BTEB2-Activated IncRNA TSPEAR-AS2 Drives GC Progression through Suppressing GJA1 Expression and Upregulating CLDN4 Expression

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Long non-coding RNAs (lncRNAs) are characterized as key layers of the genome in various cancers. TSPEAR-AS2 was highlighted to be a candidate lncRNA potentially involved in gastric cancer (GC) progression. However, the clinical significance and mechanism of TSPEAR-AS2 in GC required clarification. The clinical significance of TSPEAR-AS2 was elucidated through Kaplan-Meier Plotter. The mechanism of TSPEAR-AS2 in GC was clarified in vitro and in vivo using luciferase reporter, chromatin immunoprecipitation, RNA immunoprecipitation assays, and animal models. TSPEAR-AS2 elevation was closely correlated with overall survival of GC patients. A basic transcription element-binding protein 2 (BTEB2)-activated TSPEAR-AS2 model was first explored in this study. TSPEAR-AS2 silencing substantially reduced tumorigenic capacities of GC cells, while TSPEAR-AS2 elevation had the opposite effect. Mechanistically, TSPEAR-AS2 bound with both polycomb repressive complex 2 (PRC2) and argonaute 2 (Ago2). TSPEAR-AS2 knocked down significantly decreased H3K27me3 levels at promoter regions of gap junction protein alpha 1 (GJA1). Ago2 was recruited by TSPEAR-AS2, which was defined to sponge miR-1207-5p, contributing to the repression of claudin 4 (CLDN4) translation. The axis of EZH2/GJA1 and miR-1207-5p/CLDN4 mediated by BTEB2-activated TSPEAR-AS2 plays an important role in GC progression, suggesting a new therapeutic direction in GC treatment.

INTRODUCTION

Gastric cancer (GC) is an important issue strongly linked to public health, ranking as the third leading cause of cancer death globally.3 Despite tremendous progress in the clinical detection and treatment of GC in recent decades, the prognosis remains unsatisfactory, with a 5-year survival rate of less than 30% in most countries.2,3 The most important reasons for poor prognosis are largely due to late diagnosis, a high postoperative recurrence rate, and metastasis.4,5 Thus, a great challenge lies ahead in understanding the molecular mechanism of GC in identifying novel prognostic molecular biomarkers that can facilitate the development of appropriate therapeutic strategies earlier in GC.

Integrative genomic studies have shown that only 2% of DNA sequences can encode proteins, with more than 90% of these transcripts being actively transcribed; most of these transcripts are referred to as non-coding RNAs (ncRNAs).6 Based on size, ncRNAs are divided into two groups, long non-coding RNAs (lncRNAs) over 200 nucleotides and small ncRNAs such as microRNAs (miRNAs).6 Epigenetic modifiers, including lncRNAs and miRNAs, act to impact on human malignancies.9–11 Currently, increasing studies show that lncRNAs usually interact with RNA-binding protein (RBP) to participate in a variety of biological processes, such as chromatin remodeling, transcriptional regulation, and RNA degradation.12,13 Identification of lncRNA-dependent mechanisms of carcinogenesis is essential for understanding additional complexities of various tumors. Importantly, combined targeting of lncRNA-modulated key axes may provide a prospective rationale for cancer therapy.12,14

InoRNA TSPEAR-AS2 was previously reported to be involved in the regulation of hypoxia-induced pulmonary artery hypertension in vitro.15 In this study, TSPEAR-AS2 is defined as a GC-associated lncRNA that we identified by analyzing The Cancer Genome Atlas.
(TCGA) and Gene Expression Omnibus (GEO) datasets. Coding potential assessment tool (CPAT)\textsuperscript{16} and coding potential calculator (CPC) analyses\textsuperscript{17} predicted the low coding potential of lncRNA TSPEAR-AS2 (Figure 1B). It was shown that high TSPEAR-AS2 level closely associated with the overall survival (OS) of patients with GC, suggesting the predictive value of TSPEAR-AS2 in the prognosis of GC patients. Then, we demonstrated a mechanism by which overexpression of TSPEAR-AS2 in GC cells was activated by basic transcription element binding protein 2 (BTEB2). This mechanistic model of BTEB2/TSPEAR-AS2 was first elucidated in this research. In vitro and in vivo assays found that silencing of TSPEAR-AS2 markedly inhibited cell growth, migration, and invasion. By contrast, ectopic expression of TSPEAR-AS2 played an oncogenic role in GC progression. Based on the analysis of high-throughput RNA sequencing (RNA-seq), we identified the contribution of TSPEAR-AS2 and its key target gene gap junction protein alpha 1 (GJA1) in GC progression. Specifically, TSPEAR-AS2 epigenetically inhibited GJA1 expression through the interaction with polycomb repressive complex 2. Moreover, argonaut 2 (Ago2) was recruited by TSPEAR-AS2, which was defined to sponge miR-1207-5p, thereby contributing to the repression of claudin 4 (CLDN4) translation. In conclusion, our study aimed to characterize novel IncRNAs closely correlated with GC. The axis of enhancer of zeste 2 (EZH2)/GJA1 and miR-1207-5p/CLDN4 mediated by BTEB2-activated-TSPEAR-AS2 may provide new clues facilitating the identification of therapeutic targets as well as effective biomarkers for patients with GC.

RESULTS

IncRNA TSPEAR-AS2 Was Upregulated in GC and Tightly Associated with Survival Rates of Patients with GC

As an effort to identify IncRNAs closely correlated with GC, the publicly available data were downloaded from TCGA and GEO datasets. It was revealed that IncRNA TSPEAR-AS2 exhibited obvious up-regulation in GC tissues (n = 375), as compared with normal tissues (n = 32) (Figure 1A). Moreover, the analysis of GSE66229 datasets demonstrated that TSPEAR-AS2 was obviously increased in GC tissues (n = 300) compared with normal tissues (n = 100) (Figure 1A). Additionally, TSPEAR-AS2 was markedly upregulated in GC cells compared with GES-1 cell line (Figure S1A). SGC7901 and MGC803 cells were selected for further research owing to their remarkable elevation of TSPEAR-AS2. Next, CPAT and CPC analyses were performed to reveal the low coding potential of IncRNA TSPEAR-AS2 (Figure 1B). To explore the correlation between TSPEAR-AS2 level and OS of patients, we also analyzed the publicly available data from 631 GC patients using Kaplan-Meier Plotter (http://kmplot.com/analysis/). As shown in Figure 1C, higher TSPEAR-AS2 expression closely associated with worse OS, highlighting the prognostic value of TSPEAR-AS2 in GC (Figure 1C).

TSPEAR-AS2 Was Transcriptionally Activated by Transcription Factor BTEB2 in GC

Currently, transcription factors (TFs) have been found to be capable of driving the expression of IncRNAs with tumor-promoting functions.\textsuperscript{18,19} Therefore, we made assumptions that certain transcription factors were responsible for ectopic expression of TSPEAR-AS2. BTEB2, a zinc-finger transcription factor, has been identified to modulate activities correlated with various functions such as cell growth, proliferation, differentiation, and tumorigenesis.\textsuperscript{20–22} It was shown that BTEB2 was significantly enriched in GC tissue samples (Figure 1E). Further analysis discovered the positive correlation between TSPEAR-AS2 and BTEB2 in GC tissue samples (Figure 1D). Then, we investigated the transcriptional regulation of TSPEAR-AS2 using JASPAR (http://jaspardev.genereg.net/) databases and found that BTEB2 can possibly bind to the promoter region of the TSPEAR-AS2 gene. The predicted binding regions are represented as E1, E2, and E3, shown in Figure 1G. In this regard, we deduced that BTEB2 might activate the transcription of TSPEAR-AS2. Therefore, the protein level of BTEB2 was first examined in GC cells with BTEB2 knockdown or overexpression. Compared with the control group, the protein level of BTEB2 was dramatically increased in GC cells with transfection of pcDNA-BTEB2 (Figure S1B). By contrast, small interfering RNAs (siRNAs) against BTEB2 obviously decreased the protein level of BTEB2 in GC cells (Figure S1B). Then, qRT-PCR assays were used to test the alteration of TSPEAR-AS2 in BTEB2-depleted or BTEB2-overexpressed GC cells. As expected, BTEB2 knockdown obviously decreased TSPEAR-AS2 expression, and BTEB2 overexpression markedly increased TSPEAR-AS2 expression, identifying BTEB2 as potential upstream regulator of TSPEAR-AS2 (Figure 1F).

To assess the transcription activation of BTEB2 on the promoter of TSPEAR-AS2, we cloned the promoter region of TSPEAR-AS2 into a luciferase reporter plasmid and made deletions at the promoter of TSPEAR-AS2 (Figure 1G). Then, cotransfection was performed in HEK293T cells with pcDNA-BTEB2/empty vector and luciferase reporter vectors TSPEAR-AS2 promoter full length (F), TSPEAR-AS2...
promoter deletion 1# (D-1), TSPEAR-AS2 promoter deletion 2# (D-2), or TSPEAR-AS2 promoter deletion 3# (D-3) (Figure 1G). Dual-luciferase reporter analysis showed that D-1 caused a significant downregulation in promoter activity compared with F, D-2, and D-3 (Figure 1G). These findings elucidated the binding of BTEB2 to this region and its efficacy of luciferase activation. Meanwhile, chromatin immunoprecipitation (ChIP) experiments were implemented. Our data demonstrated that BTEB2 directly bound to their binding sites on TSPEAR-AS2 promoter region in GC cells (Figure 1H). Furthermore, downregulation or overexpression of BTEB2 decreased or increased BTEB2 enrichment within the TSPEAR-AS2 promoter, respectively (Figure 1I). Taken together, our data demonstrated that increased TSPEAR-AS2 expression could be transcriptionally activated by the key transcription factor BTEB2 in GC.

**TSPEAR-AS2 Boosted the Oncogenic Activities of GC Cells**

To illuminate the function of TSPEAR-AS2 in GC progression, we performed loss- and gain-of-function assays to effectively alter TSPEAR-AS2 expression in GC cells (Figures 2A and 2B). Knockdown of TSPEAR-AS2 obviously inhibited GC cell proliferation and impaired colony-formation ability (Figures 2C and 2E), while TSPEAR-AS2 overexpression displayed the reverse effects (Figure 2D and 2F). Moreover, Ethynyldeoxyuridine (EdU) assays verified that TSPEAR-AS2 knockdown obviously decreased the proliferative capacities of GC cells (Figure 2G). Flow cytometry assays demonstrated that inhibition of TSPEAR-AS2 significantly increased the proportion of apoptosis in GC cells (Figure 2H). Our data verified that the inhibition of cell proliferation induced by TSPEAR-AS2 knockdown could be partly attributed to activated apoptosis in GC cells. In addition, TSPEAR-AS2 repression markedly weakened the migratory and invasive capacities of GC cells (Figures 2I and S2A), and overexpression of TSPEAR-AS2 elicited the opposite impacts (Figures 4K and S2B).

To determine TSPEAR-AS2 impact on tumorigenic capacities in vivo, a xenograft tumor model was constructed. TSPEAR-AS2-stable-knockdown MGC803 cells or control cells were subcutaneously injected into male nude mice. We found that the tumors derived from the control group were markedly larger than tumors obtained from the TSPEAR-AS2-stable-knockdown group (Figure 2J). Additionally, the volume and weight of the tumors formed from TSPEAR-AS2-stable-knockdown group were significantly decreased compared with those derived from the control group, indicating the promotion effects of TSPEAR-AS2 on the tumorigenic abilities of GC cells (Figures 2K and 2L). As shown in Figures 2M and 2N, tumors derived from the control group revealed stronger staining of Ki-67 compared with tumors obtained from the TSPEAR-AS2-stable-knockdown group (Figures 2M and 2N).

**Silencing of GJA1 Was Regulated by Interaction of TSPEAR-AS2 and Polycomb Repressive Complex 2**

To uncover the underlying mechanism about how lncRNA TSPEAR-AS2 contribute to the malignant phenotype of GC, we evaluated the gene expression profiles of a control group and TSPEAR-AS2 knockdown group via RNA-seq (Figures 3A and 3B). Gene Ontology (GO) analysis of RNA-seq assays of TSPEAR-AS2 knockdown demonstrated that the alteration in gene set was closely associated with cell migration, cell proliferation, apoptotic process, and cell growth (Figure 3D). As shown in Figures 3E and 3F, Gene Set Enrichment Analysis (GSEA) was applied to further explore the pathways involved in GC pathogenesis. Enrichment plots of GSEA highlighted that the gene signatures of negative regulation of growth and positive regulative extrinsic apoptotic signaling pathway were much more involved in TSPEAR-AS2-depleted cells compared with the control group (Figures 3E and 3F). Then, qRT-PCR assays were used to identify key regulators mediated by TSPEAR-AS2 knockdown and thus further the understanding of TSPEAR-AS2-mediated GC progression. Among these aberrantly altered key genes, GJA1 displayed the highest level in TSPEAR-AS2-depleted SGC7901 cells relative to the control group (Figure 3C). Current evidence has demonstrated that GJA1 exhibits close association with cancer development, distant metastasis, and survival condition. Moreover, GJA1 was dramatically upregulated in TSPEAR-AS2-depleted GC cells, as demonstrated by qRT-PCR and western blot experiments (Figures 3G and 3H).

To further clarify TSPEAR-AS2-involved regulatory mechanisms in GC progression, we performed subcellular fractionation and RNA-fluorescence in situ hybridization (FISH) assays in GC cells. Our findings elucidated that TSPEAR-AS2 was more prevalent in the nucleus than in the cytoplasm in SGC7901 and MGC803 cells (Figures 4A and 4B). These findings may support the potential transcriptional regulation of TSPEAR-AS2 in GC progression. Currently, researchers demonstrated that IncRNAs could modulate epigenetic modification or gene silencing through binding with RBPs. To further determine the regulatory mechanism of TSPEAR-AS2-induced GJA1 silencing, bioinformatics analysis was first conducted to predict interaction possibilities of TSPEAR-AS2 and GJA1. The analysis data indicated that TSPEAR-AS2 was predicted to potentially bind with EZH2, SUZ12, EED, STA1, and Ago2, with the score of RF or SVM greater than 0.5 (Figure 4C). To verify this prediction, radioimmunoprecipitation (RIP) assays testified the binding of TSPEAR-AS2 with EZH2, SUZ12, and AGO2 in GC cells (Figure 4D).

Methyltransferase EZH2, a critical member of PRC2, enhances methylation of H3K27, leading to silencing of tumor suppressors. The amplification of EZH2 was observed in various types of cancers. Previous research also highlighted the oncogenic role of EZH2 in GC, indicating its emerging role in this active field. Methyltransferase EZH2 can dramatically upregulate GJA1 level in GC (Figure S3A). Intriguingly, knockdown of EZH2 can dramatically upregulate GJA1 level in GC (Figure S3B). Together, GJA1 may be coregulated by TSPEAR-AS2 and EZH2 in GC.

To elucidate whether TSPEAR-AS2 was involved in regulating gene transcription by recruiting PRC2, we performed ChIP assays in...
SGC7901 and MGC803 cells. It was demonstrated that TSPEAR-AS2 knockdown significantly reduced the binding of EZH2 and H3K27me3 across the GJA1 promoter region (Figure 4G). Together, our data revealed that TSPEAR-AS2 participate in the tumorigenesis of GC through the transcriptional regulation of GJA1 via binding to PRC2 in GC cells.
Figure 3. GJA1 Acted as a Key Downstream Target of TSPEAR-AS2 in GC Progression

(A) Heatmap of altered genes in GC cells transfected with TSPEAR-AS2 siRNA or Scrambled. (B) Hierarchical clustering gene transcription altered in GC cells after knockdown of TSPEAR-AS2. (C) The qRT-PCR assays were conducted to validate the level of key genes in GC cells with TSPEAR-AS2 knockdown. (D) Gene Ontology analysis for all genes with altered expressions after knockdown of TSPEAR-AS2. (E) GSEA explored the gene sets enriched by genes in response to TSPEAR-AS2 knockdown (Negative regulation of growth). (F) GSEA explored the gene sets enriched by genes in response to TSPEAR-AS2 knockdown (Positive regulation of extrinsic apoptotic signaling pathway). (G) The expression of GJA1 was determined in GC cells treated with TSPEAR-AS2 siRNA or scrambled using qRT-PCR assays. (H) The expression of GJA1 was determined in GC cells treated with TSPEAR-AS2 siRNA or scrambled using western blot assays. *p < 0.05, **p < 0.01.
Oncogenic Function of TSPEAR-AS2 by Repressing GJA1 Expression

Given the potential regulatory role of GJA1 in TSPEAR-AS2-involved GC progression, bioinformatics analysis was implicated to verify the expression pattern of TSPEAR-AS2 in specimens from patients with GC. It was shown that GJA1 was prominently downregulated in GC tissue samples (n = 300) compared with non-tumor samples (n = 100) (Figure 4E). Meanwhile, it is interesting to note the negative relationship between GJA1 level and TSPEAR-AS2 level in GC tumor specimens (n = 300) (Figure 4F).
Figure 5. Regulation Relationship between lncRNA TSPEAR-AS2 and miR-1207-5p in GC
(A) Bioinformatics databases (miRanda, pita, and RNAhybrid) were analyzed to predict potential miRNAs binding with TSPEAR-AS2. (B) Luciferase activity of HEK293T cells cotransfected with 4 various miRNA-coding plasmids and the luciferase reporter plasmids (pmirGLO-TSPEAR-AS2-WT). Data are presented as the ratio of firefly luciferase to (legend continued on next page)
In addition, the level of GJA1 was verified in GC cells treated with empty vector or pcDNA-GJA1. It can be observed that GC cells with pcDNA-GJA1 displayed remarkable upregulation compared with the control group (Figure 4H). Cell viability tests verified the inhibition impact of GJA1 overexpression on GC cell proliferation (Figure 4I).

To investigate the function of GJA1 in TSPEAR-AS2-induced promotion of GC proliferation, invasion, and migration, rescue assays were conducted in GC cells, which were cotransfected with pcDNA-TSPEAR-AS2 and pcDNA-GJA1. Of note, ectopic expression of TSPEAR-AS2 remarkably activated GC cell proliferation, migration, and invasion, and overexpression of GJA1 was capable of reversing the influence mediated by TSPEAR-AS2 (Figures 4J, 4K, and 5B). These data elucidated that the effects of TSPEAR-AS2 on GC progression may partially depend on the regulation of GJA1.

IncRNA TSPEAR-AS2 Elevated CLDN4 Expression through Competing for miR-1207-5p

Importantly, TSPEAR-AS2 may also play a post-transcriptional role in gene regulation. It is known that IncRNAs have been implicated in post-transcription regulation, such as sponging activity for miRNAs.33,34 To investigate whether TSPEAR-AS2 played such a role, we used miRanda, pita, and RNAhybrid to make a prediction of possible miRNAs targeting sites on TSPEAR-AS2 (Figure 5A). According to the prediction result and current evidence, we filtered out a number of miRNAs, which have been shown to inhibit the malignant phenotype of tumor.35–37 Therefore, the implementation of dual-luciferase reporter assays testified the interacted correlation between TSPEAR-AS2 and these miRNAs. We observed that the luciferase activity of pmir-GLO-TSPEAR-AS2 that contained full-length TSPEAR-AS2 can be significantly repressed by the transfection of miR-874-3p, miR-1207-5p, and miR-4779 (Figure 5B). Moreover, miRNA-1207-5p showed the strongest inhibition effect (Figure 5B). Thus, miR-1207-5p was selected for further analysis.

Thereafter, site-targeted mutagenesis was constructed within the speculative miR-1207-5p-binding site in the IncRNA TSPEAR-AS2 sequence (Figure 5C). It was shown that pmir-GLO-TSPEAR-AS2-mut (TSPEAR-AS2-Mut) failed to respond to miR-1207-5p, clarifying that TSPEAR-AS2 acts to sponge miR-1207-5p (Figure 5C). In addition, knockdown of TSPEAR-AS2 gave rise to an elevated level of miR-1207-5p, whereas the boosted TSPEAR-AS2 level reflected contrary impact on miR-1207-5p level in GC cells (Figure 5D). However, overexpression of miR-1207-5p displayed no significant difference on TSPEAR-AS2 expression (Figure 5C). More importantly, RIP assays were performed in GC cells to verify that TSPEAR-AS2 and miR-1207-5p were involved in the RNA-induced silencing complex (RISC). It was shown that both TSPEAR-AS2 and miR-1207-5p are drastically enriched in AGO2 immunoprecipitates compared with those in the immunoglobulin G (IgG) pellet in SGC7901 and MGC803 cells (Figure 5E). These results suggested that TSPEAR-AS2 physically existed in the AGO2-based miRNA-modulated repression complex and exhibited close association with miR-1207-5p, but miR-1207-5p did not induce TSPEAR-AS2 degradation.

Previous evidence has indicated that miR-1207-5p is of great significance in many fetal malignancies.38,39 Nevertheless, the functional biology of miR-1207-5p was not comprehensively elucidated in GC. Furthermore, the mechanistic model of miR-1207-5p in TSPEAR-AS2-mediated GC progression remains unclear. Therefore, the profile of the miR-1207-5p level was verified in paired tissue specimens from patients with GC (GSE54397) (Figure 5F). The significant downregulation of miR-1207-5p can be observed in GC tissue samples relative to matched normal tissue samples (Figure 5F). Then, significant overexpression or knockdown of miR-1207-5p level was made in GC cells using mimics or inhibitors against miR-1207-5p (Figure 5G). We gave the first evidence that the ectopic level of miR-1207-5p dramatically improves the apoptotic proportion of GC cells (Figures 5H and 5I). Consistently, knockdown of miR-1207-5p could obviously activate GC cell proliferation (Figure 5J). By contrast, elevation of miR-1207-5p impacted contrary effects on GC cell proliferation (Figure 5J). These findings highlight the critical role of miR-1207-5p in TSPEAR-AS2-correlated GC progression.

To detail the TSPEAR-AS2-involved mechanism of post-transcriptional regulation, data mining was processed in RNA-seq analysis of a control group and the TSPEAR-AS2-knockdown group. We observed that a number of key genes displayed obvious downregulation in abundance of log2FC (fold change) ≤ −1 (Figure 3B). Then, a series of verification experiments were applied to test the alteration after TSPEAR-AS2 knockdown. It was found that a number of key genes were dramatically decreased in TSPEAR-AS2-depleted GC cells, including CLDN4, solute carrier family 25 member 10 (SLC25A10), phospholipase C eta 2 (PLCH2), and sushi domain containing 2 (SUSD2), in both SGC7901 and MGC803 cells. Among these potential genes, TSPEAR-AS2 knockdown displayed the strongest inhibition effects on CLDN4 level (Figure 6A). It is known that the alteration of CLDN4 is tightly linked with the malignant progression of various malignancies and therapeutic resistance.40–42 Here, we found the obvious downregulation of CLDN4 in TSPEAR-AS2-depleted GC cells and significant upregulation of CLDN4 in TSPEAR-AS2-overexpressed GC cells, identifying that CLDN4 may play an essential role in TSPEAR-AS2-mediated GC cell progression.
be a key downstream effector of TSPEAR-AS2 in GC progression (Figure 6D).

Subsequently, we assumed that TSPEAR-AS2, miR-1207-5p, and CLDN4 were involved in a competing endogenous RNA (ceRNA) regulatory network. To verify our hypothesis, we first performed miR-1207-5p overexpression assays in GC cells and found that the elevation of miR-1207-5p resulted in the obvious decrease of CLDN4 at the mRNA level (Figure 6B). Moreover, we further revealed that the depletion or increase of miR-1207-5p can significantly upregulate or downregulate CLDN4 expression in GC cells (Figure 6D). Interestingly, the prediction analysis from miRanda database showed that miR-1207-5p can possibly bind to CLDN4 (Figure 6C). As shown in Figure 6C, a luciferase activity assay further demonstrated that miR-1207-5p elevation induced the effective suppression of luciferase activity of CLDN4-3'UTR-WT (WT) but not CLDN4-3'UTR-Mut (Figure 6C). The analysis of GSE66229 database highlighted the abundance of CLDN4 in GC tissue specimens (n = 300) compared with normal specimens (n = 100) (Figure 6E). We also detected the CLDN4 level in paired
specimens from patients with GC and indicated CLDN4 upregulation in GC (Figure 6F). Interestingly, the positive correlation can be observed between TSPEAR-AS2 and CLDN4 in GC tumor samples, highlighting the tight regulatory correlation between TSPEAR-AS2 and CLDN4 in GC progression based on data from GEO datasets (GEO: GSE65801 and GSE66229) (Figures 6G and 6H). Taken together, our findings elucidated that lncRNA TSPEAR-AS2 worked as a ceRNA for miR-1207-5p, consequently leading to a boosted level of CLDN4 in GC progression.

DISCUSSION

Mounting evidence has reported that lncRNAs are effective biological regulators rather than "transcriptional noise."\(^{11,43–45}\) Despite that lncRNAs have been considered to play important roles during cancer progression, a variety of mechanisms need to be developed and clarified in various types of cancers, especially GC. To detect lncRNAs potentially involved in GC progression, we first explored the publicly available profiling data of GC from TCGA and GEO datasets. A novel lncRNA, TSPEAR-AS2, was screened out as a candidate gene associated with GC progression. The ectopic expression of TSPEAR-AS2 exhibited close correlation with the survival condition of patients with GC. Through gain- and loss-of function assays, TSPEAR-AS2 could induce GC cell apoptosis and promote GC proliferation, migration, and invasion. Together, TSPEAR-AS2 may exhibit an oncogenic role in GC progression. To the best of our knowledge, this is the first study to systematically evaluate the role of TSPEAR-AS2 in GC initiation and development.

Current studies indicate that many transcription factors have been revealed to be highly expressed in various malignancies, contributing to the activities of transcriptional activation of lncRNAs.\(^{46,47}\) In this study, a high level of transcription factor BTEB2 was observed in GC specimens and potentially correlated with TSPEAR-AS2 abundance, contributing to TSPEAR-AS2 overexpression in GC. Both FISH and subcellular fractionation assays demonstrated that TSPEAR-AS2 was more prevalent in the nucleus of GC cells, suggesting that TSPEAR-AS2 may mediate GC progression at the transcriptional level. RNA-seq found that inhibition of TSPEAR-AS2 affected key regulators correlated with cancer, such as GJA1, HDAC9, ROS1, ANKRD1, and XAF1. The expression level of GJA1 exhibited significant upregulation in GC cells with knockdown of EZH2, which is a critical member of PRC2. Interestingly, GJA1 can be coregulated by TSPEAR-AS2/EZH2. Mechanistic assays showed that TSPEAR-AS2 may participate in the tumorigenesis of GC via transcription repression of key regulators through interaction with EZH2. Additionally, TSPEAR-AS2-induced GC proliferation, migration, and invasion can be significantly reversed by overexpression of GJA1 in GC.

Recently, a novel regulatory mechanism has been illuminated to exist between lncRNAs and miRNAs in many malignant diseases.\(^{48–50}\) Both lncRNAs and miRNAs exert dynamic function in transcriptional and translational regulation.\(^{51,52}\) lncRNAs can serve as ceRNAs to protect miRNAs through competing for their targeting miRNAs.\(^{12,53}\) In the present study, bioinformatics databases (miRanda, pita, and RNAhybrid) were analyzed to predict miRNAs, which may potentially bind with TSPEAR-AS2. Among these miRNAs, miR-1207-5p showed the strongest repressive abilities of TSPEAR-AS2-mediated luciferase activity. Furthermore, RNA-seq assays and verified assays confirmed that CLDN4 is among the most downregulated gene in GC cells with TSPEAR-AS2 knockdown. Subsequently, a series of assays were designed to determine the novel ceRNA network formed by TSPEAR-AS2, miR-1207-5p, and CLDN4. TSPEAR-AS2 knockdown or overexpression could obviously boost or impair the level of miR-1207-5p, while miR-1207-5p elevation had no impact on TSPEAR-AS2 regulation. RIP assays revealed that both TSPEAR-AS2 and miR-1207-5p were involved in the same RISC. Dual-luciferase-reporter assays clarified the direct binding ability of the predicted miR-1207-5p binding site on TSPEAR-AS2 and further demonstrated that miR-1207-5p could directly target CLDN4 in GC. These data strongly indicated that TSPEAR-AS2 could serve as a ceRNA for miR-1207-5p to regulate CLDN4 expression at the post-transcriptional level in GC. Rescue assays further revealed that CLDN4 knockdown can, at least in part, reverse the promotion of GC progression caused by overexpressing TSPEAR-AS2.

In summary, this is the first report documenting the clinical value, biological role, and mechanism of TSPEAR-AS2 in GC. The BTEB2-activated TSPEAR-AS2 model was first elucidated in this study, leading to TSPEAR-AS2 transcription promotion in GC. Meanwhile, TSPEAR-AS2 could serve as a ceRNA for miRNAs or interact with PRC2 in GC. Our data highlight the key involvements of TSPEAR-AS2 in GC progression, implicating the axis of TSPEAR-AS2/EZH2/GJA1 and TSPEAR-AS2/miR-1207-5p/CLDN4 as novel targets for GC therapeutics. Importantly, the investigation of the expression and mechanistic model of TSPEAR-AS2 in other GC cells is urgently needed in future research. More explorations are also required to detect other upstream effectors or downstream effectors of TSPEAR-AS2 in GC progression.

MATERIALS AND METHODS

Cell Culture

Human gastric adenocarcinoma cancer cell lines and normal gastric epithelium cell line (GES-1) were maintained as previously reported.\(^{53}\)

RNA Immunoprecipitation

We used EZMagna RIP Kit (Millipore, Billerica, MA, USA) to perform RIP assays in GC cell lines. The detailed information is summarized in the Supplemental Information. The details for antibodies, primers, and siRNA oligonucleotides are listed in Table S1.

Luciferase Assays

Luciferase assays were performed as previously described.\(^{53}\)

Chromatin Immunoprecipitation Assays

EZ-ChIP Kit (Millipore, Billerica, MA, USA) was used to conduct ChIP assays in accordance with the manufacturer’s instructions.
The details of ChIP procedures can be found in the Supplemental Information.

Statistical Analysis
SPSS version 25.0 software was used to assess statistical differences. The significance between groups was assessed using a paired, two-tailed Student’s t test, Wilcoxon test, or χ² test. The curves of OS were obtained using the Kaplan-Meier method. p <0.05 was indicative of significant difference.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.10.022.

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AUTHOR CONTRIBUTIONS
Z.H.M., Y.S., K.M.W., X.Z.W., and J.F.J. designed the whole study. Z.H.M. drafted the manuscript and prepared the figures. Z.H.M., Y.S., X.Y.G., and Y.Y. performed in vitro assays and data analysis. Y.S. performed in vivo assays and bioinformatics analysis. K.M.W., X.Z.W., and J.F.J. provided full guidance. All authors have read and approved the final version.

DECLARATION OF INTEREST
The authors declare no competing interests.

REFERENCES
1. Arnold, M., Park, J.Y., Camargo, M.C., Lanet, N., Forman, D., and Soorijomataram, I. (2020). Is gastric cancer becoming a rare disease? A global assessment of predicted incidence trends to 2035. Gut 69, 823–829.
2. Bray, F., Ferlay, I., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424.
3. Uno, Y. (2019). Prevention of gastric cancer by Helicobacter pylori eradication: A review from Japan. Cancer Med. 8, 3992–4000.
4. Sun, W., and Yan, L. (2016). Gastric cancer: current and evolving treatment landscape. Chin. J. Cancer 35, 83.
5. Zong, L., Abe, M., Seto, Y., and Ji, J. (2016). The challenge of screening for early gastric cancer in China. Lancet 388, 2806.
6. Ransohoff, J.D., Wei, Y., and Khavari, P.A. (2018). The functions and unique features of long intergenic non-coding RNA. Nat. Rev. Mol. Cell Biol. 19, 143–157.
7. Uszczyńska-Ratajczak, B., Lagarde, J., Frankish, A., Guigó, R., and Johnson, R. (2018). Towards a complete map of the human long non-coding RNA transcriptome. Nat. Rev. Genet. 19, 535–548.
8. Cabili, M.N., Trapnell, C., Goff, L., Kozis, M., Tazon-Vega, B., Regev, A., and Rinn, J.L. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev. 25, 1915-1927.
