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miR-644a Inhibits Cellular Proliferation and Invasion via Suppression of CtBP1 in Gastric Cancer Cells

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Epithelial–mesenchymal transition (EMT) is one of the most important mechanisms in the metastasis of various cancers, including gastric cancer (GC). In this study, we explored the putative significance of miR-644a and its role in EMT-mediated metastasis of GC. We first detected the expression of miR-644a in a cohort of 107 GC tissues using quantitative RT-PCR. The expression of miR-644a was suppressed in GC tissues and was associated with a later clinical stage and tumor metastasis. Restoring the expression of miR-644a could significantly suppress the migration and invasion of HGC-27 and SGC-7901 cells, which might be correlated to its suppressive effect on the EMT process. We also found that carboxyl-terminal-binding protein 1 (CtBP1) was a putative target gene of miR-644a in GC and might be involved in the suppressive effect. Collectively, through targeting CtBP1-mediated suppression of the EMT process, miR-644a might suppress the tumor metastasis of GC cells.

Key words: Gastric cancer (GC); Tumor metastasis; Carboxyl-terminal-binding protein 1 (CtBP1); Epithelial–mesenchymal transition (EMT) progression; miR-644a

INTRODUCTION

Gastric cancer (GC) is one of the most common cancers worldwide. Distant metastasis is the predominant reason for recurrence of GC and the most common cause of death. Although neoadjuvant treatment for GC has improved in the last decade, the survival for patients with distant metastases is still poor at less than 30%[1]. Thus, investigating the genes responsible for this process is important for clarifying the pathogenesis of GC.

Epithelial–mesenchymal transition (EMT), which makes tumor cells evade from the primary tumor site and metastasize to distant organs, plays an important role in tumor migration and metastasis[2,3]. Accumulating evidence has established that aberrant EMT activation plays a crucial role in the invasion and metastasis of various cancers, especially GC[4]. In GC, the majority of primary tumors, and even premalignant lesions, show suppressed expression of E-cadherin and increased expression of ZEB-1 and SNAI1, which represent the mesenchymal phenotype[5]. Moreover, the expression of the mesenchymal marker, N-cadherin, was positively associated with the invasive phenotype of GC, which further identified the important effect of EMT in the initiation and progression of GC[6].

Previous studies found that miRNAs are involved in the regulation of EMT in GC through multiple pathways[7,8]. For instance, miR-145-5p inhibits the invasiveness of GC through targeting N-cadherin and ZEB2 to suppress the EMT process[9], and miR-30a increases the sensitivity of GC cells to cisplatin by suppressing EMT[8]. Among these genes, miR-644a is a newly identified miRNA, which has been reported to have a close relationship to tumor metastasis[10]. In this study, we investigated the expression of miR-644a in GC and its regulation of EMT in GC cells.

MATERIALS AND METHODS

Clinical Samples

Cancerous and adjacent noncancerous tissues were obtained from 107 patients with advanced GC who had not received any adjuvant therapy. The matched normal tissues were obtained 5 cm from the tumor margin.

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Absence of disease was further confirmed by pathologists. The tumor samples were obtained with prior approval of informed consent. The study was approved by The Second Affiliated Hospital of Medical School of Xi'an Jiaotong University Institutional Ethics Committee.

Cell Culture and Cell Transfection

Human GC cell lines HGC-27 and SGC-7901 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Darmstadt, Germany), as previously described[11]. miR-644a, scramble mimic, siRNAs (specific to CtBP1), and control siRNAs were purchased from Dharmacon (Austin, TX, USA) and were then transfected into cells to a final concentration of 50 nM by DharmaFECT 1 (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions.

RNA Extraction and Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed using the First-Strand cDNA Synthesis kit (Invitrogen). Specific primers for reverse transcription of miR-644a were 5′-GTCGTATCCAGTGCAGGGTC CGAGGTATTCGCACTGG GCTCTAA-3′, for CtBP1 were 5′-TTTTTTTTTTTTTTTTTT-3′, and for U6 as described previously[11]. Quantitative real-time PCR was then performed as described previously[11], and the expression levels of U6 and GAPDH were used as an internal control for miRNA and mRNA, respectively. The primers for qRT-PCR were used as follows: miR-644a, 5′-GCGGCGAGTGTGGCTTTC-3′ (sense) and 5′-CAGT GCAGGGTCCGAGGT-3′ (antisense); CtBP1, 5′-AAAC TCGAGTACTTCAATTCCTCGGTATTG-3′ (sense) and 5′-AAATCTAGACACACTGTTCTACAGTCCAAGGC-3′ (antisense). The primers for U6 and GAPDH were used as described previously. PCR efficiencies were calculated with a relative standard curve derived from a complementary DNA mixture and given regression coefficients >0.95. The relative expression levels were evaluated using the $2^{-\Delta\Delta}$Ct method. All experiments were repeated three times to reduce curve-derived variance.

Biological Function Assays

The cell counting kit-8 (CCK-8) and Transwell assays were performed to explore the effects of miR-644a on the proliferation, migration, and invasion of GC cells. Before performing the assays, MGC-27 and SGC-7901 cells were seeded into 24-well plates at different concentrations. For analysis of proliferation, 5×10^3 cells were seeded in each well, incubated with CCK-8 (Dojindo, Kumamoto, Japan), and diluted in normal culture medium at 37°C until visual color conversion occurred. The absorbance in each well was measured with a microplate reader set at 450 and 630 nM at 0, 24, 48, and 72 h after transfection. The Transwell migration/invasion assays were performed according to the methods described in our previous article[11].

Luciferase Reporter Assays

The entire 3′-UTR of the CtBP1 gene was amplified from genomic DNA and cloned into the pGL3 vector (Promega, Madison, WI, USA) immediately downstream of the *Renilla* luciferase gene. Mutations in the 3′-UTR of the CtBP1 gene with the miR-644a target site deleted (Mut) were generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, San Diego, CA, USA). Twenty-four hours before transfection, 1×10^5 GC cells were seeded into each well of 24-well plates. Cells were cotransfected with pRL-TK *Renilla* luciferase reporter (10 ng), pRL-TK *Renilla* luciferase reporter (50 ng), and miR-644a or scramble mimic (50 nM) using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared using Passive Lysis Buffer (Promega) 48 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega). Results were normalized to the *Renilla* luciferase.

Immunoblot Assays

For the immunoblot assays, cells were harvested in ice-cold PBS 48 h after transfection and lysed on ice in a modified radioimmunoprecipitation buffer supplemented with protease inhibitors. Protein concentration was determined using the BCA Protein Assay Kit, and equal amounts of protein were analyzed by SDS-PAGE. Gels were electroblotted onto nitrocellulose membranes (Millipore, Milwaukee, WI, USA). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 2 h, membranes were incubated at 4°C overnight with primary antibodies [E-cadherin (1:1,000), N-cadherin (1:1,000), vimetin (1:1,000), and CtBP1 (1:1,000)]. GAPDH was used as a negative control (Cell Signaling Technology, Danvers, MA, USA). Membranes were then incubated with respective second antibodies and detected by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (ECL) (Millipore).

Statistical Analysis

Data were presented as the mean ± standard deviation of repeated experiments. All experiments were repeated at least three times independently. Statistical analysis was carried out using SPSS 17.0 software (Chicago,

IL, USA). Student's *t*-test was used to analyze the data. Values of $p < 0.05$ were considered significant.

RESULTS

The Expression of miR-644a Was Suppressed in GC Tissues and Correlated With Tumor Metastasis

To explore the putative significance of miR-644a in GC, its expression was detected in 107 paired GC tissues. Among these tumor tissues, 75 out of 107 GC tissues showed suppressed expression of miR-644a (70%) (Fig. 1A). We further compared the mean level of miR-644a between cancerous and noncancerous tissues. As expected, the mean level of miR-644a in cancerous tissues was significantly lower than its level in noncancerous tissues (Fig. 1B). Moreover, we explored the correlation between the expression of miR-644a and tumor local invasion. Strikingly, a statistically significant association was observed between the expression of miR-644a and the clinical stage of GC as well as metastasis. The patients with a lower level of miR-644a were

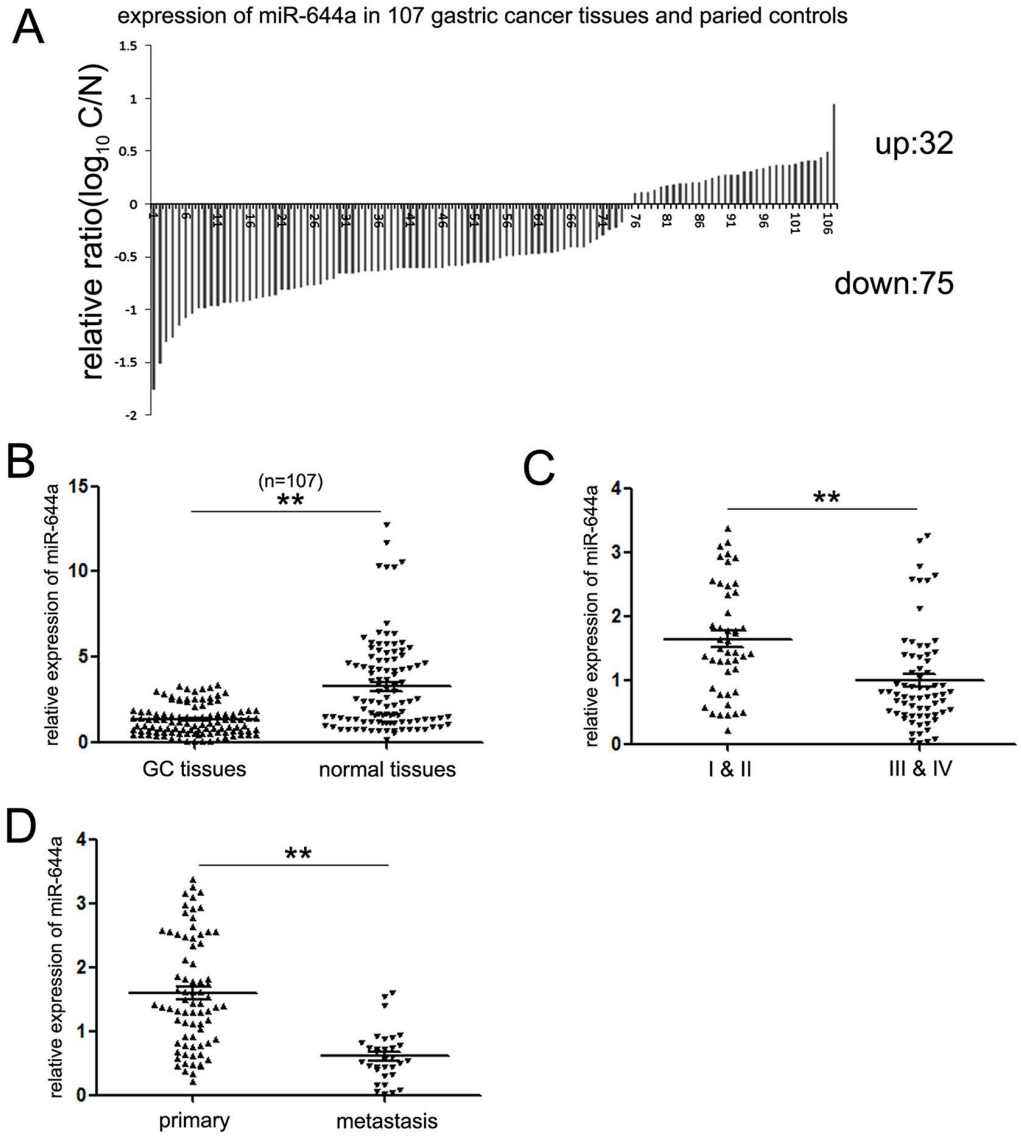

Figure 1. Expression level of miR-644a in gastric cancer tissues. (A) The expression level of miR644a was detected in 107 pairs of GC tissues using TaqMan quantitative RT-PCR. Data are shown as $\log_{10}$ of relative ratio change of esophageal cancer tissues relative to normal tissues. (B) The mean level of miR-644a expression in GC cancerous and noncancerous tissues. The expression of miR-644a was suppressed in GC tissues. (C, D) The expression of miR-644a was suppressed in cancer tissues in patients at a later clinical stage (stage III + IV vs. stage I + II) and metastasis. The expression of miR-644a was normalized to small nuclear RNA U6. $^{**}p < 0.01$ compared with scramble group.

positively associated with late-stage tumors (stages III and IV; $p<0.01$) (Fig. 1C). Moreover, when the patients were stratified on the basis of metastasis, we found that miR-644a was significantly suppressed in GC tissues with tumor metastasis ($p<0.01$) (Fig. 1D), which suggests that miR-644a is involved in the progression and metastasis of GC.

miR-644a Suppresses the Proliferation and Invasion of GC Cells In Vitro

The suppressed expression of miR-644a in cancerous tissues proposes that miR-644a performs as a tumor suppressor in GC. Thus, the miR-644a-overexpressed models in GC cell lines, HGC-27 and SGC-7901, were established through transient transfection with the miR-644a mimic. Upon transfection, the expression of miR-644a was increased about 100- and 200-fold in HGC-27 and SGC-7901 cells compared to the scramble groups (Fig. 2A).

Given that miR-644a displays a close association with tumor metastasis, we proposed that miR-644a might play a pivotal role in GC cell migration and invasion, which promotes tumor metastasis. Transwell assays were performed in order to identify the effect of miR-644a on the migratory and invasive abilities of GC cells. Transwell assays without Matrigel showed that overexpression of miR-644a inhibited GC cells passing through the chambers compared with the scramble groups (Fig. 2B). When the chambers were coated with Matrigel, which mimics the extracellular matrix (ECM) of tumor cells, similar results were found in that overexpression of miR-644a suppressed the invasive capacity of GC cells (Fig. 2C). To identify whether cell growth affected the invasive capacity, we further investigated the effect of miR-644a on the cell proliferation rate using a CCK-8 assay. Transfection with miR-644a significantly inhibited the cell growth rate of GC cells after 24 h upon transfection (Fig. 2D). However, there was no influence on the growth rate of GC cells within 24 h upon transfection, which might not affect the invasive capacity in Transwell assays. These data indicated that miR-205 could efficiently repress cell motility and invasiveness of GC cells in vitro.

miR-644a Suppresses the EMT Progression of GC Cells In Vitro

Since EMT progression is closely related to tumor metastasis, we hypothesized that it might participate in miR-644a-mediated suppression of the metastasis of GC cells. Thus, a series of variant, well-accepted epithelial and mesenchymal markers were analyzed in GC cells upon transfection with miR-644a. As expected, the mesenchymal markers N-cadherin as well as vimentin were consistently suppressed in GC cells upon treatment with miR-644a (Fig. 2E). On the other hand, the epithelial markers E-cadherin and β-catenin were upregulated when treated with miR-644a. The similarity was also found in immunofluorescence assays (Fig. 2F). These results proposed that overexpression of miR-644a suppressed the EMT progression of GC cells.

CtBP1 Is a Putative Target Gene of miR-644a in GC Cells

To identify the putative mechanisms involved in the suppressive effect of GC cells triggered by miR-644a, prediction programs were performed to search its putative targets. Among the genes being predicted, carboxyl-terminal-binding protein 1 (CtBP1) was selected as a perfect candidate because of its important effect on the regulation of EMT[12]. Although the miR-644a/CtBP1 axis has been reported to perform an important role in breast cancer[10], the interaction between miR-644a and CtBP1 has not been experimentally validated in GC. The putative miR-644a binding site within the 3′-UTR of the CtBP1 gene has been previously studied. Accordingly, the miR-644a-binding site mutant of the CtBP1 3′-UTR with a deleted putative binding site was conducted (Fig. 3A). Dual-luciferase detection assays were conducted to evaluate the effects of mutant and wild constructs. As expected, a significant repression of luciferase activities was observed in GC cells cotransfected with the wild 3′-UTR construct and the miR-644a mimic compared to the mutant construct groups (Fig. 3C), suggesting that miR-644a suppressed the transcription activity of the CtBP1 gene. Moreover, overexpression of miR-644a significantly suppressed the mRNA as protein levels of CtBP1 in GC cells (Fig. 3B and D). Together, these results indicate that miR-644a directly targets the expression of CtBP1 in GC cells.

Suppression of CtBP1 Inhibits Proliferation, Invasion, and EMT Progression in GC Cells

CtBP1 is a transcriptional corepressor that represses the expression of various tumor suppressor genes. Among these, the epithelial marker E-cadherin is an important downstream gene. By suppressing the expression of E-cadherin, CtBP1 might induce the process of EMT and thus promote tumor metastasis. However, its putative effect in GC cells is less well known. Therefore, we knocked down the expression of CtBP1 using RNA interference. Upon transfection with si-CtBP1, the mRNA and protein levels of CtBP1 were substantially suppressed in both cell lines (Fig. 4A and B). Consistent with the repressed expression of CtBP1, the expression of the aforementioned mesenchymal markers, N-cadherin and vimentin, was suppressed, while the downstream gene E-cadherin as well as the epithelial marker β-catenin were both upregulated (Fig. 4B), which promoted the suppression of CtBP1 and suppressed the EMT progression of

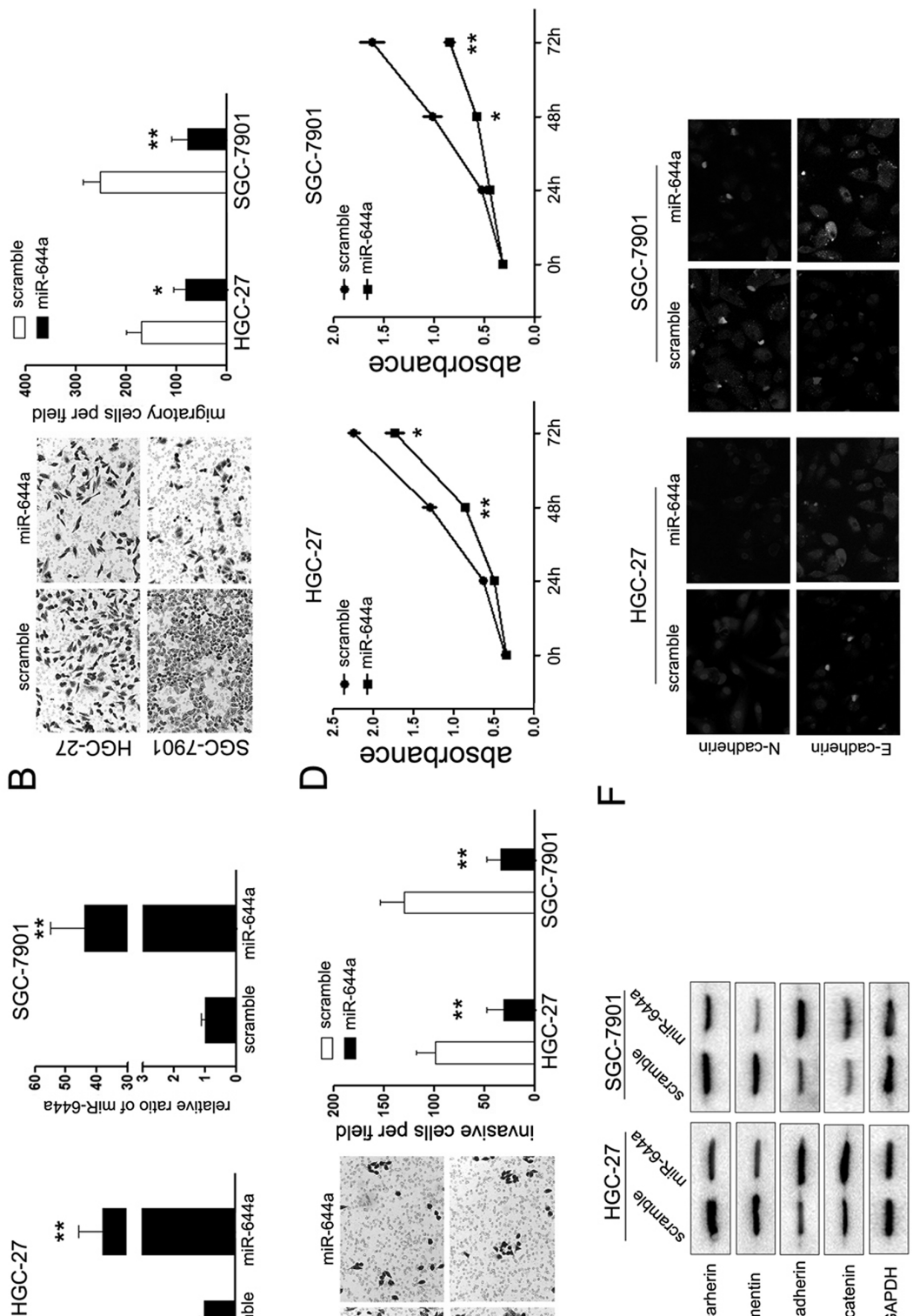

Figure 2. The effects of miR-644a on GC cell proliferation and invasion. (A) The level of miR-644a expression was detected in HGC-27 and SGC-7901 cells upon transfection with miR-644a or scramble mimic by quantitative RT-PCR. (B, C) The effects of miR-644a on cell migration and invasion of HGC-27 and SGC-7901 cells were performed by Transwell assay upon transfection with miR-644a. (Left) Cells passing through the chambers. (Right) The relative number of cells passing through per field. (D) A cell proliferation assay of GC cells was performed after transfection with miR-644a or scramble mimic by using CCK-8. (E) Western blot analysis of the expression of epithelial and mesenchymal marker proteins in GC cells transfected with miR-644a or scramble mimic was performed. Upon transfection, the epithelial marker E-cadherin was increased, and the mesenchymal markers vimentin and N-cadherin were suppressed. (F) Immunofluorescence assays further explored the expression of markers mentioned above. $*p<0.05$, $**p<0.01$ compared with the scramble group.

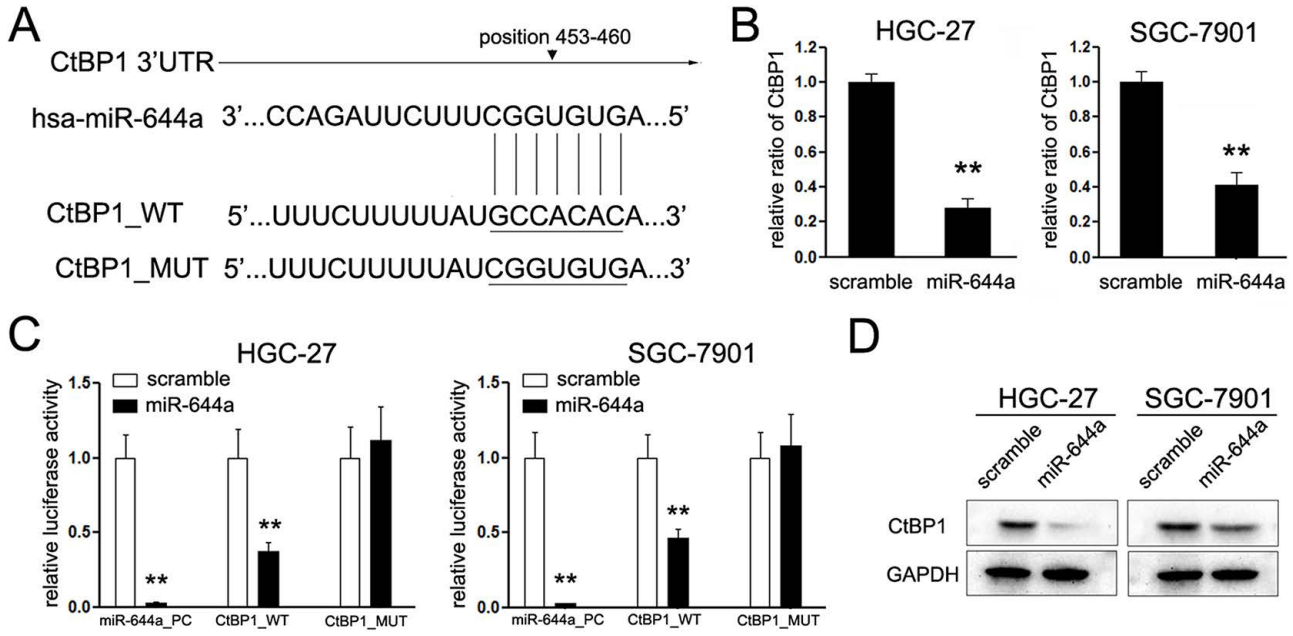

Figure 3. CtBP1 is a putative target gene of miR-644a in GC cells. (A) Schematic representation of CtBP1 3′-UTR showing the putative miR-644a target site. (B) A quantitative RT-PCR assay was performed to detect the expression of CtBP1 upon transfection with miR-644a or scramble mimic. (C) Relative luciferase activity of the indicated CtBP1 reporter construct in GC cells, cotransfected with miR-644a or scramble mimic, is shown. (D) Western blot analysis of the expression of CtBP1 protein in HGC-27 and SGC-7901 cells transfected with miR-644a or scramble mimic was performed. $^{**}p<0.01$ compared with the scramble group.

GC cells. In agreement with the suppressed effect on EMT progression, inhibited cell proliferation and invasion were also observed in GC cells transfected with si-CtBP1 at the same time (Fig. 4C and D). These results demonstrate that knockdown of CtBP1 mimics the biological function of miR-644a in GC cells. Combined with our substantial evidence that miR-644a inversely regulates CtBP1 expression, CtBP1 has been found to be a functional target gene of miR-644a and might be involved in miR-644a-mediated repression of cell invasion and EMT progression in GC cells.

DISCUSSION

Recently, previously unknown markers, microRNAs (miRNAs), have been considered to be important components of the cancer signaling network and are emerging as novel biomarkers in many diseases[13]. A series of studies have reported the pivotal effects of miRNAs in the initiation, progression, and metastases of GC[14]. In this study, we explored the expression of miR-644a in GC and found that it was suppressed in cancerous tissues of GC. Moreover, the suppression of miR-644a was related to tumor invasion and metastasis, as the lower level of miR-644a was found in the tumors in a late clinical stage and with distant metastasis. These prompted us to determine that miR-644a functions as a tumor suppressor in GC and participates in the progression of GC. Further biological function assays identified our hypothesis, as overexpression of miR-644a could significantly inhibit cell proliferation, invasion, and EMT progression of GC cells.

Tumor metastasis is a complex, multistep process that results in the dissemination of cells from the primary tumor site to distant organs where these cells are able to colonize and form a secondary lesion[15]. For carcinomas, the EMT process plays an important role in tumor migration and metastasis[16]. The EMT process is a complex program in which epithelial cells acquire a mesenchymal phenotype and move through a cascade of biological events. Herein we found that mesenchymal markers were consistently suppressed upon transfection with miR-644a, while the expression of epithelial markers was increased. Furthermore, the suppression of migration capability could further suggest that the motility of GC cells was suppressed. These data suggest that EMT might participate in the tumor metastasis of GC.

This information caused us to further identify the putative mechanisms involved in miR-644a-mediated suppression. CtBP1, which is a member of the CtBP family, plays an important role in the regulation of several essential cellular processes. In cooperation with the activated RAS oncogene, gene mutants in the CtBP-binding motif enhanced the transformation of primary rodent epithelial cells and thus tumorigenesis[17]. Moreover, Grooteclaes and Frisch showed that E1A expression in several cancer

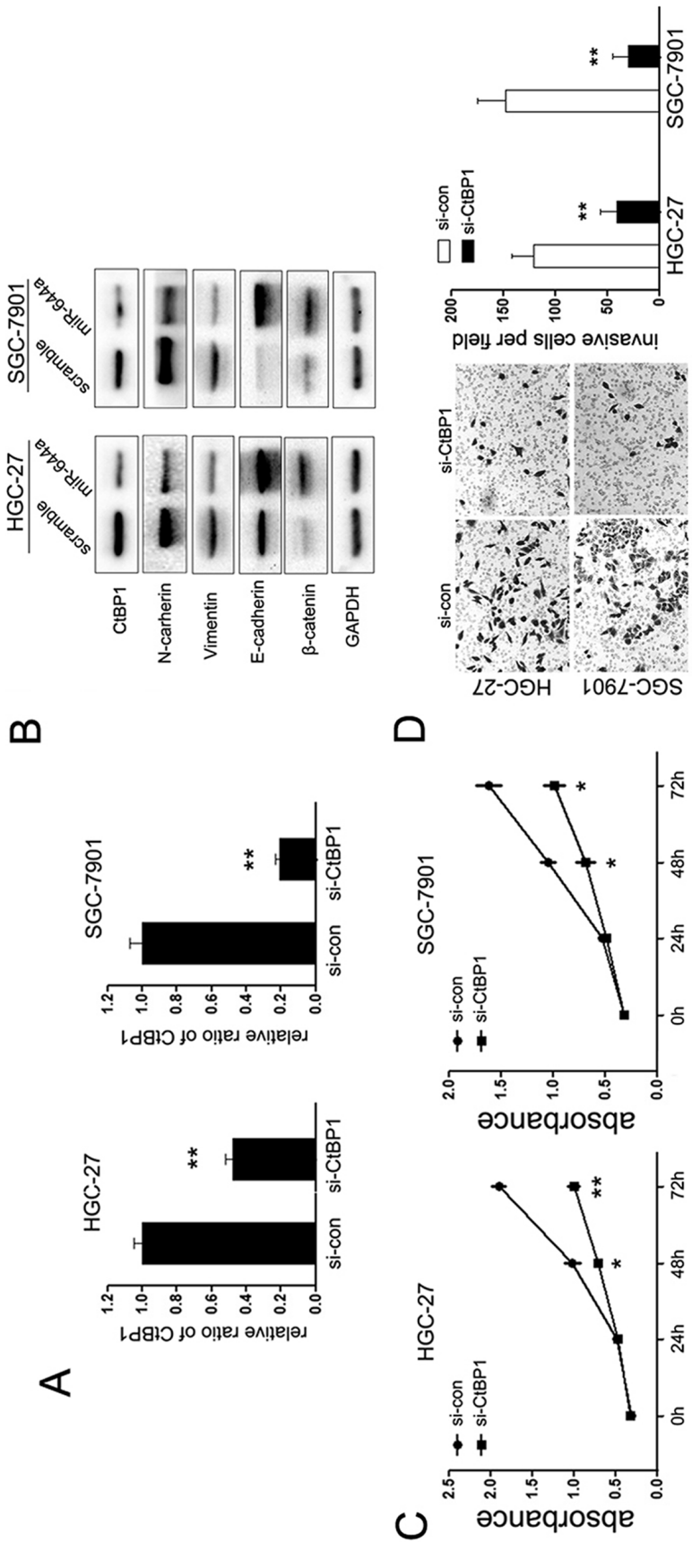

Figure 4. Knockdown of CtBP1 suppressed the proliferation and invasion of GC cells. (A) The level of CtBP1 mRNA expression was detected in HGC-27 and SGC-7901 cells upon transfection with si-CtBP1 and relative control. (B) The expression of CtBP1 protein level as well as relative markers was detected. The expression of CtBP1 and its downstream genes N-cadherin, as well as vimentin, was suppressed, while the expression of E-cadherin and β-catenin was upregulated. (C) The proliferation was suppressed upon transfection with si-CtBP1. (D) The invasion was suppressed upon transfection with si-CtBP1. $^{**}p<0.01$ compared with the scramble group.

cell lines reverses the oncogenic properties as a result of "epithelialization" by activating expression of several epithelial genes and identified CtBP as an antagonist of the epithelial phenotype[18]. We identified CtBP1 as a target gene of miR-644a. Overexpression of miR-644a suppressed the expression of CtBP1 as well as its downstream gene, E-cadherin, in GC cells. The putative effects of CtBP1 in GC cells are less well known. Thus, we further explored the biological function of CtBP1 in GC cells. As expected, knockdown of CtBP1 significantly suppressed cell proliferation, invasion, and EMT progression of GC cells, which paralleled the tumor-suppressive effects induced by miR-644a restoration. The tumorigenic role of miR-644a, combined with our substantial evidence that miR-644a targets CtBP1 directly, suggests that the antitumor function of miR-644a is achieved, at least partly, through the suppression of CtBP-mediated inhibition on cell proliferation and EMT.

Taken together, these findings offer convincing evidence that aberrant expression of miR-644a may participate in the initiation and progression of GC. Our data reveal that miR-644a may function as a tumor suppressor and modulate GC cell proliferation, invasion, and the EMT process by directly and negatively regulating CtBP1, implying that reexpression of miR-644a might be a potential therapeutic strategy for GC.

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