Abstract. Cancer cell invasion and metastasis are the leading causes of the high mortality rates in patients with malignant tumors. There is accumulating evidence to indicate that dysregulated long non-coding RNAs (lncRNAs) may be involved in the progression of tumor invasion and metastasis. However, the regulatory mechanisms of the aberrant expression of lncRNAs remain largely unknown, although the roles of lncRNAs as drivers of tumor suppressive and oncogenic functions have appeared in prevalent cancer types in recent years. In the present study, we identified that the transcription factor, activating enhancer-binding protein 4 (TFAP4), acts as a key modulator of translation regulatory long non-coding RNA 1 (TRERNA1), which has been proven to promote the invasion and metastasis of gastric cancer (GC) cells. We revealed that TRERNA1 was upregulated in gastric carcinogenesis and promoted cell migration and invasion in GC. Using bioinformatics analysis, we observed that there were several potential binding sites of TFAP4 in the promoter region of TRERNA1. The knockdown of TFAP4 significantly reduced the expression level of TRERNA1, whereas the ectopic expression of TFAP4 significantly increased the expression level of TRERNA1 in GC cell lines. Dual luciferase reporter assay combined with chromatin immunoprecipitation (ChIP) revealed that TFAP4 specifically regulated the transcriptional activity of TRERNA1 by binding to the E-box motifs in the TRERNA1 promoter. In addition, there was a positive correlation between the TFAP4 and TRERNA1 expression level in clinical GC cases, which also indicated that TFAP4 can directly modulate the expression of TRERNA1. In the present study, we provide a novel potential therapeutic target and strategy for GC.

Introduction

Gastric cancer (GC) is one of the most common human cancers and the second leading cause of cancer-related mortality worldwide, as well as specifically in China (1-3). Despite the fact that the therapeutic strategies against malignant tumors have markedly improved in recent years, the metastasis of malignant tumor cells is the cause of most cancer-related deaths and remains one of the most enigmatic aspects of cancers, including GC (2,4). Therefore, identifying metastasis-associated gene expression and the molecular regulatory mechanisms would provide a promising molecular target for the treatment of cancer.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides, that lack protein coding capacity (5). Accumulating evidence indicates that lncRNAs are involved in cellular apoptosis, cell proliferation, migration and invasion (6-8) through a variety of mechanisms, including chromosome remodeling, RNA processing, localization, mRNA stability, translation and even as a competing endogenous RNA (9-11). The dysregulation of lncRNAs in tumor formation and progression has been investigated, with a particular focus on the mechanisms of action of lncRNAs. To date, the majority of studies have analyzed the functions of lncRNAs, the underlying mechanisms of dysregulated lncRNAs in tumorigenesis and their potential roles as prognostic markers or therapeutic targets for specific cancers (12). However, little is known about the transcriptional regulatory mechanisms responsible for the aberrant transcription of lncRNAs in cancers. In the present study, we characterized the regulatory mechanisms through which the transcription factor, activating enhancer-binding protein 4 (TFAP4), activates the expression of lncRNA TRERNA1, which plays an important role in invasion and metastasis in GC, by binding to its promoter. Thus, the present study may provide a novel potential therapeutic strategy and target for GC. Our findings may also contribute...
to the improvement of individualized treatment decisions for GC patients.

Materials and methods

Tissue collection and ethics statement. A total of 48 paired fresh gastric cancer tissues and adjacent non-tumorous gastric tissues were analyzed in the present study (clinicopathological characteristics shown in Table I). The patients had undergone surgical resection between May, 2010 and December, 2014 at the Affiliated Jiangyin Hospital of Medical School (Jiangyin, Jiangsu, China) of Southeast University, China. The study was approved by the Research Ethics Committee of Southeast University, and written informed consent was obtained from all patients.

Cell culture. The GC cell lines, AGS, SGC-7901 and BGC-823, were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The MKN-74 cell line was purchased from Cellcook Biotech Co., Ltd (Guangzhou, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Wisent Inc., St. Bruno, QC, Canada), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in an incubator humidified air at 37˚C with 5% CO₂.

Reverse transcription-quantitative PCR (RT-qPCR) assay. Total RNA was first extracted from the cultured cells or tissues using TRIzol reagent (Invitrogen). Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China). Quantitative (real-time) PCR expression analyses were performed using the SYBR® Premix Ex Taq™ II kit (Takara). The results were normalized to the expression level of β-actin and all RT-qPCR data were evaluated using the 2^(-ΔΔCq) method (13), and their gene-specific primers as follows. TFAP4 forward, 5’ -GCA GGC AAT CCA GCA CAT-3’ and reverse, 5’ -GTC ACT ACA GAG CCC TCC TAT CAC GGG-3’. The RT-qPCR thermocycling conditions for TFAP4 and β-actin began with an initial hold for 2 min at 95˚C, followed by 40 cycles of denaturation at 95˚C, annealing at 60˚C and extension at 72˚C all for 30 sec. Each experiment was performed in triplicate.

Plasmid construction and cell transfection. The cDNA TRERNA1 was synthesized by Genewiz (Suzhou, China) and then cloned into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). The cDNA of TFAP4 was produced by reverse-transcription PCR (RT-PCR) and then cloned into the HindIII/EcoRI sites of the pcDNA3.1 plasmid. The primer sequences for TFAP4 PCR amplification were as follows: Forward, 5’-CCACGCTTTAGGATTTTATGCGTGC CC-3’ and reverse, 5’-GGTGAGATTTGGGCTAGTGC AGGGAA-3’. Short hairpin RNA (shRNA) targeting TFAP4 was synthesized by Genewiz, and ligated into the BglII/HindIII sites of the pSUPER-EGFP vector (OligoEngine, Seattle, WA, USA) after annealing. The sequences were as follows: 5’-GAT CCCGGTATAGGGGCTCTAGTCTACAGAGACATC AGAGGCCTCCTACTACCTTTGGGA-3’ and 5’- AGC TTTCTCAAATAAGTGATAGGAGGCTCTGATGCTCTT

Table I. Association of TFAP4 expression with clinicopathological characteristics of patients with GC.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T&gt;N</th>
<th>T≤N</th>
<th>P-value</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>8</td>
<td>2</td>
<td>0.868</td>
</tr>
<tr>
<td>≥60</td>
<td>27</td>
<td>11</td>
<td></td>
</tr>
<tr>
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<td>1.000</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>5</td>
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</tr>
<tr>
<td>Male</td>
<td>25</td>
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<td>Lauren classification</td>
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<tr>
<td>Intestinal type</td>
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<td></td>
</tr>
<tr>
<td>Diffuse type</td>
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<tr>
<td>Histological grade</td>
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<td>High</td>
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<td>Moderate</td>
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</tr>
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<td>Poor</td>
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<td>4</td>
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</tr>
<tr>
<td>Stage III/IV</td>
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<td>Lymph node metastasis</td>
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</tr>
<tr>
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<td>11</td>
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</tr>
<tr>
<td>Yes</td>
<td>24</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Chi-square test, *P<0.05. T, tumor tissue; N, non-tumor tissue.

Luciferase reporter assay. The TRERNA1 promoter fragments containing the predicted TFAP4 binding site were amplified by PCR (primers are listed in Table II), and then subcloned into the downstream of the luciferase reporter gene in the pGL3 Luciferase Reporter Vectors (Promega Corporation, Madison, WI, USA). The wild-type sequence with wild-type E2 site

TGAACTACAGAGCCTCTCATCAGGG-3’. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). For RT-qPCR assays, at 24 h post-transfection, the cells were lysed and total cellular RNA was extracted using TRIzol reagent (Invitrogen), and the transfection efficiency was determined by RT-qPCR.

Cell migration and invasion assays. Wound healing, cell migration and invasion assays were performed as previously described (14). Briefly, a scratch wound was generated using a 10 µl blunt pipette tip, and cells were cultured in serum-free medium, after being washed with phosphate-buffered saline (PBS) to remove floating cells. Cell migration and invasion assays were performed with a Transwell chamber with 8-µm pore size (EMD Millipore, Billerica, MA, USA). Cells were seeded into the upper chamber, and the cells migrating through the pores or invading through the Matrigel were fixed and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Nantong, China) following 24-36 h of incubation.

Luciferase reporter assay. The TRERNA1 promoter fragments containing the predicted TFAP4 binding site were amplified by PCR (primers are listed in Table II), and then subcloned into the downstream of the luciferase reporter gene in the pGL3 Luciferase Reporter Vectors (Promega Corporation, Madison, WI, USA). The wild-type sequence with wild-type E2 site
Table II. Primer sets designed to construct luciferase reporter vectors containing the predicted E-box sequence in the promoter region of the TRERNA1.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Primer</th>
<th>5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 E3</td>
<td>Sense</td>
<td>ATATGCTAGCTAAAGCACACACCACCTTGCC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATATAAGCTTGTCCACCCACTTCACAAA</td>
</tr>
<tr>
<td>E2</td>
<td>Sense</td>
<td>ATATGCTAGCCTAGCTTTCCCGCTTTCCC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATATAAGCTTTTAGCTGTTTTGCCCTCTT</td>
</tr>
<tr>
<td>E3</td>
<td>Sense</td>
<td>ATATGCTAGGCTGTCAGAAGGGAACCA</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATATAAGCTTCAAACAGGCGGAGGAGTA</td>
</tr>
</tbody>
</table>

Table III. Primer sets designed to amplify the conserved E-box sequence in the promoter region of the TRERNA1.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Primer</th>
<th>5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Sense (P1)</td>
<td>ATATAAGCACACACCACCTGC</td>
</tr>
<tr>
<td></td>
<td>Antisense (P1)</td>
<td>ATACCAAAAAAGAAAAACCC</td>
</tr>
<tr>
<td>E2</td>
<td>Sense (P2)</td>
<td>CACTGACTTTCCGCTTTCCC</td>
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<tr>
<td></td>
<td>Antisense (P2)</td>
<td>TTAGCAGCTTCCCTCTTCTT</td>
</tr>
<tr>
<td>E3</td>
<td>Sense (P3)</td>
<td>CAGGCGTCAAGGGAACCA</td>
</tr>
<tr>
<td></td>
<td>Antisense (P3)</td>
<td>CAACAGGCGGAGGAGGAGTA</td>
</tr>
</tbody>
</table>

(5′-CAGCTG-3′) was 5′-CTAGAGGCACTGACTTTCCGC TTTCCAGCTGTGAGGTGCTTTGCTCAATTT-3′, and the mutant-type sequence with mutant-type E2 site (5′-TGA TCA-3′) was 5′-CTAGAGGCACTGACTTTCCGGTTTGGCACTTTG GTTTGCTTTTCCCTTT-3′. These sequences were synthesized by Genewiz and subcloned into the pGL3 plasmid, separately. The luciferase activities were determined using a dual luciferase assay kit (Promega Corporation) according to the manufacturer's protocol. The relative luciferase activity was normalized to the Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed using the ChIP kit (EMD Millipore) according to the manufacturer's instructions in MKN-74 cells. Antibodies against TFAP4 [AP-4(C-18)X, dilution 1:500] and normal mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primers designed to amplify the conserved E-box in the promoter of the TRERNA1 are listed in Table III.

Statistical analysis. Statistical analyses were performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) or GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The significance of differences in the TFAP4-dependent regulation of TRERNA1 expression and transcriptional activity of E-box (E1-E3, E2 and E3) of the TRERNA1 promoter region between TFAP4 and control groups were estimated using a Student's t-test. The association between TFAP4 expression and pathological features of GC was analyzed with the Chi-square ($\chi^2$) test. The correlation between the TFAP4 and TRERNA1 expression levels was analyzed using Pearson's correlation coefficient test. All P-values presented were two-sided and P<0.05 was considered to indicate a statistically significant difference.

Results

TFAP4 upregulates the expression level of IncRNA TRERNA1. Transcription factors are the most important regulators of gene transcription by promoting or blocking specific genes (15). In order to clarify the transcription factors that may contribute to the regulation of TRERNA1, in this study, we first screened the potential transcription factors and their possible binding sites by scanning the TRERNA1 gene promoter from upstream -4 kb to the downstream +1 kb region through the online analytical website (http://gene-regulation.com/pub/databases.html). The predicted results indicated that a variety of transcription factors, such as TFAP4, CCAAT enhancer binding protein beta (CEBPB), heat shock factor 1 (HSF1), Jun proto-oncogene (JUN), nuclear factor (NFE2L2) and cAMP response element binding protein 1 (CREB1) could bind to the promoter region of TRERNA1 (data not shown). TFAP4 was considered as a priority candidate, since there were multiple binding sites in the vicinity to the transcription start site (TSS) of TRERNA1 (Fig. 1A).

In order to clarify the effect of TFAP4 on the expression of TRERNA1, we constructed TFAP4 overexpression and silencing vectors, and transfected these constructs into GC cells. As displayed in Fig. 1B, the enhanced expression of TFAP4 markedly increased the expression level of TRERNA1 gene promoter activity in SGC-7901 and BGC-823 cells, particularly in the promoter region of TRERNA1 (data not shown). TFAP4 was considered as a priority candidate, since there were multiple binding sites in the vicinity to the transcription start site (TSS) of TRERNA1 (Fig. 1A).

TFAP4-regulated transcriptional activity of TRERNA1 depends on the E-box of its promoter. To determine whether the TFAP4-dependent regulation of the expression of TRERNA1 is attributed to activated transcription, a series of constructs containing different fragments of E-box (E1-E3, E2 and E3) of the TRERNA1 promoter region were generated (Fig. 2A). As displayed in Fig. 2B, the overexpression of TFAP4 significantly enhanced TRERNA1 gene promoter activity in the SGC-7901 and BGC-823 cells, particularly in
the E1-E3 fragment containing three TFAP4 binding sites in the TRERNA1 promoter (Fig. 2B).

A previous study revealed that promoters activated by TFAP4 are located closer to the TSS than those present at TFAP4-suppressed promoters (16). In this study, to further characterize the role of the E-box motif situated on TRERNA1 as a transcriptional activator, we selected the E2 site to construct the dual luciferase reporter vector containing the motif of both the wild- (CAGCTG) and mutant-type (TGATCA) at the E2 sites (Fig. 2C). Dual-luciferase assays revealed that the overexpression or silencing of TFAP4 significantly affected the transcriptional activity of the wild-type E2 sites in SGC-7901, BGC-823 and MKN-74 cells (Fig. 2D and E). In contrast to the wild-type, TFAP4 had no significant impact on the activity of the E2-mutant TRERNA1 promoter in GC cells (Fig. 2D and E). These results also demonstrated that mutations abolished TFAP4 binding to E2. Collectively, our results revealed that TFAP4 regulation of the transcriptional activity of TRERNA1 was dependent on the CACGTG motifs in the TRERNA1 promoter.

TFAP4 specifically binds to the E-box motif of TRERNA1 promoter in vitro. Previous studies have revealed that TFAP4 is effective for both the suppression and/or activation of target genes. TFAP4 downregulates the expression level of p16 and p21 to suppress cell senescence by directly binding the putative E-box site (CAGCTG) (16,17). In this study, in order to determine whether TFAP4 promotes TRERNA1 transcriptional activity by directly binding to its promoter, we designed PCR primers (Table III) for the amplification of the three E-box sites (E1, E2 and E3) in the TRERNA1 promoter region (Fig. 3A). As displayed in Fig. 3B, the results indicated that TFAP4 bound to the promoters of TRERNA1 in the vicinity of their E-box binding motifs E1, E2 and E3, which reflected specificity of the TFAP4 binding to the E-box motifs located in the promoter of TRERNA1 (Fig. 3B). Notably, ChIP different enrichment observed among E1, E2 and E3 (Fig. 3B), indicated that the maximal binding capacity of TFAP4 to the TRERNA1 promoter presented at E2. These results demonstrated that TFAP4 directly regulated lncRNA TRERNA1 transcription by targeting the E-box of its promoter region, particularly to the E2 in GC cells.

Elevated TFAP4 is positively related to metastasis in GC cases. Numerous studies have revealed that TFAP4 plays a vital role in carcinogenesis and tumor development (16,18,19). In this study, in order to understand the expression pattern of TFAP4 in GC tissues and the association between the expression of TFAP4 with the clinicopathological features of GC patients, we analyzed the expression of TFAP4 in 48 pairs of GC tissues and non-tumorous tissues. As revealed by RT-qPCR analysis, the expression levels of TFAP4 were markedly higher in the tumor tissues compared with the adjacent non-tumor tissues (Fig. 4), and a significant association was observed between the expression level of TFAP4, lymph node metastasis and Lauren classification in GC specimens, although no other significant associations were observed concerning the TFAP4 level and GC patients (Table I). These results indicated the potential role of TFAP4 during GC development and dissemination through the regulation of TRERNA1.

Increased expression of TRERNA1 is positively related to the elevated TFAP4 level and promotes the migration and invasion ability of GC cells. Since TRERNA1 was revealed to be a direct transcriptional target of TFAP4 in GC cells, we investigated whether TFAP4 influenced the expression
Figure 2. TFAP4 stimulates the activity of the TRERNA1 promoter in GC cells. (A) A schematic diagram depicting the construction of the dual-luciferase reporter vector with truncated fragments containing different E-boxes (E1-E3, E2 and E3) in the TRERNA1 promoter. (B) Compared with the control group, TFAP4 significantly increased the transcriptional activity of TRERNA1 promoter containing different truncated E-box fragments in SGC-7901 and BGC-823 cells by dual luciferase assays. (C) Schematic representation of the structure and the oligonucleotide sequences containing the wild-type and mutated E-box2 motifs (E2) in the TRERNA1 promoter. (D and E) Compared with the control group, TFAP4 affected the transcriptional activity of wild-type E-box, however it did not affect mutant-type E-box. Luciferase activities were determined by the dual-luciferase assay system 48 h after transfection in AGS, BGC-823 and MKN-74 cells. Vertical columns and error bars represent mean ± SD of three independent experiments. *P<0.05, **P<0.01.
level of TRERNA1 in GC tissue specimens. As expected, the increased expression of TRERNA1 was observed to have a strong positive correlation with increased TFAP4 expression in GC cases (Fig. 5A). Subsequently, to assess the potential role of TRERNA1 in GC cell migration and invasion, we transfected TRERNA1 overexpression construct into BGC-823 and SGC-7901 cells, in which the expression level of TRERNA1 is relatively low among human GC cell lines (20). Wound healing assay revealed that the enforced expression of TRERNA1 in GC cells markedly enhanced the ability of the BGC-823 and SGC-7901 cells compared with the control (Fig. 5B). Similarly, the enforced expression of TRERNA1 increased the migration and invasion ability of the BGC-823 and SGC-7901 cells (Fig. 5C). These data further indicated that the increased expression level of TRERNA1 upregulated by TFAP4 promoted the migration and invasion of GC cells.

Discussion

GC is the fifth most common malignancy and the second leading cause of cancer-related mortality worldwide (21). Tumor metastasis is responsible for ~90% of cancer-associated deaths, yet this process remains poorly understood (4,5,22-26). Therefore, it is urgent to uncover the pivotal genes and underlying molecular mechanisms in cancer metastasis. With the rapid development of sequencing technology, researchers found that only ~2% of the human genome DNA includes protein-coding sequences (27). Non-coding RNAs, including IncRNAs have gained widespread attention as a potentially crucial molecule in disease onset and development in recent years (28), and hundreds of IncRNAs have been identified to be dysregulated in human cancers (29-31). Accumulating evidence has demonstrated that dysregulated IncRNAs, such as H19HOXA11-AS and UCA1 have already been confirmed to contribute to cell invasion and metastasis in GC (32-35).

TRERNA1 has been reported to act as a metastasis-promoting oncogene in lung and breast cancer (36,37). In the present study, we observed that TRERNA1 was significantly upregulated in GC tissues compared with paired normal tissues. Data from in vitro and in vivo experiments have also indicated that TRERNA1 acts as an onco-IncRNA, which promotes the metastasis and invasion of GC cells (20). A recent study demonstrated that miR-190a inhibited epithelial-mesenchymal transition (EMT) in hepatocarcinoma cells via targeting the IncRNA TRERNA1 (38). By bioinformatics analysis, in this study, it was suggested that TFAP4 could be considered as a potential regulator of TRERNA1. In addition, a loss-of-function and gain-of-function study revealed that TFAP4 regulated the expression of TRERNA1, and double luciferase activity assay revealed that TFAP4 regulated the transcriptional activity of TRERNA1. Furthermore, ChIP-PCR revealed that TFAP4 bound directly to the E-box site of the TRERNA1 promoter to regulate its expression.
We also observed that the expression level of TFAP4 was significantly related to the expression level of TRERNA1 in GC cases. These results indicated that TFAP4 regulated the expression of TRERNA1 in gastric carcinogenesis.

TFAP4 belongs to the helix-loop-helix family and plays an important regulatory role by binding to the conserved E-box (CAGCTG) sequence in transcriptional networks, thus affecting cell proliferation, differentiation, cell lineage determination and other essential biological processes (39). Previous studies have revealed that TFAP4 regulates the expression of genes such as p21 and p16 (17,40), caspase-9 (41) and HDM2 (42). According to recent research, the overexpression of TFAP4 has been reported in colorectal, breast, lung and prostate cancer. TFAP4 has been shown to induce EMT
and enhance the migration and invasion of colorectal cancer cells (16-18,40,43). In GC, research has revealed that the expression of TFAP4 is significantly higher in tumor tissue than in non-neoplastic tissue, and an elevated TFAP4 expression significantly and positively correlates with the degree of tumor differentiation, depth of tumor invasion, lymph node metastasis and TNM stage (19). The downregulation of TFAP4 has been shown to inhibit proliferation, induce cell-cycle arrest and promote the apoptosis of GC cells (44). TFAP4 performs critical functions in GC by participating in signaling pathways such as the ECM-receptor-interaction and spliceosome (45). Therefore, the function and molecular mechanisms of TFAP4 in gastric carcinogenesis warrant further investigation.

The genome-wide characterization of TFAP4 DNA binding sites and genes directly regulated by TFAP4 were identified using ChIP, next-generation sequencing and bioinformatics analysis (16). These studies on TFAP4-mediated transcriptional regulation have mainly focused on protein-coding genes; however the regulation of TFAP4 to lncRNAs has not yet been investigated. In the present study, our findings indicated that upregulated lncRNA TRERNA1 expression, which could contribute to gastric cell invasion and metastasis was induced by TFAP4 by directly binding to the CACGTG motifs in the promoter of TRERNA1. The present study clarified the mechanism of upregulated TRERNA1, which was involved in the invasion and metastasis of GC, and also revealed a new field of TFAP4 regulation of the expression of lncRNA in tumorigenesis. Furthermore, our study indicated that the TFAP4-TRERNA1 axis may be a promising novel therapeutic target for the intervention therapy of GC.

Acknowledgements

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HF conceived and designed the study. HW performed the experiments and wrote the manuscript. XL, PG and WS provided assistance for clinical sample collection, preservation, data analysis and statistical analysis. MZ, YL and ZZ were involved in interpreting the results, manuscript editing and manuscript review. All authors have read and approved the content of the manuscript.

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of Southeast University, and written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References