Regenerative Therapy 21 (2022) 25–33

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

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

Huanqin Li [1], Shuai Xue [1], Xiaohong Zhang, Fan Li, Songhua Bei, Li Feng*

Endoscopy Center, Minhang Hospital, Fudan University, 170 Xin-Song Road, Shanghai 201199, PR China

ARTICLE INFO

Article history:
Received 22 April 2021
Received in revised form
5 January 2022
Accepted 19 February 2022

Keywords:
Gastric cancer
CircPVT1
miR-423-5p
Smad3 pathway

ABSTRACT

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 cicRNA 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

* Corresponding author.
E-mail addresses: 176545456@qq.com (H. Li), 19211360003@fudan.edu.cn (S. Xue), xixi0348@163.com (X. Zhang), 18918169316@163.com (F. Li), beisonghua@126.com (S. Bei), feng_li@fudan.edu.cn (L. Feng).
Peer review under responsibility of the Japanese Society for Regenerative Medicine.
[1] These authors contributed to this article equally.

https://doi.org/10.1016/j.reth.2022.02.003

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, circNRIP1 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 EIF4A1 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/SMAD3 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/SMAD3 axis in GC.

Then we validated the interactions among CircPVT1/miR-423-5p/SMAD3 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 (ThermoFisher, 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 iQ5 Real-Time PCR Detection System (Bio-Rad, CA, USA). $2^{-\triangle\triangle}C_t$ method was applied 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 assessment. Matrigel enveloped the upper chambers in the invasion 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 fixated 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=lncRNA) 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′-GGTTCCACCAGCGTTATTC-3′	5′-CAACTTCCTTTGGGTCTCC-3′
miR-423-5p	5′-GCCTGAGGGGCAGAGAGC-3′	5′-CCACGTGTCGTGGAGTC-3′
SMAD3	5′-GTAGCTCGTGGTGGCTGTG-3′	5′-ACGTCAACACCAAGTGCAC-3′
GAPDH	5′-GAGTCAACG GATTTGGTCGTATTG-3′	5′-CCTGGAAGA TGGTGATGGGATT-3′
U6	5′-TGCGGGTGC TCGCTTCGGCAGC-3′	5′-CCAGTGCAG GGTCCGAGGT-3′

sapiens. On GEPIA database (http://gepia.cancer-pku.cn/), differential expression of SMAD3 in gastric cancer tissues and normal ones were visualized, based on which we further analyzed the disease-free survival rate and overall survival rate (insignificant, P>>0.05, not listed in the figures) in association with SMAD3 expression in tumor tissues.

2.8. Luciferase reporter assays

We acquired the predicted binding sites of CircPVT1 and SMAD3, based on which, we mutated the base sequences and generated the mutant type (MT) of CircPVT1 or SMAD3. The luciferase reporter vectors were generated by inserting the wild-type (WT) or MT of CircPVT1 or WT-SMAD3 or MT-SMAD3 into the luciferase reporter pmirGLO vector (Promega, CA, USA). Then the vectors were transfected into HEK-293T cells together with miR-423 mimic or inhibitor or NC. At 48 h, the Dual-Luciferase Reporter Assay Kit was used to detect the luciferase activity in each group (Promega). All experiments in each group were repeated for three times.

2.9. Western blot

Total proteins were extracted from cells in each group using RIPA (Beyotime). Proteins were separated by using SDS-PAGE (10%) and transferred onto PVDF membranes and skimmed milk was added for blocking. After 2h, the membranes were incubated in primary antibodies after recommended dilution at 4 °C overnight. On the second day, the membranes were incubated in the secondary antibody for another 2 h. Beta-actin was used as an internal control. The antibodies were listed in Table 2.

2.10. Immunofluorescence (IF) method for EMT-related proteins in cells

Cells were cultured in 24-well plates and then fixed using 4% paraformaldehyde for 30 min. After cells were washed using cold PBS for 3 times, 0.5%Triton-X 100 was added. Diluted donkey serum (10%) was used to block the cells for 30 min. The primary antibodies were diluted using 1% donkey serum and were then added into cells. The samples were incubated at 4 °C overnight. On the second day, the secondary antibody was added to incubate the cell samples for 2 h in the dark. DAPI was added to stain the nuclei for 5 min. Then the cells were observed under a fluorescent microscope (Nikon, Japan) and fluorescent images were taken at 200X. Antibodies used in IF were included in Table 3.

2.11. Statistical analysis

All results from experiments were displayed in figures as means and standardized errors. Statistical analysis and figure 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.

Table 2
Antibodies used in western blot.

Antibodies	Dilution rate	Item, source
E-cadherin	1:20000	ab40772, Abcam, Shanghai, China
Vimentin	1:2000	ab137321, Abcam, Shanghai, China
phospho S423 + S425 Smad3	1:2000	ab52903, Abcam, Shanghai, China
Smad2/3	1:1000	ab202445, Abcam, Shanghai, China
Beta-actin	1:2500	ab8227, Abcam, Shanghai, China
IgG H&L (HRP)	1:25000	ab205718, Abcam, Shanghai, China
Snai1	1:1000	13099-1-AP, Proteintech, Wuhan, China
Twist1	1:1000	25465-1-AP, Proteintech, Wuhan, China
ZEB1	1:1000	21544-1-AP, Proteintech, Wuhan, China

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

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 line GES-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 methods examined the effect of CircPVT1 expression alteration on the cell migration and invasion properties. Results showed that CircPVT1 upregulation also improved cell migration and invasiveness in both GC cell lines (Fig. 1F–I). 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 & 2). 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 upregulation and was enhanced after miR-423-5p downregulation 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).

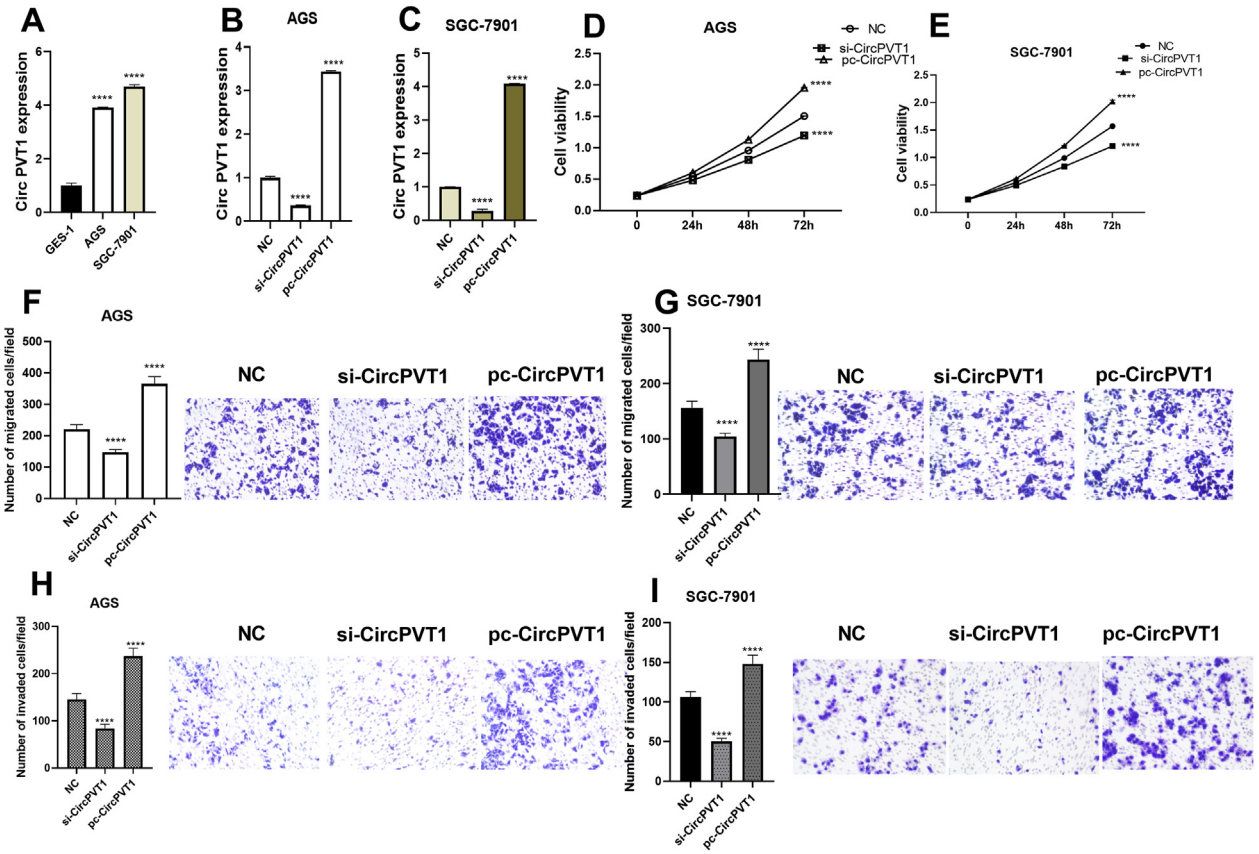

Fig. 1. Upregulation of CircPVT1 induces cell viability, invasion and migration in gastric cancer. (A) RT-PCR examined the CircPVT1 expression patterns in AGS, SGC-7901 and GES-1 cells. (B–C) Both GC cell lines were transfected with si-CircPVT1 or pc-CircPVT1. NC, negative control, was the cell group 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).

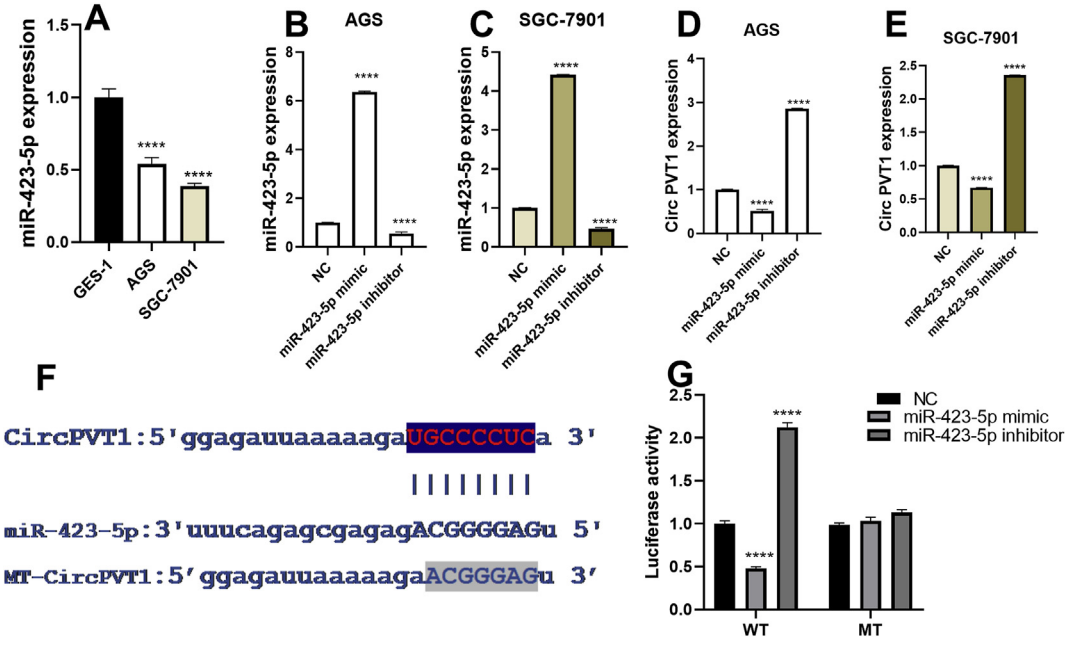

Fig. 2. miR-423-5p was inhibited in GC cells and sponged by CircPVT1. (A) RT-PCR examined the miR-423-5p expression. (B–E) AGS and SGC-7901 cells were transfected with the miR-423-5p mimic or miR-423-5p inhibitor. RT-PCR checked the miR-423-5p expression and CircPVT1 expression. (F) StarBase2.0 predictions 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-SMAD group and was inhibited after the introduction of miR-423-5p mimic or si-CircPVT1 (Fig. 5 & Supp 6). In addition, the knockdown 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

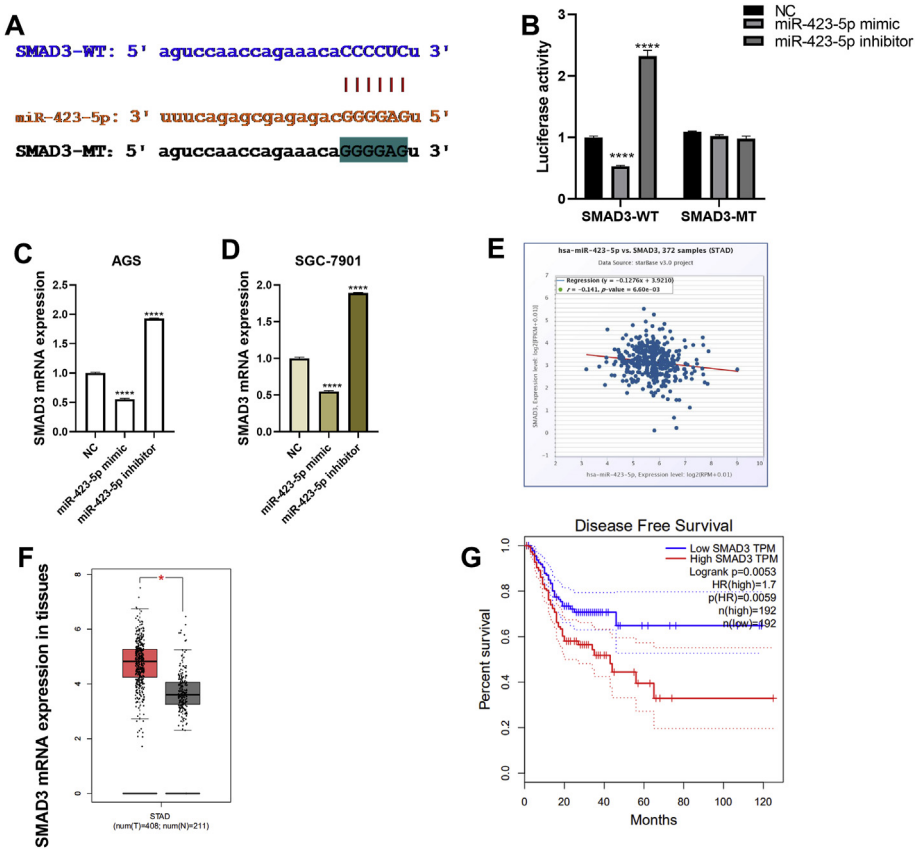

Fig. 3. SMAD3 was negatively regulated by miR-423-5p in GC. (A) starBase and targetscan online tools showed the predicted sites between miR-423-5p and SMAD3. (B) Luciferase reporter experiments measured the relative luciferase activity. (C–D) RT-PCR measured the regulations of miR-423-5p alteration on the SMAD3 in mRNA level in GC cells. (E) Pearson's association analysis was performed in starBase v3.0 in 372 gastric cancer samples. (F–G) GEPIA visualized the relative expression of SMAD3 in GC tissues with para-tumor tissues as control. Further, the disease-free survival analysis was performed. ****P < 0.0001, versus NC. All experiments were repeated for three times. Mean and sd values were shown in the subfigures.

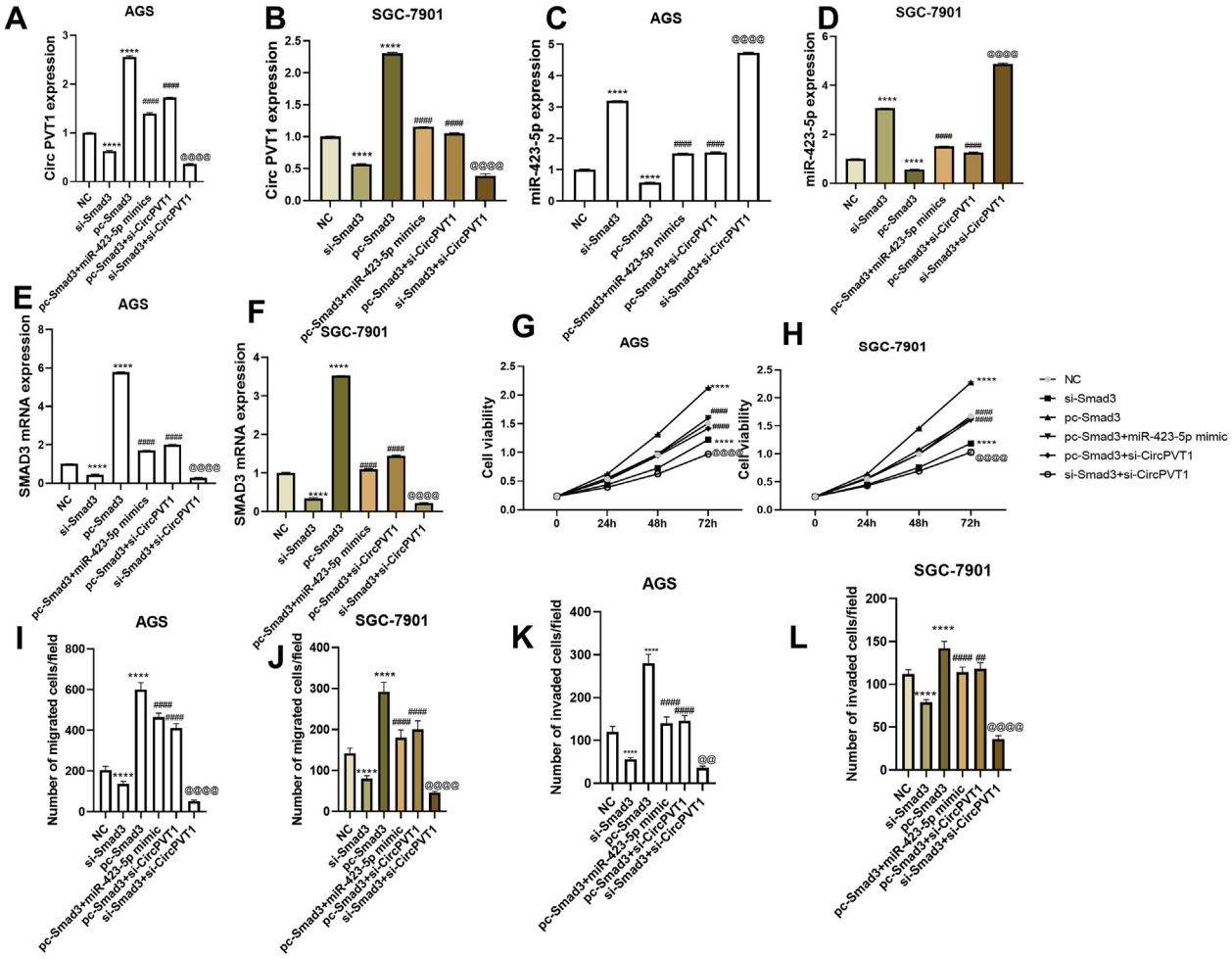

Fig. 4. CircPVT1/miR-423-5p/SMAD3 regulated cell viability, migration and invasion. GC cells were transfected with si-SMAD3/pc-SMAD3 alone, or were co-transfected with miR-423-5p mimic and pc-SMAD3, pc-SMAD3 and si-CircPVT1, si-SMAD3 and si-CircPVT1. (A–B) CircPVT1 expression. (C–D) SMAD3 mRNA expression. (E–F) miR-423-5p expression. (G–H) CCK8. (I–L) Transwell for migration and invasion. All experiments were repeated for three times. Mean and sd values were shown in the subfigures. ****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.

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

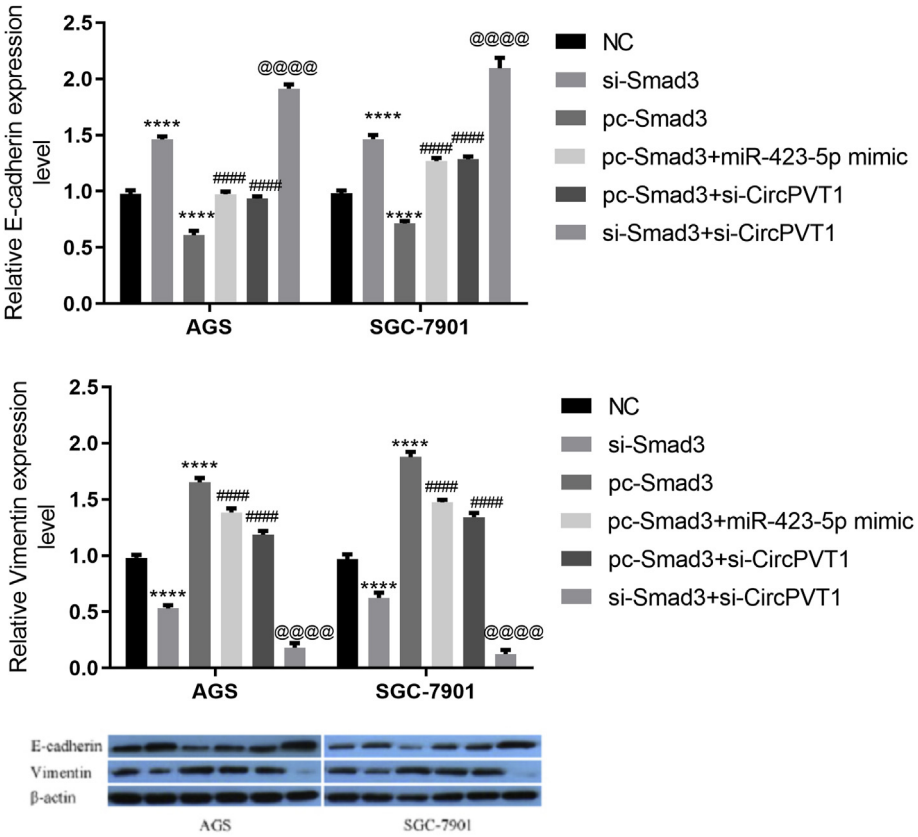

Fig. 5. CircPVT1/miR-423-5p/SMAD3 regulated EMT proteins in GC cells. Western blot methods analyzed the protein expression of E-cadherin and Vimentin in response to the interplays of CircPVT1/miR-423-5p/SMAD3 in GC cells. ****P < 0.0001, vs NC; ##P=<0.002, ###P=<0.0002, ####P < 0.0001, vs pc-SMAD3 group;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

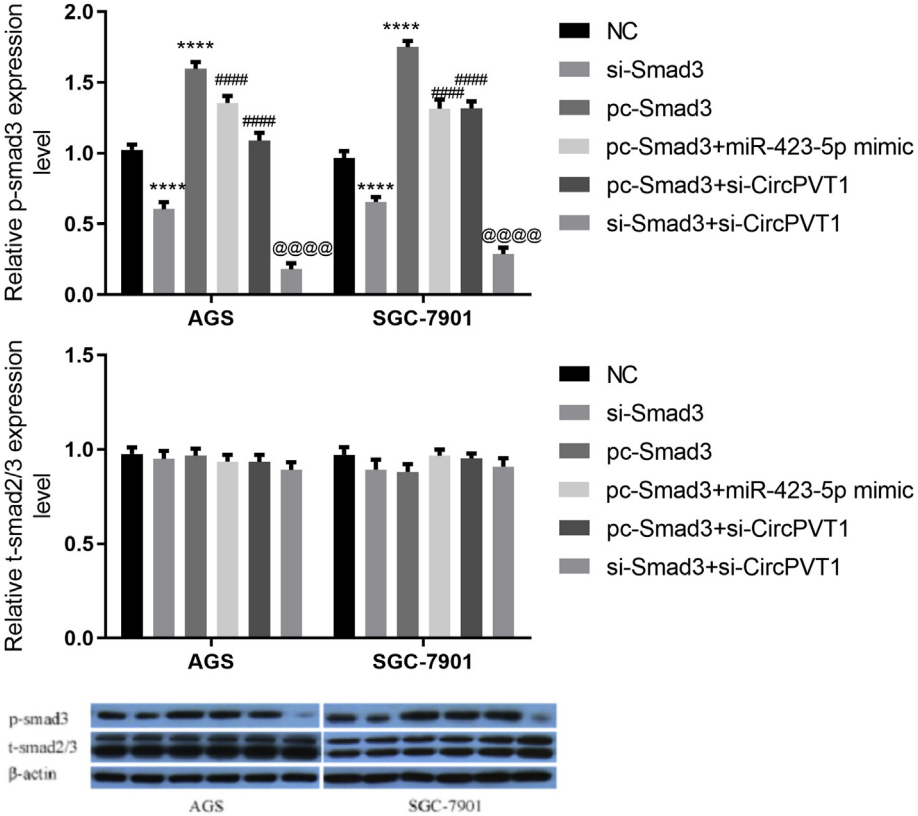

Fig. 6. CircPVT1/miR-423-5p regulated Smad3 signaling pathway. Western blot methods analyzed the protein expression of phosphorylated Smad3 and total Smad2/3 in response to the interplays of CircPVT1/miR-423-5p/SMAD3 in GC cells. ****P < 0.0001, vs NC; ##P=<0.002, ###P=<0.0002, ####P < 0.0001, vs pc-SMAD3 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 and EMT process 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 chemotherapy resistance, metastasis through TGF-β/SMAD pathway in prostate cancer [40]. SMAD3, as a key effector of TGF-β, is involved in regulatory mechanism of CAF in cancers [41]. In gastric cancer, CAF exosomes are of significance in the tumorigenesis [42], drug resistance [43], and metastasis [42]. Peritoneal dissemination is the most frequent metastatic pattern of gastric cancer [44]. The interactions between CAF and cancer cells regulate the tumor microenvironment and impact on the peritoneal metastasis in gastric cancer patients [45]. Previously, a study revealed that coculturing CAF and gastric cancer cells could enhance the DDR1 expression, which was associated with the high peritoneal metastasis rate in patients [46].

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.

Funding

1 Minhang District University Building Project (2017MWDXK03).
2 Minhang District Talent Development Special Fund Project (2017).
3 Minhang District Subject Pilot Talent Project.

Declaration of competing interest

None.

Acknowledgement

None.

Appendix A. Supplementary data

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

References

[1] Petryszyn P, Chapelle N, Matysiak-Budnik T. Gastric cancer: where are we heading? Dig Dis (Basel, Switzerland) 2020;38(4):280–5.
[2] Eusebi LH, Telese A, Marasco G, Bazzoli F, Zagari RM. Gastric cancer prevention strategies: a global perspective. J Gastroenterol Hepatol 2020;35(9):1495–502.
[3] Das M. Neoadjuvant chemotherapy: survival benefit in gastric cancer. Lancet Oncol 2017;18(6):e307.
[4] Pellino A, Riello E, Nappo F. Targeted therapies in metastatic gastric cancer: current knowledge and future perspectives. World J Gastroenterol 2019;25(38):5773–88.
[5] Shan C, Zhang Y, Hao X, Gao J, Chen X, Wang K. Biogenesis, functions and clinical significance of circRNAs in gastric cancer. Mol Cancer 2019;18(1):136.
[6] Necula L, Matei L, Dragu D, Gao J, Chen X, Wang K, et al. Recent advances in gastric cancer early diagnosis. World J Gastroenterol 2019;25(17):2029–44.
[7] Ruan Y, Li Z, Shen Y, Li T, Zhang H, Guo J. Functions of circular RNAs and their potential applications in gastric cancer. Expert Rev Gastroenterol Hepatol 2020;14(2):85–92.
[8] Liang M, Huang G, Liu Z, Wang Q, Yu Z, Liu Z, et al. Elevated levels of hsa_circ_006100 in gastric cancer promote cell growth and metastasis via miR-195/GPRC5A signalling. Cell Prolif 2019;52(5):e12661.
[9] Zhang X, Wang S, Wang H, Cao J, Huang X, Chen Z, et al. Circular RNA circNRIP1 acts as a microRNA-149-5p sponge to promote gastric cancer progression via the AKT1/mTOR pathway. Mol Cancer 2019;18(1):20.
[10] Lu J, Wang YH, Yoon C, Xu Y, Xie JW, Wang JB, et al. Circular RNA circ-RanGAP1 regulates VEGFA expression by targeting miR-877-3p to facilitate gastric cancer invasion and metastasis. Cancer Lett 2020;471:38–48.
[11] Li C, Tian Y, Liang Y, Li Q. Circ_0008035 contributes to cell proliferation and inhibits apoptosis and ferroptosis in gastric cancer via miR-599/EIF4A1 axis. Cancer Cell Int 2020;20:84.
[12] Chen J, Li Y, Zheng Q, Bao C, He J, Chen B, et al. Circular RNA profile identifies circPVT1 as a proliferative factor and prognostic marker in gastric cancer. Cancer Lett 2017;388:208–19.
[13] Liu YP, Wan J, Long F, Tian J, Zhang C. circPVT1 facilitates invasion and metastasis by regulating miR-205-5p/c-FLIP Axis in osteosarcoma. Cancer Manag Res 2020;12:1229–40.
[14] Sun G, Ding X, Bi N, Wu L, Wang J, Zhang W, et al. MiR-423-5p in brain metastasis: potential role in diagnostics and molecular biology. Cell Death Dis 2018;9(10):936.
[15] Luo Y, Tan W, Jia W, Liu Z, Ye P, Fu Z, et al. The long non-coding RNA LINC01606 contributes to the metastasis and invasion of human gastric cancer and is associated with Wnt/β-catenin signaling. Int J Biochem Cell Biol 2018;103:125–34.
[16] Liu J, Wang X, Yang X, Liu Y, Shi Y, Ren J, et al. miRNA423-5p regulates cell proliferation and invasion by targeting trefoil factor 1 in gastric cancer cells. Cancer Lett 2014;347(1):98–104.
[17] Lu Q, Liu T, Feng H, Yang R, Zhao X, Chen W, et al. Circular RNA circSLC8A1 acts as a sponge of miR-130b/miR-494 in suppressing bladder cancer progression via regulating PTEN. Mol Cancer 2019;18(1):111.
[18] Wang J, Zhao X, Wang Y, Ren F, Sun F, Yan Y, et al. circRNA-002178 act as a ceRNA to promote PDL1/PD1 expression in lung adenocarcinoma. Cell Death Dis 2020;11(1):32.
[19] Wang J, Huang K, Shi L, Zhang Q, Zhang S. CircPVT1 promoted the progression of breast cancer by regulating MiR-29a-3p-mediated AGR2-HIF-1α pathway. Cancer Manag Res 2020;12:11477–90.
[20] Adhikary J, Chakraborty S, Dalal S, Basu S, Dey A, Ghosh A. Circular PVT1: an oncogenic non-coding RNA with emerging clinical importance. J Clin Pathol 2019;72(8):513–9.
[21] Li X, Zhang Z, Jiang H, Li Q, Wang R, Pan H, et al. Circular RNA circPVT1 promotes proliferation and invasion through sponging miR-125b and activating E2F2 signaling in non-small cell lung cancer. Cell Physiol Biochem 2018;51(5):2324–40.
[22] Wang Z, Su M, Xiang B, Zhao K, Qin B. Circular RNA PVT1 promotes metastasis via miR-145 sponging in CRC. Biochem Biophys Res Commun 2019;512(4):716–22.

[23] Yang W, Feng W, Wu F, Gao Y, Sun Q, Hu N, et al. MiR-135-5p inhibits TGF-β-induced epithelial-mesenchymal transition and metastasis by targeting SMAD3 in breast cancer. J Cancer 2020;11(21):6402–12.
[24] Li J, Xu X, Meng S, Liang Z, Wang X, Xu M, et al. MET/SMAD3/SNAIL circuit mediated by miR-323a-3p is involved in regulating epithelial-mesenchymal transition progression in bladder cancer. Cell Death Dis 2017;8(8):e3010.
[25] Shi Y, Zhang J, Liu M, Huang Y, Yin L. SMAD3 inducing the transcription of STYK1 to promote the EMT process and improve the tolerance of ovarian carcinoma cells to paclitaxel. J Cell Biochem 2019;120(6):10796–811.
[26] Roberts AB, Tian F, Byfield SD, Stuelten C, Ooshima A, Saika S, et al. Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. Cytokine Growth Factor Rev 2006;17(1–2):19–27.
[27] Liu X, Zhang Y, Zhou GJ, Hou Y, Kong Q, Lu JJ, et al. Natural alkaloid 8-oxo-epiberberine inhibited TGF-β1-triggred epithelial-mesenchymal transition by interfering Smad3. Toxicol Appl Pharmacol 2020;404:115179.
[28] Katsuno Y, Lamouille S, Derynck R. TGF-β signaling and epithelial-mesenchymal transition in cancer progression. Curr Opin Oncol 2013;25(1):76–84.
[29] Huang F, Shi Q, Li Y, Xu L, Xu C, Chen F, et al. HER2/EGFR-AKT signaling switches TGFβ from inhibiting cell proliferation to promoting cell migration in breast cancer. Cancer Res 2018;78(21):6073–85.
[30] Jiang X, Zhang Z, Song C, Deng H, Yang R, Zhou L, et al. Glaucocalyxin A reverses EMT and TGF-β1-induced EMT by inhibiting TGF-β1/Smad2/3 signaling pathway in osteosarcoma. Chem Biol Interact 2019;307:158–66.
[31] Tang Y, Xuan Y, Qiao G. MDM2 promotes epithelial-mesenchymal transition through activation of Smad2/3 signaling pathway in lung adenocarcinoma. OncoTargets Ther 2019;12:2247–58.
[32] Goossens S, Vandamme N, Van Vlierberghe P, Berx G. EMT transcription factors in cancer development re-evaluated: beyond EMT and MET. Biochim Biophys Acta Rev Cancer 2017;1868(2):584–91.
[33] Suarez-Carmona M, Lesage J, Cataldo D, Gilles C. EMT and inflammation: inseparable actors of cancer progression. Mol Oncol 2017;11(7):805–23.
[34] Pastushenko I, Blanpain C. EMT transition states during tumor progression and metastasis. Trends Cell Biol 2019;29(3):212–26.
[35] Mittal V. Epithelial mesenchymal transition in tumor metastasis. Ann Rev Pathol 2018;13:395–412.
[36] Bure IV, Nemtsova MV, Zaletaev DV. Roles of E-cadherin and noncoding RNAs in the epithelial-mesenchymal transition and progression in gastric cancer. Int J Mol Sci 2019;20(12).
[37] Wang R, Sun Y, Yu W, Yan Y, Qiao M, Jiang R, et al. Downregulation of miRNA-214 in cancer-associated fibroblasts contributes to migration and invasion of gastric cancer cells through targeting FGF9 and inducing EMT. J Exp Clin Cancer Res 2019;38(1):20.
[38] Lin X, Huang C, Chen Z, Wang H, Zeng Y. CircRNA_100876 is upregulated in gastric cancer (GC) and promotes the GC cells' growth, migration and invasion via miR-665/YAP1 signaling. Front Genet 2020;11:546275.
[39] Fiori ME, Di Franco S, Villanova L, Bianca P, Stassi G, De Maria R. Cancer-associated fibroblasts as abettors of tumor progression at the crossroads of EMT and therapy resistance. Mol Cancer 2019;18(1):70.
[40] Shan G, Gu J, Zhou D, Li L, Cheng W, Wang Y, et al. Cancer-associated fibroblast-secreted exosomal miR-423-5p promotes chemotherapy resistance in prostate cancer by targeting GREM2 through the TGF-β signaling pathway. Exp Mol Med 2020;52(11):1809–22.
[41] Ren J, Smid M, Iaria J, Salvatori DCF, van Dam H, Zhu HJ, et al. Cancer-associated fibroblast-derived Gremlin 1 promotes breast cancer progression. Breast Cancer Res 2019;21(1):109.
[42] Yan Y, Wang LF, Wang RF. Role of cancer-associated fibroblasts in invasion and metastasis of gastric cancer. World J Gastroenterol 2015;21(33):9717–26.
[43] Uchihara T, Miyake K, Yonemura A, Komohara Y, Itoyama R, Koiwa M, et al. Extracellular vesicles from cancer-associated fibroblasts containing annexin A6 induces FAK-YAP activation by stabilizing β1 integrin, enhancing drug resistance. Cancer Res 2020;80(16):3222–35.
[44] Sun F, Feng M, Guan W. Mechanisms of peritoneal dissemination in gastric cancer. Oncol Lett 2017;14(6):6991–8.
[45] Miki Y, Yashiro M, Moyano-Galceran L, Sugimoto A, Ohira M, Lehti K. Crosstalk between cancer associated fibroblasts and cancer cells in scirrhous type gastric cancer. Front Oncol 2020;10:568557.
[46] Jin H, Ham IH, Oh HJ, Bae CA, Lee D, Kim YB, et al. Inhibition of discoidin domain receptor 1 prevents stroma-induced peritoneal metastasis in gastric carcinoma. Mol Cancer Res: MCR 2018;16(10):1590–600.