Transcription Factor CTCFL Promotes cell Proliferation, Migration and Invasion in Gastric Cancer Via Activating DPPA2

Haibo Yao  
Zhejiang Provincial People's Hospital

Qinshu Shao  
Zhejiang Provincial People's Hospital

Yanfei Shao  (shaoyanfeimd@163.com)  
Zhejiang Provincial People's Hospital (People's Hospital of Hangzhou Medical College)  
https://orcid.org/0000-0002-8796-726X

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Abstract

Background: The purpose of this study was to explore the relationship between CTCFL and DPPA2, and validate the positive role of CTCFL/DPPA2 in cell proliferation, migration and invasion in gastric cancer.

Methods: Bioinformatics methods were applied for the prediction of gastric cancer-related transcription factors and corresponding target mRNAs. qRT-PCR and western blot were performed to test the levels of CTCFL and DPPA2. Then a series of in vitro experiments were conducted to assay the cell biological behaviors, including CCK-8, colony formation assay, wound healing assay and Transwell invasion assay. CHIP was carried out for assessment of the targeted relationship between CTCFL and DPPA2.

Results: CTCFL and DPPA2 were both highly expressed in gastric cancer cells, and high CTCFL and DPPA2 could promote cell proliferation, migration and invasion. CHIP validated that DPPA2 was a target of CTCFL. In addition, high DPPA2 could reverse the inhibitory effect of CTCFL silencing on the cell proliferation, migration and invasion in gastric cancer.

Conclusion: The transcription factor CTCFL promotes cell proliferation, migration and invasion in gastric cancer via activating DPPA2.

Highlight

1. CTCFL was identified to be able to positively regulate DPPA2;
2. CTCFL was validated to play a positive role in cell proliferation, migration and invasion in gastric cancer;
3. CTCFL was confirmed to mediate cell proliferation, migration and invasion in gastric cancer via targeting DPPA2.

Introduction

Gastric cancer is one of the most common gastrointestinal malignancies and the second cause of the cancer death worldwide\(^1\). Although great advance has been achieved towards cancer treatment, it is still a big challenge for gastric cancer treatment that metastasis occurs after the disease is radically cured\(^2\). Hence, it is necessary to perform in-depth research on the molecular mechanism underlying the metastasis of gastric cancer, so as to provide potential therapeutic strategies.

CTCF (CCCTC-binding factor) is a highly conserved protein exerting diverse functions on transcriptional regulation as well as chromatin architecture and it can serve as a transcription factor mediating the insulation and cycling of chromatin, in short, CTCF is a necessity for life maintenance\(^3,4\). The combination of CTCF with DNA sequences is predominantly realized via the 11-zinc finger region, which is beneficial for the protein-protein interactions. CTCFL is a homology of CTCF harboring a nearly identical 11-zinc finger region\(^5\). Meanwhile, these two proteins have similar binding specificity to DNA
sequences due to the difference in the sequences on the amino and carboxyl terminals, but the protein functions are different\textsuperscript{5}. In the current public literatures, CTCFL can mediate the occurrence and development of various cancers, such as liver cancer and neuroblastoma\textsuperscript{6,7}, yet no relevant efforts have been made in gastric cancer.

In recent years, DPPA2 has been found to be specifically expressed in pluripotent cells and some cancer tissues\textsuperscript{8,9}. It is involved in the pluripotent maintenance of embryonic stem cells and plays an important role in early embryonic development and the reprogramming of somatic cells into induced pluripotent stem cells\textsuperscript{10–12}. It has been reported that DPPA2 is differentially expressed in diverse cancer types and can be used as a specific therapeutic target in some tumors, such as non-small cell lung cancer, ovarian cancer, colon cancer, lymphoma and melanoma\textsuperscript{13}. In addition, the role of DPPA2 in gastric cancer has been explored in some reports, yet further verification needs to be further carried out\textsuperscript{14}.

In the present study, we described the differential expression of CTCFL and DPPA2 in gastric cancer tissues. Meanwhile, we investigated the role of CTCFL/DPPA2 in the cell proliferation, migration and invasion, and also validated the targeted relationship between CTCFL and DPPA2. In short, our study provides a novel therapeutic target for gastric cancer treatment.

Materials And Methods

Bioinformatics analysis

Gene expression files of STAD included in TCGA database were accessed and then processed for gene ID transformation using the GTF (GRCh38.p5) files for getting the data of the mRNA expression profile. The profile contains 32 normal samples and 373 tissue samples of gastric cancer. The “edgeR” package in the R language was used for identifying the differentially expressed mRNAs (DE mRNAs) with the critical value set to |logFC|>2 and adj.pvalue < 0.01. Afterwards, the sequences on the upstream 500 bp of the DE mRNAs were applied as putative promoter sequences, which were then used for the extraction of the DE transcription factors (TF) with the JASPAR database (http://jaspar.genereg.net/). The TFs were firstly subjected to the FIMO software (http://meme-suite.org/tools/fimo) for predicting the target mRNAs and then processed for enrichment analysis in DE mRNAs (cor > 0.3, p < 0.05). The TFs with q_value < 0.05 were identified as candidate TFs. Pearson correlation analysis was performed for analyzing the relationship between the target TF and mRNA.

Clinical samples

15 pairs of human gastric cancer tissues and corresponding adjacent normal tissues (margin > 5 cm) from June 2015 to June 2019 were procured from the Zhejiang Provincial People's Hospital with the approval of all subjects. All cancer samples were pathologically diagnosed and immediately frozen in liquid nitrogen and preserved at -80°C after being isolated. All subjects had never received any
preoperative treatment like chemotherapy or radiotherapy. Our study had been approved by the Ethic Committee of the Zhejiang Provincial People's Hospital.

**Cell culture**

Human normal gastric epithelial cell line GES-1 (No: CBP60512) and gastric cancer cell lines AGS (No: CBP60476), SGC-7901 (No: CBP60500), HGC-27 (No: CBP60480) and BGC-823 (No: CBP60477) were all purchased from the Cell Bank of the China Center for Type Culture Collection, Chinese Academy of Sciences (CTCC; Shanghai, China). All cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Inc., USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and then maintained in a 37 °C incubator containing 5% CO₂.

**Cell transfection**

Vectors oe-CTCFL, sh-CTCFL, oe-DPPA2, sh-DPPA2 and their matched negative controls (oe-NC and sh-NC) were synthesized by GenePharma (Shanghai, China). Cells (1 × 10⁵) before transfection were firstly incubated in 12-well plates. LipoFiter assay kit (Hanbio, Shanghai, China) was applied for conducting transfection process per the manufacturer's protocols. Total RNA and proteins were extracted after 48 h of transfection.

**qRT-PCR**

Total RNA was isolated from cells using the Trizol (Invitrogen, Carlsbad, USA) and then used for the synthesis of the cDNA with the reverse transcription assay kit (Invitrogen, Carlsbad, USA), following the standard process. qRT-PCR was run on the ABI 7900HT instrument (Applied Biosystems, USA) with the miScript SYBR Green PCR Kit (Qiagen, Germany) under the following thermal cycling conditions: predenaturation at 95°C for 10 min, 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 2 min. The results were normalized to GAPDH level with the $2^{-\Delta\Delta Ct}$ method. The primers were designed as below:

**CTCFL**

Forward: 5'-AAAACCTTCCGTACGGTCACTCT-3';

Reverse: 5'-TGTTGCAGTCGTTACACTTGTAGG-3';

**DPPA2**

Forward: 5'-AAGGAGGAGGAGGAGCCAAAC-3';

Reverse: 5'-TGGTTGGGTGTTTGATTCCAGC-3';

**GAPDH**

Forward: 5'- TCCATGACAACCTTTGGCATTG-3';

Reverse: 5'-CAGTCTTTCTGGGTGGCAGTG-3'.
Western blot

RIPA lysate buffer containing 1% protease inhibitor (Beyotime, Shanghai, China) was used for the isolation of total proteins from cells, and the BCA protein assay kit (Beyotime, Shanghai, China) was applied for quantification, according to the manufacturer’s instructions. After being denatured at a high-temperature, the protein samples (30 µg/pore) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto the polyvinylidene fluoride (PVDF; Millipore) membranes. 5% skim milk was used to block the membranes for 2 h. Thereafter, the membranes were incubated with primary rabbit polyclonal antibodies overnight at 4°C. The primary antibodies were comprised of CTCFL (ab126766, 1:1000; abcam, China), DPPA2 (ab91318, 1:100; abcam, China) and GAPDH (ab137321, 1:10000; abcam, China). On the following day, the secondary antibody horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG was added onto the membranes for hybridization at room temperature for 120 min. 1 × TBST (Solarbio, Beijing, China) was used to wash the membranes three times. After reaction, the enhanced chemiluminescence (ECL) assay kit (Solarbio, Beijing, China) was employed for the visualization of the protein bands, and then images were captured.

CCK-8

96-well plates were used for cell incubation (200 µl, 1 × 10^4 cells/ml). At 0, 24, 48 and 72 h, the reagent (20 µg/well) supplied by the cell counting kit-8 (Yeasen) was added into the cells for 4 h of incubation at 37 °C in 5% CO₂. SpectraMax M5 (Molecular Devices, MD, USA) was used to measure the absorbance values at 450 nm.

Colony formation assay

A measure of 2 ml cell suspension was seeded into 6-well plates at a density of 2 × 10^2 cells/ml. The mediums were replaced every 4 days. After 3 rounds, the cells were fixed with 4% paraformaldehyde (Invitrogen, China) and then stained in 0.1% crystal violet (Thermo Scientific™ RA Lamb, China). The stained cells were photographed and calculated.

Wound healing assay

Cells (2 ml, 2.5 × 10^5 cells/ml) were inoculated into 6-well plates until the confluence reached 90%. Then the cells were wounded with the tip of a sterile pipette, and sequentially washed with PBS and suspended by the FBS-free mediums at 37°C in 5% CO₂. The wound areas at 0 and 72 h were observed and photographed under an inverted microscope.

Transwell invasion assay

Transwell inserts (sigma, China) that were pre-coated with Matrigel matrix (BD, USA) were put into 24-well plates. 200 µl of cells (1 × 10^5 cells/ml) suspended by FBS-free mediums were planted into the inserts, and 10% FBS-supplemented mediums were added into the plates. After 24 h of incubation at 37°C in 5% CO₂, the cells invaded to the plates were exposed to 4% paraformaldehyde for fixation for 30 min,
followed by 0.1% crystal violet for staining for further 30 min. Cells still in the inserts were softly wiped off with a wet cotton swab. Five fields of the view were randomly selected using an inverted microscope, and then photographed for cell count.

**Chromatin immunoprecipitation (ChIP)-PCR**

The EZ-Magna ChIP assay kit (Millipore) was used for ChIP assay. The specific procedures were as below: 1% formaldehyde solution was used to induce the cross-linking of cells and 140 mM glycine was added for the reaction termination. After the cells were lysed, the nucleoprotein complexes were sheared to the 200–500 bp, and then the obtained DNA fragments were incubated with the antibody for immunoprecipitation overnight at 4°C. Then the samples were washed with 1 × low salt buffer, 1 × high salt buffer, 1 × LiCl buffer and 2 × TE buffer, and sequentially eluted with 200 µl of elution buffer at 37°C for 15 min. Thereafter, the samples were incubated with 5M NaCl for the reversal of cross-linking overnight at 65°C, and then treated with RNase and protease K. qRT-PCR was performed for identifying the combination of CTCFL and the DPPA2 promoter region.

**Statistical analysis**

All data were analyzed under the GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA). Measurement data were presented as mean ± standard deviation. Comparisons between two groups and among multiple groups were analyzed by Student’s t test and one-way analysis of variance, respectively. Each result was representative of at least three independent experiments. $P < 0.05$ was set to be a threshold for statistical significance.

**Results**

**Bioinformatics analysis results**

Totally 1645 DE mRNAs (Fig. 1A) and 62 DE TFs (Supplementary Table 1) were obtained. The DE TFs were used for prediction of the target mRNAs using the FIMO software and then subjected to enrichment analysis in DE mRNAs. Among the DE TFs, 3 TFs with $q_{-}value < 0.05$ were identified, including TFAP2B, CTCFL and SP8, and the mRNAs meeting $cor > 0.3$ and $p < 0.05$ were then projected onto corresponding TF regulatory networks (Fig. 1F). It has been reported that CTCFL (BORIS) is an important DNA binding protein involved in tumor regulation and it also serves as a vital immunotherapeutic target. Besides, Pearson correlation analysis was conducted and found that there was a positive correlation between CTCFL and DPPA2 (Fig. 1E). Hence, we selected CTCFL as our research object. Bioinformatics analysis revealed that CTCFL and DPPA2 were both highly expressed in tumor tissues relative to the normal tissues in the TCGA-STAD dataset (Fig. 1B-C). In addition, survival analysis suggested that high DPPA2 was significantly associated with poor prognosis of patients with gastric cancer (Fig. 1D). As DPPA2 has been reported to be elevated in cancer cells and implicated with cell metastasis in gastric cancer, we reasoned that the TF CTCFL functions on cell proliferation and metastasis in gastric cancer via targeting DPPA2.
CTCFL and DPPA2 are highly expressed in gastric cancer cells

To be much clearer on the levels of CTCFL and DPPA2 in gastric cancer, clinical tissue samples (tumor and adjacent normal), human normal gastric epithelial cell line GES-1 and 4 cancer cell lines AGS, SGC-7901, HGC-27, BGC-823 were selected for further verification. qRT-PCR and western blot were performed and revealed that CTCFL and DPPA2 were both significantly elevated in mRNA and protein levels in cancer cases relative to the corresponding controls (Fig. 2A-F), which showed a good consistence with the result of the above bioinformatics analysis.

Silencing CTCFL inhibits cell proliferation, migration and invasion in gastric cancer

To gain more insight into the role of CTCFL in gastric cancer, sh-CTCFL and sh-NC were transfected into cells for construction of CTCFL silencing cell line (Fig. 3A). Then a series of experiments including CCK-8, colony formation assay, wound healing assay and Transwell invasion assay were performed to test the cell biological behaviors. As shown in Fig. 3B-E, silencing CTCFL suppressed cell proliferation, migration and invasion abilities. These results collectively demonstrated that CTCFL could potentiate cell proliferation, migration and invasion in gastric cancer.

Silencing DPPA2 suppresses cell proliferation, migration and invasion in gastric cancer

Similarly, DPPA2 was silenced for further investigation (Fig. 4A). CCK-8 and colony formation assay suggested that cell proliferation was significantly reduced in sh-DPPA2 transfected cells relative to the NC, and cell migration and invasion were as well decreased as evidenced by wound healing assay and Transwell (Fig. 4B-E). Taken together, it could be seen that DPPA2 played a promotive role in cell proliferation, migration and invasion in gastric cancer.

CTCFL positively regulates the expression of DPPA2

As abovementioned, CTCFL and DPPA2 both could promote the cell proliferation, migration and invasion in gastric cancer. Besides, potential binding sites of CTCFL on DPPA2 were predicted using the bioinformatics analysis (Fig. 5A). To know more about the relationship between CTCFL and DPPA2, sh-CTCFL, oe-CTCFL and matched negative controls were transfected into cancer cells. qRT-PCR was carried out and found that CTCFL silencing decreased DPPA2 level (Fig. 5B). Reversely, CTCFL overexpression increased DPPA2 level (Fig. 5C). In addition, ChIP-PCR was conducted for further verification of the interaction between CTCFL and DPPA2 promoter (Fig. 5D). Moreover, correlation analysis indicated that there was a positive correlation between the levels of CTCFL and DPPA2 (Fig. 5E). Overall, these findings elucidated that DPPA2 was positively regulated by CTCFL.
The inhibitory effect of CTCFL silencing on cell proliferation, migration and invasion in gastric cancer can be reversed by DPPA2 overexpression

As we had confirmed that CTCFL could positively mediate DPPA2, to clearly clarify the underlying mechanism in gastric cancer, rescue experiments were further conducted. All cells were classified into 3 groups: sh-NC + oe-NC, sh-CTCFL + oe-NC and sh-CTCFL + oe-DPPA2. qRT-PCR was performed for the assessment of the transfection efficiency (Fig. 6A). Then CCK-8 and colony formation assay were conducted and showed that CTCFL silencing inhibited cell viability and decreased cell colony formation ability, but such inhibitory effect was attenuated when DPPA2 was simultaneously overexpressed (Fig. 6B-C). Meanwhile, similar result could be seen on cell migration and invasion as detected by wound healing assay and Transwell invasion assay (Fig. 6D-E). Thus, we could conclude that DPPA2 overexpression suppressed the negative effect of CTCFL silencing on cell proliferation, migration and invasion in gastric cancer.

Discussion

Transcription factors (TF) are proteins that are able to bind with specific DNA sequences so as to ensure that their target genes can be expressed at a certain time and space with a certain intensity, and their dysfunction is the crucial pathological cause leading to the occurrence of malignant tumors. For example, the TF E2F1 induces the TINCR transcriptional activity and accelerates gastric cancer progression via activating the TINCR/STAU1/CDKN2B signaling axis. The TF TFAP4 induces the activation of the PI3K/AKT signaling pathway to potentiate cell metastasis in hepatocellular carcinoma (HCC). And the TF Nrf2 promotes the occurrence and development of bladder urothelial carcinoma by interacting with TUG1. CTCFL has been reported to be intimately correlated with various cancer types. In HCC, for instance, CTCFL up-regulates OCT4 via histone methylation to potentiate cancer stem cell-like properties. And in breast cancer, CTCFL mediates the tumor occurrence and development in the way of inducing the activation of progesterone and estrogen receptor genes. However, no study has focused on the role of CTCFL in gastric cancer as well as the corresponding regulatory mechanisms.

In the present study, we used the bioinformatics analysis to know that CTCFL and DPPA2 were both differentially expressed in gastric cancer. To be more receivable, the levels of CTCFL and DPPA2 were detected in clinical tumor tissue samples and matched adjacent normal tissue samples. It was found that CTCFL and DPPA2 were highly expressed in cancer cases, which demonstrated that these two genes might be implicated with the cell characteristics in gastric cancer. Subsequently, some in vitro experiments were performed to assess the effect of CTCFL or DPPA2 on gastric cancer cell biological behaviors. CCK-8 and colony formation assays revealed that overexpressing CTCFL or DPPA2 promoted cell proliferation, and wound healing assay and Transwell invasion assay showed that the cell migration and invasion could also be increased with high CTCFL or DPPA2. It has been reported that CTCFL or DPPA2 exhibits a tight correlation with cell proliferation and metastasis in tumors. For example, DPPA2 knockdown plays an inhibitory role in the proliferation of mouse stem cells, and is able to decreased the
metastasis of cancer stem cells in neuroblastoma cell lines. In view of these, we could see that overexpressing CTCFL or DPPA2 promotively functions on cell proliferation and metastasis in gastric cancer.

Moreover, to further validate the targeted relationship between CTCFL and DPPA2, CTCFL silencing and overexpression cell lines were constructed. qRT-PCR was performed and found that the level of DPPA2 was positively altered with the level of CTCFL, showing that there was a certain relationship between the two genes. Hence, we conducted ChIP-PCR for further verification. As expected, CTCFL could targeted binding with DPPA2. Furthermore, rescue experiments were used to clarify the mechanism of CTCFL/DPPA2 in gastric cancer. The results revealed that overexpressing DPPA2 could attenuate the inhibitory effect of CTCFL silencing on cell biological behaviors. Collectively, it could be concluded that the TF CTCFL activates DPPA2 to promote cell proliferation, migration and invasion in gastric cancer.

In sum, this study finds that CTCFL and DPPA2 are valuable prognostic biomarkers for gastric cancer and can be used for identifying the possibility of the occurrence of tumor metastasis and relapse. Meanwhile, our study confirms the targeted relationship between DPPA2 and CTCFL, which may help to develop a novel strategy towards gastric cancer prevention and treatment.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare no conflicts of interest.

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Authors' contributions

YHB contributed to the study design and wrote the article. SQS conducted the literature search and acquired the data. SYF revised the article and gave the final approval of the version to be submitted.

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Figures
Figure 1

Bioinformatics analysis results (A) Differential analysis was performed to identify the DE mRNAs in the TCGA-STAD dataset. (B) CTCFL and (C) DPPA2 levels were test in the TCGA-STAD dataset (red: normal; blue: tumor). Then, (D) Kaplan-Meier survival analysis was conducted on the DPPA2 in the TCGA-STAD dataset (red: high expression; blue: low expression) and (E) the relationship between the levels of CTCFL
and DPPA2 was analyzed by Pearson correlation analysis. (F) The regulatory networks of CTCFL, TFAP2B and SP8.

Figure 2

CTCFL and DPPA2 are highly expressed in gastric cancer cells qRT-PCR and western blot showed the mRNA level of (A) CTCFL and (B) DPPA2 in clinical tissue samples (tumor and adjacent normal), and
displayed the mRNA and protein levels of (C, D) CTCFL and (E, F) DPPA2 in human normal gastric epithelial cell line GES-1 and 4 cancer cell lines AGS, SGC-7901, HGC-27, BGC-823. (* p<0.05)

Figure 3

Silencing CTCFL inhibits cell proliferation, migration and invasion in gastric cancer. Sh-CTCFL and sh-NC were transfected into cancer cells. (A) qRT-PCR was conducted to test the transfection efficiency. Then the
transfected cells were harvested for (B) CCK-8, (C) colony formation assay, (D) wound healing assay and (E) Transwell for determining cell biological behaviors. (* p<0.05)

Figure 4

Silencing DPPA2 suppresses cell proliferation, migration and invasion in gastric cancer Sh-DPPA2 and sh-NC were transfected into cancer cells. (A) qRT-PCR was conducted to test the transfection efficiency. Then
the transfected cells were harvested for (B) CCK-8, (C) colony formation assay, (D) wound healing assay and (E) Transwell for determining cell biological behaviors. (* p<0.05)

Figure 5

CTCFL promotes the expression of DPPA2 in gastric cancer (A) Bioinformatics analysis was performed and discovered that there were potential binding sites of CTCFL on DPPA2 promoter. (B, C) qRT-PCR was carried out to determine the level of DPPA2 mRNA in cells transfected with (B) sh-CTCFL and (C) oe-
CTCFL. (D) ChIP-PCR was conducted to further validate the relationship between CTCFL and DPPA2, and (E) correlation analysis was performed on the levels of CTCFL and DPPA2 in the 15 gastric cancer tissue samples. (* p<0.05)

**Figure 6**

The inhibitory effect of CTCFL silencing on cell proliferation, migration and invasion in gastric cancer can be reversed by DPPA2 overexpression Sh-NC+oe-NC, sh-CTCFL+oe-NC and sh-CTCFL+oe-DPPA2 were
transfected into cells. (A) qRT-PCR was performed to detect the transfection efficiency. Then the cells were collected for assessing the cell biological behaviors using the (B) CCK-8, (C) colony formation assay, (D) wound healing assay and (E) Transwell invasion assay. (* p<0.05)

Supplementary Files

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