Volume 22, No 2 | International Journal of Radiation Research, April 2024

Cortactin enhances invadopodium formation to promote invasion and metastasis of gastric cancer cells via matrix metalloproteinases

Y. Wu[1#], M. Peng[2#], Q. Tang[3], P. Guo[1], P. Nie[1], Y. Cui[1], J. Yu[4*]

[1]Section for Gastrointestinal Surgery, Department of General Surgery, The Third People's Hospital of Chengdu, Affiliated Hospital of Southwest Jiaotong University & The Second Affiliated Hospital of Chengdu, Chongqing Medical University, Chengdu, 610031, China

[2]Department of Gastrointestinal surgery, The First Affiliated Hospital of Chengdu Medical College, Chengdu, 610500, China

[3]Department of Emergency, The Third People's Hospital of Chengdu, Chengdu, Sichuan 610031, China

[4]Center of Gastrointestinal and Minimally Invasive Surgery, Department of General Surgery, The Third People's Hospital of Chengdu, Affiliated Hospital of Southwest Jiaotong University & The Second Affiliated Hospital of Chengdu, Chongqing Medical University, Chengdu, 610031, China

► *Original article*

Corresponding author:
Jiahui Yu, Ph.D.,
E-mail: *dr_jiahuiyu@163.com*

Received: November 2023
Final revised: December 2023
Accepted: January 2024

Int. J. Radiat. Res., April 2024; 22(2): 487-494

DOI: 10.61186/ijrr.22.2.487

Keywords: *Gastric cancer, cortactin, invadopodium, metastasis, matrix metalloproteinases.*

#these authors are contributed equally to this work.

ABSTRACT

Background: One prevalent malignant tumor in the digestive system is gastric cancer (GC). Cortactin is an intracellular cytoskeleton protein and exerts the crucial function in GC development. However, the roles and mechanisms of cortactin in the invasion and metastasis of GC need further exploration. ***Materials and Methods:*** Cortactin expression in GC tissues and cells via western blot and quantitative reverse transcription PCR. Cell migration and invasion were detected by the Transwell assays. Immunofluorescence staining and extracellular matrix (ECM) degradation assays verified the ability to invadopodium formation and ECM degradation.We then used gelatin zymography to identify the relationship between cortactin and matrix metalloproteinases (MMPs). The xenograft tumor model proved that cortactin can accelerate tumor growth and intraperitoneal metastasis in mice. ***Results:*** We found that cortactin is overexpressed in GC. cortactin overexpression facilitated cell migration and invasion, whereas cortactin silencing exerted the opposite function. cortactin can facilitate invadopodium formation and ECM degradation in GC cells. Cortactin can positively regulate matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) levels. Furthermore, Cortactin accelerate GC progression in vivo. ***Conclusion:*** In short, this study confirmed that cortactin enhanced invadopodium formation to accelerate GC development through upregulating MMP2 and MMP9.

INTRODUCTION

One prevalent malignant tumor in the digestive system is gastric cancer (GC) [1]. Although there are new therapies such as immunotherapy and targeted therapy for GC, the prognosis of GC patients is still far from satisfactory [2, 3]. This is because GC lacks early specific diagnostic biomarkers, making it difficult for most patients to diagnose the cancer early, resulting in poor treatment efficacy and a 5-year overall survival rate of less than 30% [4, 5]. Metastasis is the main cause of cancer death [6-8]. Therefore, for the purpose of creating cutting-edge and successful therapy and diagnostic approaches, a deeper comprehension of the probable mechanism of GC metastasis is essential.

In order to fulfill their objective of proliferating in distant organs, cancer cells must undergo metastasis, which is the separation of the tumor from the surrounding extracellular matrix (ECM) and subsequent dissemination throughout the body via the lymphatic or circulatory systems [9]. The degradation and remodeling of ECM are considered important steps in cancer cell metastasis and dissemination [10]. Tumor cells can release exosomes to recombine ECM and secrete matrix metalloproteinases (MMPs) to promote migration and invasion [11]. Cell adhesion, ECM proteolysis, and cell migration are the three steps in the multi-step process of cancer cell invasion across the extracellular matrix (ECM) barrier [12, 13]. Recent studies have shown cancer cell invasion can be mediated via invadopodium. Invadopodium is the actin-rich cancer cell protrusion with proteolytic activity, which can promote MMPs-mediated ECM degradation and cell movement [14]. There are

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[DOI: 10.61186/ijrr.22.2.487]

currently studies that use the mechanisms related to invadopodium as an auxiliary means for treatment methods such as surgery, radiotherapy, and chemotherapy, as well as potential therapeutic targets for glioma invasion [15]. They are enriched in assorted proteins which modulate actin polymerization such as cortactin, N-WASp, Arp2/3, and cofilin [16]. The crucial role of invadopodium in tumor metastasis has been confirmed by multiple studies [17]. PLXDC2 interacts with PTP1B to promote invadopodium formation to strengthen GC cell metastasis [18]. Smad4 promotes fascin expression to facilitate invadopodium formation and cell invasion in breast cancer [19]. However, the specific molecular mechanism regulating the invadopodium formation is still unclear.

Cortactin, an intracellular cytoskeleton protein, was initially identified as the substrate of Src kinase [20]. Cortactin exerts the crucial function in actin assembly, scaffold, cytoskeletal arrangement, and membrane trafficking [21]. Under external stimulation, cortactin can undergo tyrosine phosphorylation, and promote the polymerization and assembly of actin filaments required for cell migration [22]. After stimulation, cortactin activates Arp2/3 complex to promote the polymerization of branched chain actin around cells [23]. Cortactin is an essential protein component for the core formation of invadopodium precursors, which is crucial for the invadopodium formation, ECM degradation, cell invasion, and tumor metastasis [24]. In addition, cortactin overexpression is frequently observed in assorted cancer types, which is closely related to poor prognosis and low survival rate [25]. Cortactin is reported to facilitate cell migration and invasion of glioma via modulating lamellipodia formation through combining with Arp2/3 complex [26]. Cortactin facilitates colorectal cancer cell growth through the EGFR-MAPK pathway [27]. Furthermore, cortactin can act as the potential marker for dural-targeted therapy in GC [28], and it accelerates GC cell migration and invasion [29]. MMPs take part in protein degradation in ECM, and they are closely associated with tumor development and metastasis [30]. However, there have been no reports on the relationship between cortactin and MMPs in the study of GC invasion and metastasis.

This study mainly explores the specific impacts and potential regulatory mechanism of alterations in cortactin expression on GC metastasis and invadopodium formation. It is hoped that targeting invadopodia-mediated gastric cancer cell invasion and metastasis will provide new insights and develop new targeted treatment strategies in the future.

MATERIALS AND METHODS

Tissue collection

Twenty paraffin-embedded tumor tissues and matched surrounding tissues as well as 16 fresh frozen tumor and paired normal tissues were collected from GC patients received gastrectomy at The Third People's Hospital of Chengdu, Affiliated Hospital of Southwest Jiaotong University & The Second Affiliated Hospital of Chengdu. This study was approved by The Third People's Hospital of Chengdu, Affiliated Hospital of Southwest Jiaotong University & the Second Affiliated Hospital of Chengdu. All patients have signed informed consent (CL2020-L-109).

Table 1. Clinicopathological features from gastric cancer patients.

Variables	GC patients (n=36)
Age	63.1 (37-62)
Sex	
Male	21
Female	15
Growth pattern	
Expansive	7
Infiltrative	22
Indeterminate	7
TNM stage	
I/II	12
III/IV	24

Immunohistochemical staining (IHC)

After being fixed in paraffin, the tissues were sectioned into 5 μm pieces and rehydrated and dewaxed. Next, they were soaked in EDTA and microwaved for antigenic retrieval. Then, the endogenous peroxidase was blockaded by the treatment of 0.3% hydrogen peroxide. After culturing with 1% bovine serum, sections were cultured with anti-cortactin (ab81208, Abcam, UK) at the temperature of 4°C for a whole night. After incubation with secondary antibody (ab6112, Abcam), sections were dyed by DAB (Dako, Denmark).

Cell culture

GC cell lines HGC27 and AGS as well as the normal stomach mucosa epithelium cell line GES-1 were obtained from ATCC (Manassas, USA). Cells were incubated in RPMI-1640 medium 10% FBS (Gibco, USA) at 37 °C with 5% CO_2.

Cell transfection

The short hairpin RNAs (shRNAs; GenePharma, China) specifically targeted to cortactin was constructed, with nonspecific shRNAs as negative control (NC). To overexpress cortactin, the whole sequence of cortactin was cloned into pcDNA3.1 vector (Geenseed Biotech, China), with the empty vector as NC. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was utilized for cell transfection.

RT-qPCR

The total RNAs extracted from cells were acquired utilizing Trizol reagent (Invitrogen, USA) for the synthesis of first cDNA template in line with the

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instruction (Takara, Kyoto, Japan). After that, SYBR Green PCR Master Mix (Takara, Japan) was applied for quantitative analysis with the Step-One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The processed outcomes with $2^{-\Delta\Delta CT}$ method were standardized GAPDH.

Western blot

The protein extraction kit (Key Gene, China) was used to extract all of the proteins. The protein was deposited onto PVDF membranes (Invitrogen, USA) and blocked with 5% nonfat milk following electrophoresis on 10% SDS-PAGE. Subsequently, they underwent one night of 4°C probing with anti-cortactin (ab81208, Abcam, USA), anti-MMP2 (ab92536, Abcam, USA), and anti-MMP9 (ab76003, Abcam, USA). After that, the membranes were incubated for two hours with secondary antibodies (Abcam, USA). The ECL kit was used to test the protein band (Millipore, USA).

Transwell assays

Transwell chambers from Corning, New York, were used for experiments involving cell invasion and migration. For 24 hours, cells in 200 milliliters of serum-free media have been placed into the top chamber. The Matrigel-coated upper chamber was ready for the invasion test. A 600 ml full medium supplement was added to the lower chamber. Cells were fixed after 48 hours, and 5% crystal violet was used to color them. An Olympus microscope was used for observation and photography.

Immunofluorescence (IF) assay

Cells were placed on covering slides in 24-well plates coated with 0.2% gelatin, then fixed with 4% PFA and permeabilized for 30 minutes with 0.5% Triton X-100 (Thermo, USA). After blockading with 10% of goat serum, slides were cultured with anti-MMP14 (ab51074, Abcam, USA) at the temperature of 4 °C for a whole night. Slides were cultured with secondary antibody (Abcam, USA) for one hour following PBS rinsing. F-actin were dyed by Alexa 594 phalloidin (A12381, Thermo Fisher scientific) for 1 h. DAPI (Thermo, USA) was utilized for staining nucleus. The inverted microscopy (Olympus, Japan) was applied for observation.

Gelatin zymography

The cell supernatant was subjected to concentration and mix with the non-reducing sample buffer (Invitrogen, USA). Protein extracts were loaded onto the SDS-PAGE comprising with 1 mg/mL gelatin. Then, gels were rinsed for half an hour in 2.5% Triton X-100 and cultured in development solution (Bio-Rad) for 15 h. Gels were dyed by Coomassie Brilliant Blue for testing the activities of MMP2 and MMP9.

ECM degradation assay

For twenty minutes, the abacteria coverslips were coated with 0.1 mg/ ml^{-1} poly-d-lysine. Following three PBS rinses, they were cultured for fifteen minutes with 0.4% glutaraldehyde. Oregon Green™ gelatin 488 (Invitrogen, USA) was utilized for coating coverslips for 1 h. Next, coverslips were cultured with sodium borohydride for 1 min and 70% ethanol for 20 min. RPMI-1640 was supplemented into the coverslips for 1 h prior to plating. Cells were put on coverslip and cultured for 8 h, followed by fixing with 4% PFA and permeabilizing with 0.5% Triton X-100. F-actin (Thermo Fisher Scientific, USA) was applied for incubating the coverslips for 1 h. DAPI was applied for visualizing nuclei.

ECM adhesion assay

Human fibronectin, laminin, or vitronectin were coated on 96-well plates for one night at the temperature of 4 °C and blockaded with 1% bovine serum albumin for 1 h at the temperature of 37 °C. Then, cells were put in the plates for 2 h of incubation. After rinsing with PBS, to measure absorbance, serum-free media was added to the plates, and the CCK-8 assay kit (Invitrogen, USA) was used.

Animal experiments

The female 6-week-old BALB/C nude mice were purchased from The Third People's Hospital of Chengdu, Affiliated Hospital of Southwest Jiaotong University & the Second Affiliated Hospital of Chengdu. The animal assays were also approved by the hospital. The transfected AGS cells (2×10^5) stably overexpressing or silencing cortactin were subcutaneously injected into root of right thigh of mice to establishing the xenograft tumor model (n=6 each group), or intraperitoneally injected into mice for establishing the metastasis model (n=6 each group). After 35 days of injection, mice were euthanized. The tumors were removed and weighted. The quantities of metastatic nodules were calculated.

HE staining

Tumors were fixed by 10% buffered formalin for 48 h and embedded in paraffin. Then they were cut into 5 μm thick sections. Sections were dyed by hematoxylin and eosin (Beyotime, China), and then analyzed by microscopic (Olympus, Japan).

Statistical analysis

Each experiment in our research was carried out over three times. Data was displayed as mean ± SD. Besides, the information was assessed by GraphPad PRISM 6 using the Student's t-test or one-way ANOVA. Comparisons were regarded as significantly different at the value of $p < 0.05$.

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RESULTS

Cortactin is overexpressed in GC

First, we investigated the trend of cortactin expression in GC. Through IHC staining, cortactin exhibited low or no expression in normal adjacent tissues, but exhibited high expression in tumor tissues and metastatic lymph nodes, and the positive area of cortactin in tumor tissues increased with the depth of invasion (figure 1A). RT-qPCR results illustrated the high mRNA expression of cortactin in tumor tissues (figure 1B). For further confirming the IHC outcomes, we determined cortactin mRNA and protein levels in fresh GC tumor tissues and adjacent tissues through RT-qPCR and western blot. We observed that cortactin levels were notably raised in tumor tissues in comparison of normal controls (figure 1C-D). In GC cell line AGS and HGC27, we also observed the overexpression of cortactin (figure 1E).

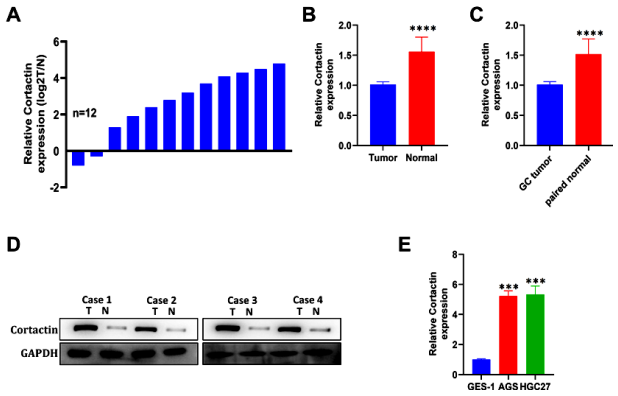

Figure 1. Cortactin is overexpressed in GC. **(A)** IHC staining of relative cortactin expression in normal tissues, GC tissues, and metastasis lymph nodes (n=12). **(B)** RT-qPCR of cortactin expression in normal and tumor tissues (n=20). **(C)** RT-qPCR of cortactin expression in fresh GC tumor tissues and the paired normal tissues (n=12). **(D)** Western blot of cortactin expression in fresh GC tumor tissues and the paired normal tissues (n=6). **(E)** RT-qPCR of cortactin expression in GC cells. ***P<0.001, ****P<0.0001.

Cortactin facilitates the migration and invasion of GC cells

We tested the impacts of cortactin expression on GC cell migration and invasion. Cortactin expression was silenced in AGS and HGC27 cells by transfecting with sh-cortactin plasmids, and overexpressed in cells by transfecting with pcDNA3.1-cortactin vector. Then, through transwell assays, we discovered that cortactin depletion markedly reduced the migratory and invasive capabilities of AGS and HGC27 cells, while cortactin upregulation promoted these capabilities (figure 2A-H).

Cortactin modulates invadopodium formation and ECM degradation

Cortactin depletion notably declined the ratio of invadopodium-positive cells in AGS and HGC27 cells, while cortactin upregulation elevated the ratio of invadopodium-positive cells (figure 3A-D). Next, gelatin degradation assay was applied for testing the effect of cortactin on nidus matrix degradation. Cortactin deficiency resulted in the notable decrease on cell capability of degrading focal ECM. The ability of cortactin silenced cells to degrade ECM was notably reduced. On the contrary, cortactin upregulation resulted in the enhanced ECM degradation (figure 3E-H).

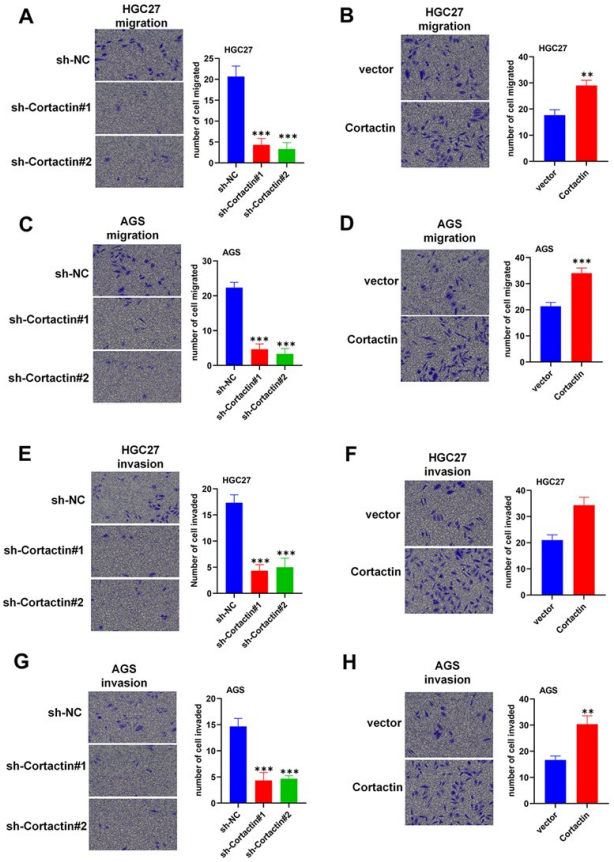

Figure 2. Cortactin facilitates GC cell migration and invasion. **(A-D)** Transwell assays were implemented for testing the impacts of cortactin depletion or overexpression on HGC27 and AGS cell migration. **(E-H)** Transwell assays were implemented for testing the impacts of cortactin depletion or overexpression on HGC27 and AGS cell invasion. **P<0.01, ***P<0.001.

Cortactin promotes ECM adhesion of GC cells

Cell-ECM adhesion is the vital process in tumor metastasis [31]. We further estimated the adhesion capability of GC cells to ECM components (fibronectin, laminin and vitronectin) utilizing CCK-8 assay. We discovered that, in comparison of control cells, cortactin silenced cells illustrated the lower adhesion capability to ECM components. Nevertheless, cortactin overexpression markedly elevated the quantity of adhered cells (figure 4A-C).

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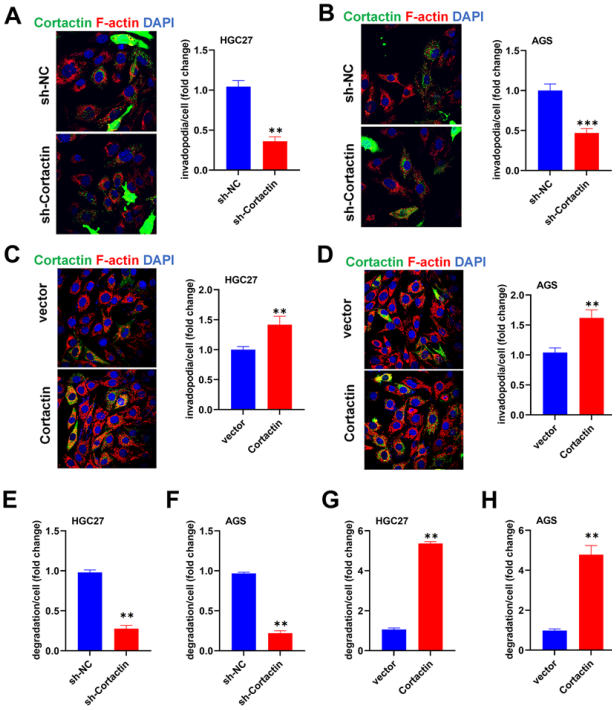

Figure 3. Cortactin modulates invadopodium formation and ECM degradation. **(A-B)** IF staining assay was applied for testing the co-localization of MMP14 and F-actin in HGC27 and AGS cells when cortactin was silenced. **(C-D)** IF staining assay was applied for testing the co-localization of MMP14 and F-actin in HGC27 and AGS cells when cortactin was overexpression. **(E-F)** ECM degradation assay was performed in HGC27 and AGS cells when cortactin was silenced. **(G-H)** ECM degradation assay was performed in HGC27 and AGS cells when cortactin was overexpression. **P<0.01, ***P<0.001.

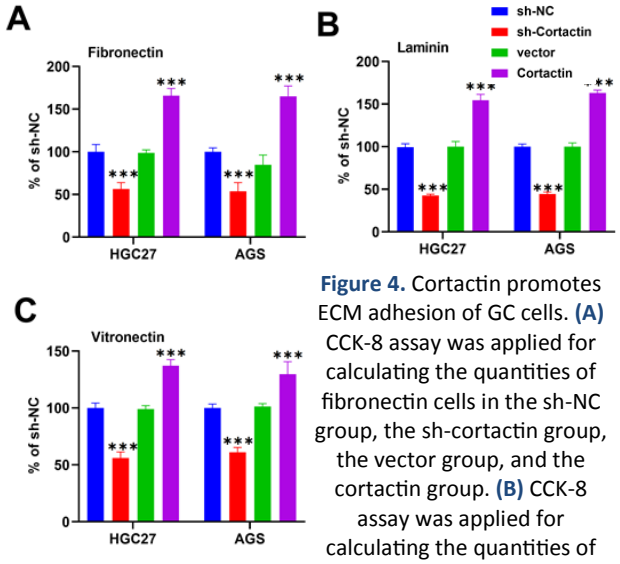

Figure 4. Cortactin promotes ECM adhesion of GC cells. **(A)** CCK-8 assay was applied for calculating the quantities of fibronectin cells in the sh-NC group, the sh-cortactin group, the vector group, and the cortactin group. **(B)** CCK-8 assay was applied for calculating the quantities of laminin cells in groups. **(C)** CCK-8 assay was applied for calculating the quantities of vitronectin cells in groups. ***P<0.001.

Cortactin elevates the expression and activity of MMPs

MMPs are involved in the degradation of various proteins in ECM, and the elevated levels of MMPs are closely related to tumor invasion [30]. Thus, we detected the influence of cortactin on MMP2 and MMP9. We found that cortactin shortage limited the mRNA and protein levels of MMP2 and MMP9, but cortactin overexpression increased them (figure 5A-C). For further determining whether MMP2 and MMP9 activation was raised by cortactin, we implemented gelatin zymography. Cortactin silenced cells showed the low levels of activated MMP2 and MMP9, while cortactin overexpression promoted their levels (figure 5D-E).

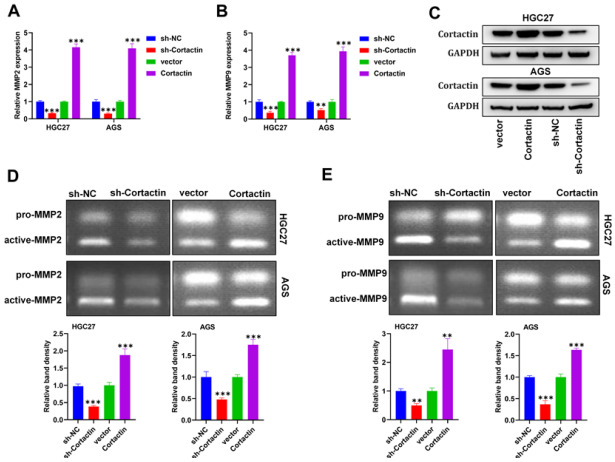

Figure 5. Cortactin elevates the expression and activity of MMPs. **(A-B)** RT-qPCR of cortactin levels in cells transfected with sh-NC, sh-cortactin, pcDNA3.1-vector, or pcDNA3.1-cortactin. **(C)** Western blot of cortactin levels in cells transfected with sh-NC, sh-cortactin, pcDNA3.1-vector, or pcDNA3.1-cortactin. **(D)** Gelatin zymography was applied for testing elevated the activity of MMP2 in different cells. **(E)** Gelatin zymography was applied for testing elevated the activity of MMP9 in different cells. **P<0.01, ***P<0.001.

Cortactin promotes tumor growth and metastasis in GC

For evaluating cortactin function in tumor growth and metastasis, we implemented the animal experiments utilizing nude mice. AGS cells stably transfecting sh-NC, sh-cortactin, vector, and cortactin were injected subcutaneously into mice. After mice were killed, tumors were removed and the tumor images were represented in figure 6A. The measurement results manifested that cortactin deficiency restrained tumor growth and weight, while cortactin overexpression promoted them (figure 6A-B). Then, we proved that cortactin, MMP2, and MMP9 expressions in tumor tissues were suppressed by cortactin depletion and raised by cortactin overexpression (figure 6C-E). Next, the intraperitoneal metastasis mouse model was established. We discovered that, in comparison of control mice, mice injected with sh-cortactin cells possessed the less quantity of metastatic nodules, while mice injected with cortactin overexpressed cells possessed a large quantity of metastatic nodules (figure 6F-G). HE staining further proved the impacts of cortactin on metastatic nodules (figure 6H-I).

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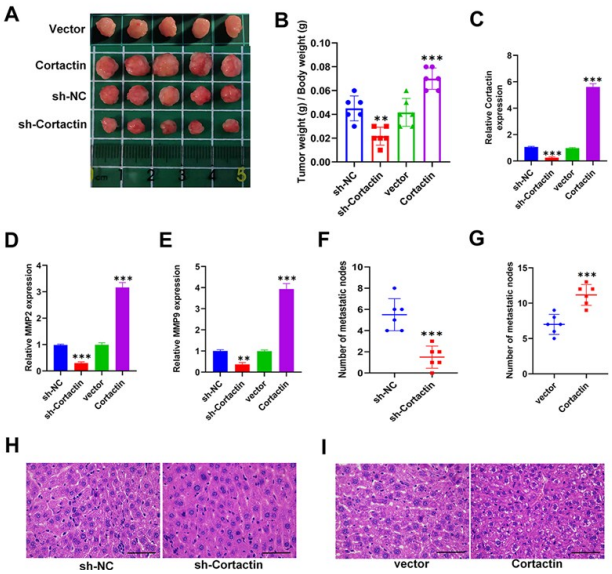

Figure 6. Cortactin promotes tumor growth and metastasis in GC. **(A)** The tumor images and weight in the sh-NC group, the sh-cortactin group, the vector group, and the cortactin group. **(B)** The tumor weight / body weight in the sh-NC group, the sh -cortactin group, the vector group, and the cortactin group. **(C-E)** RT-qPCR of cortactin, MMP2, and MMP9 expression in tumor tissues. **(F-G)** The quantification of quantity of metastatic nodes. **(H-I)** HE staining was utilized to test the metastatic nodes. Scale bars = 50 μm, **P<0.01, ***P<0.001.

DISCUSSION

Metastasis is the major cause of poor prognosis of most malignant tumor patients [32]. Cortactin was initially found as a substrate of Src kinase [33]. Afterwards, studies confirm that cortactin is an F-actin binding protein which can stimulate cell migration and metastasis in assorted cells [34]. The carcinogenic properties of cortactin has been proved in assorted solid tumors including GC, colorectal cancer, breast cancer, and liver cancer [25, 35-37]. Similarly, we found that cortactin was abundantly produced in GC tissues, and that the degree of expression increased with infiltration depth. Functional assays manifested that cortactin overexpression promoted GC cell migration and invasion, while cortactin deficiency inhibited migration and invasion. In addition, animal experiments proved that cortactin overexpression promoted tumor growth and metastasis. Cortactin downregulation weakened the tumor growth rate and the quantity of metastatic nodules in mice. Thus, we confirmed the carcinogenic characteristics of cortactin in GC. Chuma *et al.* have supported that cortactin overexpression facilitates migration and metastasis of hepatocellular carcinoma [38]. Nakane et al. suggest that cortactin depletion can attenuate cell migration and invasion in prostate cancer [39]. Wei *et al.* confirm that cortactin exerts the promoting function on cell proliferation, invasion, and tumor metastasis in GC [29]. These studies once again confirm our findings.

ECM remodeling is a prerequisite for malignant tumor metastasis [40]. Cancer cells possessing the capability of degrading ECM can invade ambient tissues, leading to the spread and metastasis of primary tumors [41]. The invasive cells complete the metastasis procedure by invadopodia, which are the actin-rich subcellular protrusions and can degrade ECM and promote cell migration [42]). Invadopodia have gradually become a marker of cancer cell invasion and metastasis [43]. Invadopodium formation has been found in various malignant tumors, such as GC [18], hepatocellular carcinoma [44], breast cancer [45], melanoma [46], and so on. Cortactin participates in four different stages of the invadopodium lifecycle and is a vital regulatory factor for invadopodium formation [24]. Cortactin can combine with invadopodia proteins such as Arp2/3 and N-WASP to facilitate actin assembly and exert the scaffolding function in invadopodia [47], suggesting that cortactin also acts as a scaffolding function in invadopodia. Xue *et al.* have suggested that Cav2.2 enhances cortactin stability to accelerate ECM degradation, invadopodium formation, and metastasis in breast cancer [48]. Li *et al.* have revealed that cortactin promotes invadopodium formation and metastasis of lung cancer [49]. In addition, cell-ECM adhesion is the vital process in tumor metastasis [31]. Our study found that cortactin deficiency resulted in the notable decrease on cell capability of degrading focal ECM, and reduced the adhesion capability of GC cells to ECM components (fibronectin, laminin and vitronectin). These findings indicated that cortactin promotes invadopodium formation, ECM degradation, and ECM adhesion.

MMPs are a class of zinc-dependent endopeptidases that can destroy ECM constituents [30]. MMPs participate in all steps of tumor development and affect various biological functions, including cell proliferation, migration, tumor growth, immune response, etc. [50]. The matrix degradation activity of invadopodia is achieved through the secretion of matrix degrading enzymes including MMPs [50]. Accumulating studies have shown that MMP2, MMP9, and MMP14 are enriched in invadopodia, and promote the development and metastasis of assorted cancers [51]. MMP2 and MMP9 belong to the gelatinase family in MMPs and have the capability to hydrolyze basement membrane [52]. The increase on their activity can result in ECM degradation and metastasis of cancer cells to distant organs [53]. It is reported that cortactin is a crucial modulator of MMP secretion and ECM degradation in invadopodia, and MMP2 and MMP9 secretion is closely associated with cortactin expression [54]. In this study, we proved that cortactin can increase mRNA and protein levels, as well as MMP2 and MMP9 activity in GC cells. It proved that cortactin

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facilitated invadopodium formation by promoting the expression and activity of MMPs.

CONCLUSION

Taken together, our study proved that cortactin enhanced invadopodium formation and ECM degradation to accelerate metastasis of GC through regulating MMPs. The molecular mechanism of invadopodium mediating the invasion of gastric cancer cells still needs further research in the future, and our study provides new insights into targeted therapy strategies.

***Funding*:** This work was supported by the Foundation of Science and Technology of Chengdu City (No. 2021-YF05-00843-SN).

***Conflicts of interests*:** No potential conflict of interest was reported by the authors.

***Ethical consideration*:** All patients signed a documented, voluntarily informed consent form. All methods were carried out in compliance with the Helsinki Declaration criteria, and this study was authorized by our institution's Ethics Committee (CL2020-L-109).

***Author contribution*:** J.Y. conceived and designed the experiments. M.P. and Q.T. contributed significantly to the experiments and arranging data. P.G., P.N. and Y.C. performed data analyses. Y.W. wrote the draft manuscript. Y.W. and M.P. revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENT

None.

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