected and centrifuged (200 x g for 5 min), rinsed twice with PBS and fixed with 70% ethanol at 4°C overnight. The following day, the cells were treated with 50 mg/l propidium iodide (PI) (Beyotime) for 30 min in the condition of 4°C in the dark. For apoptosis analyses, the cells were washed, collected, resuspended in PBS, stained with Annexin V-FITC (BD Pharmingen, San, Jose, CA, USA) and PI, and analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Wound-healing assay. The cells were cultured in 6-well plates. Using a sterile tip to scratched a straight linear wound when the cultured cells were confluent. After rinsing with PBS, the cells were incubated with fresh growth medium. Images of the cells were captured with a 10X objective lens (Nikon, Tokyo, Japan) at 0, 24 and 48 h after wounding for the SGC7901 cells and at 0, 12 and 24 h for the MKN45 cells. The experiments were performed in triplicate.

Migration and invasion assay. The migratory and invasive abilities of the GC cells were also investigated using Transwell chambers (8 µm pore size; Corning, New York, NY, USA) with or without a Matrigel (BD Biosciences) barrier added to the top chamber. The MKN45 and SGC7901 cells transfected with the ZNF382 overexpression vector or the control vector were collected, washed twice in serum-free medium, and seeded into the upper Transwell chamber. Approximately 800 µl of medium with 10% FBS was added to the lower chambers. Following incubation at 37°C and 5% CO₂ for 24 h, the cells were fixed in 4% paraformaldehyde for 30 min and stained with crystal violet (DC079; Genview, Beijing, China) for 20 min at room temperature. Cells on the upper side of the chamber were wiped off with a cotton bud. Cells from 6 random fields were captured and counted under a microscope (x100 magnification, CTR4000; Leica, Wetzlar, Germany).

Immunofluorescence staining. The MKN45 and SGC7901 cells were cultured in 24-well plates with glass coverslips in the wells and transiently transfected with pcDNA3.1-Flag-ZNF382. Forty-eight hours later, the cells were fixed in 4% paraformaldehyde for 30 min, then permeabilized in 0.1% Triton X-100 for 5 min, followed by blocking with 1% bovine serum albumin (BSA) in PBS for 1 h. The cells were then incubated with primary antibodies against ZNF382 (HPA049259, 1:1,000 dilution; Sigma-Aldrich), E-cadherin (sc-8426, 1:1,000 dilution), Vimentin (sc-6260, 1:1,000 dilution) (both from Santa Cruz Biotechnology, Inc.), SNAIL1 (ab35708, 1:1,000 dilution; Abcam, Cambridge, UK), NOTCH1 (sc-376403, 1:1,000 dilution), NOTCH3 (sc-515825, 1:1,000 dilution), HES-1 (sc-166378, 1:1,000 dilution) and SOX2 (sc-365823, 1:1,000 dilution); GAPDH (sc-47724, 1:1,000 dilution) (all from Santa Cruz Biotechnology, Inc.) was used as a control. Anti-rabbit IgG (#7074, 1:3,000 dilution) and anti-mouse IgG (#7076, 1:3,000 dilution) (both from Cell Signaling Technology, Danvers, MA, USA) horseradish peroxidase conjugate secondary antibodies were used. The membranes were visualized using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Western blot analysis. This assay was conducted as previously described (27). Protein extraction reagent (Thermo Fisher Scientific, Inc.), containing phenylmethanesulfonyl fluoride, protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich), was used for cell lysis (all proteins were extracted from cells). Following disruption using the Ultrasonic Cell Disruptor (Ningbo Scientechnology Co., Ltd., Ningbo, China), the cell suspensions were centrifuged (200 x g for 4°C), and the supernatant was collected. To determine the concentration of proteins, a BCA protein kit (Thermo Fisher Scientific, Inc.) was used. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was conducted for the separation of a total of 40 mg protein lysate. Proteins were then transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Membranes were then incubated with blocking buffer (PBS with 5% non-fat milk and 0.1% Tween-20) for 1 h at room temperature. Several primary antibodies were used: ZNF382 (HPA049259, 1:1,000 dilution; Sigma-Aldrich), E-cadherin (sc-8426, 1:1,000 dilution), Vimentin (sc-6260, 1:1,000 dilution) (both from Santa Cruz Biotechnology, Inc.), SNAIL1 (ab35708, 1:1,000 dilution; Abcam, Cambridge, UK), NOTCH1 (sc-376403, 1:1,000 dilution), NOTCH3 (sc-515825, 1:1,000 dilution), HES-1 (sc-166378, 1:1,000 dilution) and SOX2 (sc-365823, 1:1,000 dilution); GAPDH (sc-47724, 1:1,000 dilution) (all from Santa Cruz Biotechnology, Inc.) was used as a control. Anti-rabbit IgG (#7074, 1:3,000 dilution) and anti-mouse IgG (#7076, 1:3,000 dilution) (both from Cell Signaling Technology, Danvers, MA, USA) horseradish peroxidase conjugate secondary antibodies were used. The membranes were visualized using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis. All data were analyzed with the use of SPSS software, version 19.0 (SPSS Inc., Chicago, IL, USA). The Chi-square (also termed χ²) test was used for the analysis of the results of immunohistochemistry. The Student's t-tests, Chi-square test and Fisher's exact test were used for the comparison of the methylation status and clinicopathological characteristics of the patients with GC. For all assays, a value of P<0.05 was considered to indicate a statistically significant difference.

Results

ZNF382 expression is downregulated in both GC cell lines and primary GC tissues. Semi-quantitative RT-PCR was carried out to examine the expression of ZNF382 in several gastric tumor cell lines and 5 normal gastric tissues. ZNF382 expression was significantly suppressed in 3 of the 5 GC cell lines, and ZNF382 was faintly expressed in the AGS and MKN28 cell lines. By contrast, ZNF382 was strongly expressed in the 5 normal gastric tissues (Fig. 2A). ZNF382 expression in the GC tissue samples and paired tumor adjacent samples was then assayed by qPCR and immunohistochemistry. A total of 55 GC tissues and 29 matched adjacent non-tumor tissues were collected to determine the ZNF382 protein levels by immunohistochemistry. We found that ZNF382 was located predominantly in the nucleus (Fig. 1A). The majority of the tumor tissue samples (51/55) had a lower level of ZNF382, while the adjacent non-tumor samples (21/29) exhibited...
a higher ZNF382 level (P<0.0001) (Fig. 1B). The mRNA expression level of ZNF382 in 13 additional GC samples was markedly decreased in comparison with the paired tumor adjacent tissues (P<0.05) (Fig. 1C). These findings indicated that ZNF382 expression was downregulated in both GC cells and primary GC tissues. No association was observed between the ZNF382 expression level and the clinicopathological characteristics of the patients with GC (Table II).

ZNF382 downregulation in GC cell lines by promoter CpG methylation. We then determined whether promoter CpG methylation is the primary cause for the downregulation of ZNF382 in GC cell lines. The ZNF382 promoter methylation status in 4 GC cell lines was detected with the use of MSP. The hypermethylation of the ZNF382 promoter was observed in 3 of the 4 gastric tumor cell lines (Fig. 2A). To examine whether ZNF382 suppression was due directly to promoter methylation, we treated the MKN45 and SGC7901 cells with Aza and TSA, and then performed RT-PCR. The restoration of ZNF382 expression was observed following treatment with Aza and TSA (Fig. 2B).

We further assayed the methylation of the ZNF382 promoter in 70 GC tissues, as well as 22 matched adjacent non-tumor gastric tissues. The results revealed that ZNF382 was methylated in 100% (70/70) of primary GC tissues (Figs. 2C and 3), and the methylation level was significantly higher in the majority of the GC tissues compared with the adjacent non-tumor tissues (Fig. 3). However, we failed to identify any inter-relation between ZNF382 promoter methylation and the patient clinicopathological characteristics (data not shown).

Table II. Association between the clinicopathological characteristics of the patients with gastric cancer and ZNF382 expression.

| Parameter          | No. | None | Low  | Moderate | High | P-value |
|--------------------|-----|------|------|----------|------|---------|
| Sex                |     |      |      |          |      |         |
| Female             | 15  | 5    | 10   | 0        | 0    | 0.283   |
| Male               | 40  | 20   | 16   | 2        | 2    |         |
| Age (years)        |     |      |      |          |      |         |
| ≤60                | 28  | 13   | 14   | 1        | 0    | 0.504   |
| >60                | 27  | 13   | 11   | 1        | 2    |         |
| Tumor size (cm)    |     |      |      |          |      |         |
| ≤3                 | 12  | 6    | 5    | 1        | 0    | 0.668   |
| >3                 | 43  | 20   | 20   | 1        | 2    |         |
| Metastasis         |     |      |      |          |      |         |
| None               | 37  | 17   | 17   | 2        | 1    | 0.731   |
| Yes                | 18  | 9    | 8    | 0        | 1    |         |
| Grade              |     |      |      |          |      |         |
| G2                 | 14  | 6    | 7    | 1        | 0    | 0.793   |
| G3                 | 41  | 20   | 17   | 2        | 2    |         |
| T Stage            |     |      |      |          |      |         |
| Ta-T2              | 8   | 3    | 5    | 0        | 0    | 0.714   |
| T3-T4              | 47  | 24   | 20   | 2        | 1    |         |

P-values were calculated using the $\chi^2$ test.
ZNF382 inhibits colony formation and proliferation, and induces cell cycle arrest and the apoptosis of GC cell lines. Several methods were used to determine the function of ZNF382 in GC cells. To investigate whether ZNF382 affects
cell growth in GC, colony formation and CCK8 assays were carried out using stably transfected MKN45 and SGC7901 cells. ZNF382 expression in the cell lines was verified by RT-PCR and western blot analysis (Fig. 4A and B). The ectopic expression of ZNF382 markedly reduced the ability of the GC cells to form colonies compared with the controls (P<0.001) (Fig. 4C and D). Cell viability also decreased markedly at 48 and 72 h (P<0.01) (Fig. 4E).

In addition, flow cytometry was used to determine whether ZNF382 affects the cell cycle and apoptosis of GC cells. It was found that a greater number of ZNF382-expressing cells had accumulated in the G0/G1 phase of the cell cycle compared with the controls (P<0.001) (Fig. 5A). Subsequently, Annexin V-FITC/PI staining assay was performed to assess the rate of apoptosis. We found that ZNF382 exerted a pro-apoptotic effect on these two GC cell lines (P<0.001) (Fig. 5B), suggesting that ZNF382 acts as a potential tumor suppressor in GC.

ZNF382 suppresses cell migration and invasion in gastric tumor cells. The effects of ZNF382 on the migration and invasion of GC cells were investigated using wound-healing and Transwell assays. The results of wound-healing assay revealed that ZNF382-expressing SGC7901 cells were less able to migrate along the edges of wounds at 24 and 48 h compared with the controls, while the same phenomenon was observed in the MKN45 cells at 12 and 24 h (P<0.001) (Fig. 6A and B). Furthermore, the results of Transwell assay illustrated that the number of migrated cells was markedly decreased in the ZNF382-transfected cells compared with the controls (P<0.001) (Fig. 6C and D). In the Transwell assay, which included a Matrigel barrier, ZNF382 overexpression was associated with the inhibition of GC cell invasion through the Matrigel before traversing the Transwell chamber membrane (P<0.05, P<0.001) (Fig. 6E and F), indicating that ZNF382 inhibits the migration and invasion of GC cells.

ZNF382 can reverse EMT through NOTCH signaling in GC cells. We then examined whether ZNF382 can affect EMT in GC cells. The results indicated that ectopic ZNF382 expression reversed EMT to mesenchymal-to-epithelial transition in both cell lines examined (MKN45 and SGC7901).
results of western blot analysis and qPCR confirmed that E-cadherin expression was increased in the cells transfected with ZNF382, and the expression of SNAIL1, Twist and Vimentin was decreased (Fig. 7A-C). Moreover, immunofluorescence revealed increased staining for E-cadherin and decreased staining for Vimentin in the ZNF382-expressing cells (Fig. 7D), indicating that ZNF382 suppressed EMT in GC cells.

Recent studies have illustrated that NOTCH signaling plays a role in promoting EMT in multiple carcinoma types (14,16-18). In this study, we thus examined whether ZNF382 is related to this pathway. The results of RT-PCR and qPCR revealed that the ectopic expression of ZNF382 downregulated the important receptor and ligand markers of the NOTCH signaling pathway (e.g., NOTCH1, NOTCH2, NOTCH3, NOTCH4 and JAG1) in the MKN45 and SGC7901 cells (Fig. 8A-C). The results were verified by western blot analysis, revealing that the NOTCH signaling downstream target, HES-1, was also downregulated in ZNF382-expressing cells (Fig. 8D). Thus, these findings suggest that ZNF382 reverses the EMT process by antagonizing NOTCH signaling, although this requires further investigation.

Finally, cells that have stem-like properties are tightly connected with EMT in tumor cells. Thus, we investigated whether ZNF382 suppresses stemness in GC cells. Several markers of cell stemness, such as NANOG, octamer-binding transcription factor 4 (OCT4) and SOX2, were downregulated in ZNF382-expressing cells (Fig. 8A-C). These results were confirmed by western blot analysis (Fig. 8D), illustrating that ZNF382 suppresses both EMT and stemness in GC cells.

Discussion

Previous research has revealed that ZNF382 is commonly silenced by the methylation of its promoter, and that ZNF382 exists in multiple carcinoma types, including colorectal,
nasopharyngeal, gastric and breast carcinomas as a tumor suppressor (9). However, as a novel member of the KRAB-ZFP family, little is known about its role in GC. Thus, it is worth elucidating the direct association between ZNF382 and GC. In this study, we observed that ZNF382 expression was decreased in several GC cell lines and GC tissues. We also noted that ZNF382 expression was decreased in the AGS and MKN28 cells, while it was silent in the BGC823, MKN45 and SGC7901 cells. MSP and demethylation treatment revealed that the downregulation of ZNF382 in the GC cell lines and GC tumors was a result of promoter methylation. We then examined the tumor-repressive function of ZNF382 in the MKN45 and SGC7901 cells. The ectopic expression of ZNF382 in these two cell lines markedly repressed clonogenicity, suppressed cell proliferation, restrained migration and invasion, and induced apoptosis; these data illustrate that ZNF382 functions as a tumor suppressor in GC cells.

Moreover, ZNF382 binds to target promoters and acts as a transcriptional repressor. Therefore, investigating the target genes affected by ZNF382 may prove to be pivotal for revealing the underlying mechanisms of its suppressive effect. As such, RT-PCR and qPCR assays were carried out to screen the downstream target genes of ZNF382. Our results revealed that the ectopic expression of ZNF382 significantly reversed EMT.

Figure 6. Ectopic expression of ZNF382 inhibits the migration and invasion of gastric cancer cells. (A and B) The cellular migration abilities (MKN45 and SGC7901) were examined by wound healing assays. Images were captured at 0, 24 or 48 h. The ratio of wound healing was calculated (right panels) (**P<0.001). (C-F) ZNF382 inhibited the migration and invasion of the gastric carcinoma cells, x400 magnification (*P<0.05 and **P<0.001). Each experiment was performed 3 times.
to a mesenchymal-to-epithelial transition in both the MKN45 and SGC7901 cells, evidenced by the increased expression of the epithelial marker, E-cadherin, and the decreased expression of the mesenchymal markers, Vimentin, SNAIL1 and Twist. These findings indicate that ZNF382 may serve as a transcriptional repressor, reversing EMT in GC cells.

NOTCH is bound by its ligands, which is followed by the cleavage and release of the NOTCH intracellular domain (NICD). NICD regulates downstream target genes by translocating to the nucleus and binding specific transcriptional regulators (12,28). As recently reported, the NOTCH signaling pathway promotes EMT in multiple carcinoma types, including GC (12,14). However, it remains unclear as to whether ZNF382 is associated with the NOTCH signaling pathway in GC. RT-PCR and qPCR assays revealed that the ectopic expression of ZNF382 downregulated the expression of NOTCH1, NOTCH2, NOTCH3, NOTCH4, HES-1 and JAG1, as well as that of several stem cell markers (OCT4, SOX2 and NANOG). Some of these results were confirmed by western blot analysis, which indicated that ZNF382 overexpression downregulates NOTCH1, NOTCH3 and its downstream target, HES-1, in the MKN45 and SGC7901 cells. Thus, we hypothesized that ZNF382 may reverse EMT by antagonizing NOTCH signaling; however, further investigations are required to determine the exact mechanisms through which ZNF382 regulates EMT via NOTCH signaling. Moreover, further studies such as sphere forming assay are warranted to determine whether ZNF382 suppresses stemness properties.

In conclusion, we found that promoter methylation is a key mechanism contributing to the downregulation of ZNF382 in GC cells. We further confirmed that ZNF382 is a functional TSG in GC by inducing cell apoptosis and suppressing tumor cell growth and metastasis and may be considered as a tumor marker for GC.
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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TX and GR made substantial contributions to the conception and design of the study. LP, SL, XH and RS performed the experiments and analyzed the data; QX was involved in the acquisition of the samples and the interpretation of the data for the study; LP and TX prepared the figures and drafted the manuscript; TX and LP revised the study critically for important intellectual content; TX and GR finalized the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, and all patients provided signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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