CircRNA PVT1 modulated cell migration and invasion through Epithelial-Mesenchymal Transition (EMT) mediation in gastric cancer through miR-423-5p/Smad3 pathway

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Background: Gastric cancer (GC) progression is related with gene regulations.
Objectives: This study explored underlying regulatory axis of circRNA PVT1 (circPVT1) in GC.
Methods: GC cell lines were detected for circPVT1 expression with the normal mucous epithelial cell GES-1 as control. After regulation of circPVT1, miR-423-5p and SMAD3 expression through transfection, CCK8 evaluated the cell viability, Transwell measured the migratory and invasive capability of cells. Luciferase verified the paired bindings between miR-423-5p and CircPVT1 or SMAD3. The functions of CircPVT1/miR-423-5p/SMAD3 were evaluated using RT-PCR, CCK8, Transwell assays. Western blot analyzed EMT-related proteins and phosphorylation of Smad3 in GC cells. Immunofluorescence method was used to evaluate the EMT-related proteins as well.
Results: CircPVT1 displayed higher expression in GC cells and knockdown led to decrease in cell growth, invasion and migration. CircPVT1 was targeted by miR-423-5p as a ceRNA of SMAD3. miR-423-5p upregulation suppressed both circRNA PVT1 and SMAD3 in GC cells. Decrease in SMAD3 expression suppressed CircPVT1 by releasing miR-423-5p in cells, inhibiting cell growth, invasion and migration and suppressing the EMT process.
Conclusion: CircPVT1 modulated cell growth, invasion and migration through EMT mediation in gastric cancer through miR-423-5p/Smad3 pathway.

1. Introduction

Gastric cancer (GC) has been long listed as one of the most common death causes among all cancers. The risk factors for pathogenesis of GC include Helicobacter pylori, Epstein Barr Virus [1], unhealthy diet habit and lifestyles [2]. The current major therapies for gastric cancer at early stage is resection of primary tumors while for gastric cancer at late stages, chemotherapies remain major options yet prognosis stays poor [3]. Recently, targeted therapy drugs including trastuzumab, ramucirumab (anti-angiogenesis), and nivolumab or pembrolizumab (anti-PD-1) have posed great potential in the improvement of the prognosis of GC patients at advanced stages although some of the previous randomly-controlled trials failed to meet the expectations [4]. Such development also encourages more and more basic research on exploration into new potential therapeutic targets and related mechanisms.

In the past 10 years, researches that focused on molecular regulations in gastric cancer reported that non-coding RNAs including circular (Circ) RNAs, long non-coding RNAs and micro RNAs, etc. [5] are engaged in the tumor genesis and progression. CircRNAs were increasingly discovered to display abnormally high or low expression in tumor tissues or cell lines compared to their normal counterparts [5], which might enable certain genes to act as biomarkers at early diagnosis [6]. Further studies also show that CircRNAs could regulate transcription through competitively sponging miRNAs and further engage in the protein mediation [7]. The CircRNA-miRNA-mRNA mechanisms have been demonstrated in earlier studies in gastric cancer. To list a few, hsa_circ_006100 upregulation (targeted and inhibited by miR-195) in GC cells induced high cell
proliferation, migration and invasive levels, which was inhibited by the miR-195 upregulation in cells while GPRC5A, an mRNA targeted by miR-195, restored the cell functions mentioned as a competitively endogenous RNA (ceRNA) [8]. Similarly, circRIP1 mediated the GC development through sponging miR-149-5p, which further targeted AKT1 and modulated the mTOR signaling pathway in vitro and in vivo [9]. Likewise, circ-RanGAP1 downregulation could inhibit GC cell migration and invasiveness by inhibiting VEGFA through sponging miR-877-3p [10]. Likewise, the inhibition of circ_0008035 in GC cells decreased cell proliferation as a sponge of miR-599, which targeted and mediated EPL1A further [11]. Previously, it was also reported that CircPVT1 promoted GC cell proliferation as a sponge of miR-125b, yet the further regulatory mechanisms concerning mRNA and canonical pathways remain to be revealed [12]. However, in other cancers, the regulatory mechanisms related to CircPVT1 were veiled. For instance, in Osteosarcoma, CircPVT1 induced the cell invasion and migration and upregulated c-FLIP indirectly by sponging miR-205-5p [13]. In liver cancer, CircPVT1/miR-377/TRIM23 axis regulated cell proliferation and apoptosis [11].

Therefore, in this study, we resorted to bioinformatic tools and predicted the bindings between CircPVT1/SMA3 and miR-423-5p. Previously, there were a few researches revealing the dual roles of miR-423-5p in cancers. In lung cancer, miR-423-5p was associated with the brain metastasis and cell migration and invasion [14]. In gastric cancer, miR-423-5p was downregulated in GC and targeted and inhibited LINC01606, and was associated with GC metastasis [15]. An earlier research discovered that miR-423-5p was linked to the regulation of GC progression in vitro [16]. However, no previous studies explored the CircPVT1/miR-423-5p/SMA3 axis in GC.

Then we validated the interactions among CircPVT1/miR-423-5p/SMA3 in gastric cancer cells as well as regulations in EMT in vitro.

2. Methods

2.1. Ethical statement

No human samples nor animals were engaged in this research. The experiments were completed by adhering to Minhang Hospital of Fudan University lab regulations.

2.2. Cell culture

GES-1, AGS, SGC-7901 and HEK-293T cells were provided by Procell (Wuhan, China). GES-1 is human normal gastric mucosal epithelial cell line. AGS cell line, derived from a tumor from a gastric adenocarcinoma patient without any treatment. SGC-7901 was from a gastric adenocarcinoma patient with lymphatic metastasis. GES-1 and SGC-7901 cells were cultured in RPMI-1640 containing 10% FBS (Gibco, Shanghai, China) and 1% Penicillin-Streptomycin Solution (Procell, Wuhan, China). AGS cells were cultured in Ham’s F-12 (Procell, Wuhan, China) with addition of 10% FBS and 1% Penicillin-Streptomycin Solution. HEK-293T cell line, embryonic kidney cells, was used for luciferase assays in this study. HEK-293T cells were cultured using DMEM with supplementation of 10% FBS. All the cells were cultured with 5% CO2 at 37 °C. Cells were used for further experiments at log phase.

2.3. Cell transfection

The gastric cell lines, AGS and SGC-7901, were selected for transfection so as to manipulate the CircPVT1, miR-423-5p and SMAD3 expression levels in cells. The plasmids of pc-CircPVT1 and pc-SMAD3, si-CircPVT1 and si-SMAD3 were synthesized by GenePharma (Suzhou, China). So were the plasmids of miR-423-5p mimic and miR-423-5p inhibitor. Lipofectamine 2000 (Thermo-Fisher, Shanghai, China) was used to achieve the transfection in cells. The transfected cells underwent RT-PCR examinations for relative gene expression.

2.4. RT-PCR

Total RNAs were extracted by using Trizol (Invitrogen, Shanghai, China). cDNA of CircPVT1 and SMAD3 was synthesized using PrimeScript Reverse Transcription Kits (TaKara, Japan). The cDNA of miR-423-5p was synthesized using the MicroRNA Reverse Transcription Kit (TaqMan, Shanghai, China). The SYBR Green (ThermoFisher, Shanghai, China) was used to measure the relative expression of genes mentioned above on q5 Real-Time PCR Detection System (Bio-Rad, CA, USA). 2−ΔΔCt method was used to calculate the relative expression of CircPVT1 and SMAD3 with normalization to GAPDH and of miR-423-5p with normalization to U6. The primer sequences were listed (Table 1). Three repetitions were carried out for each group.

2.5. CCK8

The Cell Counting Kit-8 (Beyotime, Shanghai, China) was applied for the cell proliferation capability in each group. Cells at log phase were seeded onto the 96-well plates (2000 cell count per well). At 0, 24, 48 and 72 h, 10 ul CCK8 solution was used in each well and then the cells were incubated for another hour. OD values were read using a lab microplate reader at the wavelength of 450 nm. All the experiments in each cell group were repeated for 3 times.

2.6. Transwell for migration and invasion

Cells were collected at log phase and placed into the serum-free upper chambers (Corning, Shanghai, China) for migration experiments (Corning, Shanghai, China). The lower chambers were supplemented using 10% FBS (Gibco, Shanghai, China). After 24 h’s incubation, the cells migrating from the upper chamber or invading through the Matrigel onto the membranes were fixed using 4% paraformaldehyde and stained using crystal violet (Beyotime, Shanghai, China). The inverted microscope was used to take photos of the stained cells from six different fields in each group. The stained migrated and invaded cells were counted.

2.7. Bioinformatics

starbase v3.0 (http://starbase.sysu.edu.cn/agoClipRNA.php?source=IncRNA) were used to predict the potential microRNAs that target CircPVT1 and potential ceRNAs in homo sapiens. On Targetscan (http://www.targetscan.org/vert_72/), potential bindings between the miR-423-5p and SMAD3 were predicted in homo

| Table 1  | Primer sequences. |
|----------|------------------|
| **Forward** | **Reverse** |
| CircPVT1    | 5'-GGTTCACCCAGTGTTATTC-3'  | 5'-CACTCTTCTGCTGTTC-3' |
| miR-423-5p  | 5'-GCCTGAGCCCAGAGAC-3'    | 5'-CCAGTGCCTGTGAGT-3' |
| SMAD3       | 5'-GATCCAGGTCTGATGAGC-3'  | 5'-GCTCAACCACTGTCAC-3' |
| GAPDH       | 5'-GACTAACC-3'            | 5'-GCTCCAGAC-3'        |
| U6          | 5'-TCCGGGTGC              | 5'-TCACGTCG-3'         |
|            | TCCCGCGCCAG-3'           | GTTCCGCGGT-3'         |
generation were completed on GraphPad Prism 8 Software (GraphPad, CA, USA). One-way ANOVA and Tukey’s correction were used to analyze the difference among the groups. CCK8 results were analyzed using the two-way ANOVA method and Sidak’s correction. The general alpha value in the statistical system is 0.05.

3. Results

3.1. Upregulation of CircPVT1 promotes cell viability, migration and invasion and EMT in gastric cancer in vitro.

CircPVT1 was upregulated in GC cell lines AGS and SGC-7901 compared to the human stomach mucous epithelial cell lineGES-1 (Fig. 1A). CircPVT1 expression was regulated in AGS and SGC-7901 cells after transfection and RT-PCR verified that CircPVT1 was significantly upregulated in pc-CircPVT1 groups and downregulated in si-CircPVT1 groups (Fig. 1B and C). Furthermore, CCK8 assays found that upregulation of CircPVT1 enhanced the cell viability in both gastric cancer cell lines (Fig. 1D and E). Transwell assays found that upregulation of CircPVT1 enhanced the cell migration and invasive property. Results showed that CircPVT1 upregulation also improved cell migration and invasiveness in both GC cell lines (Fig. 1F). Thereafter, Western Blot methods analyzed the resultant changes in EMT protein markers, which witnessed the growth in the protein expression of Vimentin, Snai1, Twist1 and Zeb1 after the upregulation of CircPVT1 and a decrease in the protein expression of epithelial marker E-cadherin, suggesting a facilitative impact on EMT process (Supp 1). On the contrary, the downregulation of CircPVT1 displayed an opposite effect to the cellular functions compared to CircPVT1 upregulation (Fig. 1C-I, Supp 1 & 2). On the other hand, immunofluorescence results further confirmed that the upregulation of CircPVT1 inhibited E-cadherin and promoted Vimentin, Snai1, Twist1 and Zeb1 in both GC cell lines (Supp 3 & 4).

3.2. miR-423-5p was downregulated in GC cells and targeted CircPVT1

According to RT-PCR findings in this study, miR-423-5p was downregulated in GC cells in compared to GES-1 cell line (Fig. 2A). Furthermore, GC cells were transfected with miR-423-5p mimic or inhibitor and underwent RT-PCR evaluation, which validated an increase of miR-423-5p expression in miR-423-5p mimic groups and a decrease in the miR-423-5p inhibitor groups (Fig. 2B and C). CircPVT1 expression was reduced after miR-423-5p downregulation and was enhanced after miR-423-5p upregulation in GC cells (Fig. 2D and E). Bioinformatic analysis predicted the bindings between miR-423-5p and CircPVT1 in homo sapiens (Fig. 2F). Luciferase reporter experiments were performed to measure the relative luciferase activity in human cells HEK-293T. It was found that relative luciferase activity was inhibited in the co-transfected group of miR-423-5p mimic and WT-CircPVT1 and was enhanced in the group of miR-423-5p inhibitor and WT-CircPVT1 (Fig. 2G).

Table 3
Antibodies used in immunofluorescence.

| Antibodies | Dilution rate | Item, source                  |
|------------|---------------|-------------------------------|
| E-cadherin | 1:100         | 20874-1-AP, Proteintech, Wuhan, China |
| Vimentin   | 1:100         | 10366-1-AP, Proteintech, Wuhan, China |
| IgG H&L (FITC) | 1:1000 | ab6717, Abcam, Shanghai, China |
| Snai1      | 1:100         | 13099-1-AP, Proteintech, Wuhan, China |
| Twist1     | 1:100         | 25465-1-AP, Proteintech, Wuhan, China |
| Zeb1       | 1:100         | 21544-1-AP, Proteintech, Wuhan, China |

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Fig. 1. Upregulation of CircPVT1 induces cell viability, invasion and migration in gastric cancer. (A) RT-PCR examined the CircPVT1 expression patterns in AGS, SGC-7901 and GES-1 cells. (B–C) Both GC cell lines were transfected with si-CircPVT1 or pc-CircPVT1. NC, negative control, was the cell group without transfection. RT-PCR quantified the relative expression of CircPVT1 in each group. (D–E) Cell viability in all groups were evaluated using CCK8 method. (F–I) Transwell experiments were conducted to detect the cell migration and invasiveness in response to the alteration of CircPVT1 expression in GC cell lines. All experiments were repeated thrice. Mean and standard deviation (sd) values were shown in the subfigures. ****P < 0.0001, compared to the GES-1 group (A), NC groups (B–I).

Fig. 2. miR-423-5p was inhibited in GC cells and sponged by CircPVT1. (A) RT-PCR examined the miR-423-5p expression. (B–E) AGS and SGC-7901 cells were transfected with the miR-423-5p mimic or miR-423-5p inhibitor. RT-PCR checked the miR-423-5p expression and CircPVT1 expression. (F) StarBase2.0 predictions on targeted sites between CircPVT1 and miR-423-5p. (G) Luciferase activity. ****P < 0.0001, versus NC groups.
3.3. SMAD3 was targeted and negatively regulated by miR-423-5p in gastric cancer

Online bioinformatics was made to predict the binding sites between miR-423-5p and SMAD3 (Fig. 3A). Luciferase assays detected a decrease in the group transfected with SMAD3-WT and miR-423-5p mimic and an increase in the group transfected with miR-423-5p inhibitor and SMAD3-WT in HEK-293T cells (Fig. 3B). Further, RT-PCR proved that in GC cells, upregulation of miR-423-5p inhibited the SMAD3 mRNA expression while downregulation of miR-423-5p increased the SMAD3 in mRNA level (Fig. 3C and D). Based on the online analysis tool, starBase v3.0, miR-423-4p expression was negatively associated with that of CircPVT1 in gastric cancer tissues (Fig. 3E). Furthermore, online database GEPIA disclosed that in gastric cancer tissues, SMAD3 expression was upregulated in comparison with the non-tumor tissues (Fig. 3F). Furthermore, disease free survival analysis on GEPIA revealed a correlation between the low expression of SMAD3 and high disease-free survival rate (Fig. 3G).

3.4. CircPVT1/miR-423-5p regulated cell viability, migration and invasion and EMT process through Smad3 pathway in GC cells.

The interactions among CircPVT1/miR-423-5p/SMAD3 were analyzed. GC cells were transfected with si-SMAD3/pc-SMAD3 alone, or were co-transfected with miR-423-5p mimic and pc-SMAD3, pc-SMAD3 and si-CircPVT1, si-SMAD3 and si-CircPVT1. RT-PCR tests found that inhibition of SMAD3 resulted in a decrease in CircPVT1 expression, upregulation of SMAD3 induced CircPVT1 expression and upregulation of miR-423-5p lowered CircPVT1 expression in GC cells (Fig. 4A and B). miR-423-5p expression was provoked by inhibition of SMAD3 and was reduced by the upregulation of SMAD3 in GC cells (Fig. 4C and D). The downregulation of CircPVT1 recovered the miR-423-5p expression in GC cells (Fig. 4D and E). On the other hand, SMAD3 expression was inhibited in si-SMAD3 groups and provoked in pc-SMAD3 groups, was inhibited by the upregulation of miR-423-5p and inhibited by the downregulation of CircPVT1 in GC cells (Fig. 4E and F). The upregulation of SMAD3 led to a significant increase in cell viability and downregulation of SMAD3 inhibited cell viability in GC cells (Fig. 4G and H). Inhibition of CircPVT1 or upregulation of miR-423-5p decreased the cell viability in GC cells (Fig. 4G and H). Transwell assays showed similar findings to results in cell viability in each group (Fig. 4I–L, Supp 5), which demonstrated the promotive role of SMAD3 and CircPVT1 and inhibitory function of miR-423-5p in mediating cell migration and invasion. Western blot evaluated the protein changes in EMT biomarkers. EMT process, featuring decrease in E-cadherin and increase in Vimentin, Snai1, Twist1 and Zeb1 protein expressions, was promoted when SMAD3 was upregulated in pc-SMAD3 group and was inhibited after the introduction of miR-423-5p mimic or si-CircPVT1 (Fig. 5 & Supp 6). In addition, the knock-down of SMAD3 led to the inhibition of the EMT process (Fig. 5 & Supp 6). The phosphorylated Smad3 was examined by Western blot methods, showing the inactivation of Smad3 pathway in si-SMAD3.
group and activation in pc-SMAD3 group (Fig. 6). Additionally, the immunofluorescence in cells showed that downregulation of SMAD3 could inhibit the Vimentin, Snai1, Twist1 and Zeb1 proteins and promote E-cadherin, decreasing EMT (Supp 3 & 4). To note, the promoted EMT after upregulation of CircPVT1 could be partly counteracted by miR-423-5p upregulation according to the IF results (Supp 3 & 4).

4. Discussion

In this study, we examined the interactions among CircPVT1, miR-423-5p and SMAD3 in gastric cancer cells (Supp 3). CircPVT1 sponged miR-423-5p and inhibited its expression and upregulated SMAD3 while miR-423-5p targeted and inhibited SMAD3 expression in GC cells, demonstrating a typical circRNA-miRNA-mRNA regulatory mechanism in GC [17,18]. In functional modulation, CircPVT1 upregulation could enhance the cell growth, invasion and migration through promoting SMAD3 expression as a sponge and ceRNA to miR-423-5p. In accordance with previous studies, CircPVT1 was also an oncogene as it was verified [19–22] and different from the ones reported by others earlier, our study reveals a new modulatory system about CircPVT1 through interaction with miR-423-5p and SMAD3 in gastric cancer, which has never been reported before. Furthermore, it was also discovered that CircPVT1 upregulation or miR-423-5p inhibition could induce SMAD3 mRNA expression in cells, and eventually promoted the phosphorylation of Smad3, activating the Smad3 pathway.

Furthermore, we also found through Western blot analysis that EMT process was inhibited when SMAD3 mRNA expression was knocked down and was promoted when SMAD3 was upregulated in GC cells. Similar finding was revealed in breast cancer both in vitro and in vivo [23]. Also, it was also reported that SMAD3/SNAIL could be regulated by miR-323a-3p and impacted on the EMT process in bladder cancer [24]. In ovarian cancer, SMAD3 was discovered to facilitate EMT process and cell migration and invasion through enhancing the STYK1 transcription [25]. Smad3, induced by TGF-β, is closely involved in cell migration and invasion and EMT process in cancers [26–28]. Previous study also suggested that Smad3 inhibitors could curb EMT [26]. In breast cancer, the HER2/EGFR-AKT pathway altered TGF-β and regulated phosphorylation of Smad3, thus mediating cell migration [29]. In osteosarcoma, Smad3 phosphorylation could be inhibited by Glaucocalyxin A, suppressing EMT process [30]. In lung cancer, EMT process could be promoted through the activation of Smad2/3 signaling pathway and inhibited by the downregulation of SMAD2 or SMAD3 gene expression [31].

EMT process, which features the loss of epithelial marker proteins including E-cadherin and gain of mesenchymal markers like
Fig. 5. CircPVT1/miR-423-5p/SMAD3 regulated EMT proteins in GC cells. Western blot methods analyzed the protein expression of E-cadherin and Vimentin in response to the interplays of CircPVT1/miR-423-5p/SMAD3 in GC cells. ****P < 0.0001, vs NC; ##P =< 0.002, ###P =< 0.0002, ####P < 0.0001, vs pc-SMAD3 group; @@P =< 0.002, @@@@P < 0.0001, vs si-SMAD3 group.

Fig. 6. CircPVT1/miR-423-5p regulated Smad3 signaling pathway. Western blot methods analyzed the protein expression of phosphorylated Smad3 and total Smad2/3 in response to the interplays of CircPVT1/miR-423-5p/SMAD3 in GC cells. ****P < 0.0001, vs NC; ##P =< 0.002, ###P =< 0.0002, ####P < 0.0001, vs pc-SMAD3 group; @@P =< 0.002, @@@@P < 0.0001, vs si-SMAD3 group.
Vimentin and in addition, transcriptional factors including SNAI, ZEB and TWIST participate in the regulation of EMT process [32,33]. EMT is regarded as a key element of tumor metastasis and cell migration and invasion [34,35]. In gastric cancer, previous studies reveal that noncoding RNAs including miRNAs, circ RNAs, lncRNAs and mRNAs can regulate EMT-related proteins, leading to the alterations in GC cell migration and invasion and tumor metastasis in vivo [36]. Downregulation of miR-214 promoted GC cell migration and invasion through promoting EMT process [37]. CircRNA 100876 upregulation could result in the promotion of GC cell proliferation, migration and invasion by sponging miR-665 and upregulating YAP-1 [38]. Cancer-associated fibroblasts (CAF) exosome plays a critical role in tumor development and therapy resistance through modulating the proliferation, invasion, and stemness of cancer cells, etc [39]. Through TGF-β, CAF exosomes could induce EMT in cancers [40]. Previously, miR-423-5p, derived from CAF exosome, was discovered to regulate chemotherapeutic resistance, metastasis through TGF-β/SMAD pathway in prostate cancer [40]. SMAD3, as a key effector of TGF-β/Smad signaling axis, is critically important for important intellectual contents: Li Feng.

This current study reveals the regulatory system of CircPVT1, miR-423-5p and SMAD3 in EMT, cell migration and invasion and proliferation in GC for the first time, which enriches the knowledge of EMT regulation in GC further. The interaction between CircPVT1/miR-423-5p and SMAD3 in GC cells in this study also connected the changes in cell migration and invasion to EMT process, which was that when EMT process was curbed, cell migration and invasion functions were inhibited as well.

In conclusion, this study demonstrated that a new regulatory axis, CircPVT1/miR-423-5p/SMAD3, in gastric cancer, could mediate cellular proliferation, invasion, migration and EMT through Smad3 signaling pathway.

Ethic approval

Not Applicable.

Authors contributions

Conception and design of study: Huanqin Li, Shuai Xue. Acquisition of data: Xiaohong Zhang. Fan Li analysis and/or interpretation of data: Huanqin Li, Li Feng. Xiaohong Zhang. Drafting the manuscript: Shuai Xue, Songhua Bei, Fan Li revising the manuscript critically for important intellectual content: Li Feng.

Data availability

All the data are presented in the text.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.02.003.

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