circRNA-CER mediates malignant progression of breast cancer through targeting the miR-136/MMP13 axis

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Abstract. Chondrocyte extracellular matrix-related circular RNAs (circRNA-CER) have been demonstrated to be involved in various diseases. However, its role in the development of human breast cancer is not clearly understood. The aims of the present study were to assess circRNA-CER expression in paired cancer tissue and adjacent non-tumor tissue from 24 patients with breast cancer, and to explore the roles and mechanisms by which circRNA-CER mediates the malignant progression of breast cancer cells. The results revealed that circRNA-CER and matrix metalloproteinase 13 (MMP13) were upregulated, whereas miR-136 was downregulated in breast cancer tissues compared with adjacent non-tumor tissues. In vitro silencing of circRNA-CER using small interfering RNA (siRNA) had inhibitory effects on MCF-7 breast cancer cell proliferation and migration, and similar results were obtained following overexpression of microRNA (miR)-136 in MCF-7 cells by transfection with miR-136 mimics. The subsequent mechanistic study revealed that the expression levels of MMP13 were significantly lower in MCF-7 cells following transfection with miR-136 mimics, and silencing of circRNA-CER enhanced miR-136 and decreased MMP13 expression levels. Furthermore, silencing of miR-136 by transfection with miR-136 inhibitors resulted in an increase in MCF-7 cell proliferation and migration. miR-136 inhibitor-derived biological effects were reversed by co-transfection of cells with miR-136 inhibitors and circRNA-CER siRNA. Taken together, the present results suggested that circRNA-CER may serve an important role in the progression of breast cancer by regulating the activity of the miR-136/MMP13 axis, and may be a potential biomarker for the prediction and treatment of breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer type and the fifth most common cause of mortality among women worldwide (1). It was estimated that there were 268,600 newly diagnosed cases and 69,500 patients that succumbed to this disease in China in 2015, which accounts for 15.1% of all cancer incidences and 6.9% of cancer-related mortalities in women (2). Although the mortality rates due to breast cancer have declined, the incidence rates of this disease have increased in China over the past decades (3). Breast cancer is a heterogeneous disease that can be classified into several forms based on histological, clinical and genetic features. Numerous attempts of categorizing this heterogeneous disease have led to a molecular classification into five subgroups by gene-expression profiling (4,5).

Circular RNAs (circRNAs) are large non-coding RNAs that are present ubiquitously in the cytoplasm of eukaryotic cells and function as competing endogenous RNAs (6). Numerous circRNAs serve important ‘sponge’ roles through their effect on microRNA (miR/miRNA) activity. For example, circRNA ciRS-7 is a newly identified human circRNA that is involved in resistance to miRNA-mediated target destabilization, by strongly binding and suppressing the activity of miR-7 (7). A previous report also revealed the crucial role of circRNAs in regulating gene transcription (8). Accumulating evidences have demonstrated that circRNAs have remarkable regulatory roles in the generation and development of various diseases, including atherosclerosis, nervous system disorders and cancers, including leukemia and solid tumors (9-11). A recent study demonstrated that chondrocyte extracellular matrix-related circRNA (circRNA-CER) expression increases with increased levels of interleukin-1 and tumor necrosis factor in chondrocytes, and silencing of circRNA-CER using small interfering RNA (siRNA) suppresses matrix metalloproteinase 13 (MMP13) expression and increases extracellular matrix formation (12). Yao et al suggested that the expression of circRNA-CER is significantly associated with lymph node metastasis, overall survival and tumor stage in non-small cell lung cancer (13). However, the function of circRNA-CER in breast cancer remains largely unknown and requires further investigation.

The present study aimed to evaluate the expression profiles of circRNA-CER in breast cancer and investigate their potential role in the disease. The data demonstrated that the expression levels of circRNA-CER in breast cancer tissue were
significantly higher compared with in adjacent non-tumor tissue, and that circRNA-CER may function as a competing endogenous RNA to regulate the expression of MMP13 by competing with miR-136 in breast cancer cells. To the best of our knowledge the present study demonstrated for the first time a positive circRNA-CER/MMP13 association, and crosstalk among miR-136, circRNA-CER and MMP13, which provides novel insight into the treatment of breast cancer.

Materials and methods

Patients and tissue samples. A total of 24 surgically resected tissue specimens from female patients with breast cancer were collected from the First Affiliated Hospital of Jiamusi University (Jiamusi, China) from April 2016 to March 2018. Patients who had received local or systemic therapy prior to surgery were excluded from the study, and the ages of the patients ranged from 38-73 years old. Pathological examination of the tissue specimens was conducted independently by three pathologists, without prior knowledge of the patients’ medical records. The present study was approved by the Ethics Committee of Jiamusi University (approval no. JMSU-215) and all patients provided written informed consent prior to enrolment.

Immunohistochemistry. The tissue samples were fixed in 10% buffered formalin at room temperature for 24 h, embedded in paraffin, and cut into 5 μm-thick sections. Following deparaffinization in xylene at 37°C for 30 min, the sections were dehydrated in a graded series of ethanol, subjected to antigen retrieval with 0.01 M citrate buffer (cat. no. AR0024; Wuhan Boster Biological Technology, Ltd., Wuhan, China) at 100°C for 18 min in a high-pressure cooker and washed in PBS (cat. no. AR0030; Wuhan Boster Biological Technology, Ltd.) at room temperature for 5 min. Endogenous peroxidase activity was subsequently quenched by incubating the sections with 3% hydrogen peroxide in PBS for 15 min at room temperature, followed by blocking with 5% bovine serum albumin (BSA, cat. no. AR0004; Wuhan Boster Biological Technology, Ltd.) at 37°C for 60 min. Then, the slides were incubated with a rabbit polyclonal anti-MMP13 primary antibody (1:150; cat. no. ab39012; Abcam, Cambridge, MA, USA) overnight at 4°C. As a negative control, the primary antibody was replaced with the same amount of normal rabbit serum (cat. no. AR0010; Wuhan Boster Biological Technology, Ltd.) at room temperature for 5 min. Following several washes in PBS (3x3 min) to remove the excess of antibody, a biotin-conjugated goat anti-rabbit secondary antibody (cat. no. SA1022; Wuhan Boster Biological Technology, Ltd.) was applied at 37°C for 20 min, followed by washing of slides in PBS (3x3 min). Next, the slides were incubated in avidin-biotin peroxidase complex (cat. no. SA1022; Wuhan Boster Biological Technology, Ltd.) for 20 min at 37°C. Then, 3,3'-diaminobenzidine (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) was used for chromogen detection and slides were counterstained with hematoxylin at room temperature for 30 min for cell nuclear detection.

MMP13 protein expression was quantified based on the staining intensity and proportion of stained cells, according to Li et al (14) with slight modifications. Briefly, MMP13 staining intensity was scored from 0 to 3 as follows: 0, absent immunopositivity; 1, low immunopositivity; 2, moderate immunopositivity; 3, intense immunopositivity. The extent of total staining was scored from 0 to 4 as follows: 0, negative; 1, 1-25% of cells; 2, 26-50% of cells; 3, 51-75% of cells; and 4, 76-100% of cells. On average, three random fields were observed for each tissue at x400 magnification under an Olympus BX-60 fluorescence microscope (Olympus Corporation, Tokyo, Japan). The final staining score was the sum of the intensity and extent scores.

Cell lines, cell culture and transfection. The MCF-7 and ZR-75-30 breast cancer cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ in a humidified atmosphere. miR-136 mimics, miR-136 inhibitors and the corresponding negative controls were designed and constructed by Shanghai Gene Pharma Co., Ltd. (Shanghai, China). siRNA targeting circRNA-CER (si-circRNA-CER) and its negative controls were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences for miR-136 mimics, miR-136 inhibitors and the corresponding negative controls were designed and constructed by Shanghai Gene Pharma Co., Ltd. (Shanghai, China). The sequences for miR-136 mimics, miR-136 inhibitors and the corresponding negative controls were designed and constructed by Shanghai Gene Pharma Co., Ltd. (Shanghai, China). The sequences for miR-136 mimics, miR-136 inhibitors and the corresponding negative controls were designed and constructed by Shanghai Gene Pharma Co., Ltd. (Shanghai, China). The sequences for miR-136 mimics, miR-136 inhibitors and the corresponding negative controls were designed and constructed by Shanghai Gene Pharma Co., Ltd. (Shanghai, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Specific primers for circRNA-CER, β-actin, miRNA-136 and U6 were purchased from GeneCopoeia, Inc. (Rockville, MD, USA). The primers used in this investigation were: circRNA-CER forward: 5'-CTGGTGTCAG TGGAGACGAG-3', reverse: 5'-CGACCCTTCAATGCT CTCTTC-3'; β-actin forward: 5'-CTCCATCTGGCTTGGT-3', reverse: 5'-CTGGTGACCATTTACCGTTCC-3'; miRNA-136 forward: 5'-ACUCAUUUUGUUUGAUGA-3', reverse: 5'-UCACAAUCAAAACAAAUGAGU-3', U6 forward: 5'-GCTGTTCGAGCATGACTAAAT-3', reverse: 5'-GGTTTCAATGTTTGCTG-3'. For detection of circRNA-CER, total RNA from cells and tissues was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reverse transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocols. circRNA-CER PCR amplifications were performed using a Super Real Pre Mix Color (SYBR Green) Real Time PCR kit (cat. no. FP215; Tiangen Biotech Co., Ltd., Beijing, China) with the following conditions: Denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 10 sec. Relative expression of miR-136 was determined with the All-in-One™ miRNA qRT-PCR Detection kit based on SYBR Green dye method (cat. no. AOMD-Q020; GeneCopoeia, Inc.), according to the manufacturer's protocol. miR-136 PCR amplifications were performed using the following conditions:
Denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 12 sec. The expression levels of circRNA-CER and miR-136 were evaluated using the comparative quantification method (15), and normalized to β-actin and U6 expression levels, respectively.

Cell proliferation assay. Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8; cat. no. AR1160; Wuhan Boster Biological Technology, Ltd.), according to the manufacturer's protocols. Briefly, cells were plated in a 96-well plate (1x10^4 cells/well) and cultured in 100 µl RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Following incubation at 37˚C for 24, 48, 72 or 96 h, 10 µl CCK-8 reagent was added to each well and cells were further incubated for 1 h. Cell proliferation analysis was subsequently performed by measuring absorbance at wavelength 450 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Cell migration assay. Migration of MCF-7 cells was evaluated using Transwell inserts (pore size, 8 µm; Corning, Inc., Corning, NY, USA) in a 24-well plate. Briefly, 2x10^4 cells (for miR-136 mimics and si-circRNA-CER assays) or 5x10^3 cells (for miR-136 inhibitors assays) were resuspended in 100 µl serum-free RPMI-1640 medium and seeded in the top chamber of each Transwell insert in triplicate. A total of 600 µl RPMI-1640 medium containing 10% FBS was added to the lower chamber. After 48 h of incubation at 37˚C, cells in the top chamber were removed by scraping with a cotton swab, and the inserts were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.5% crystal violet at room temperature for 15 min. Cells were visualized under a light microscope at x100 magnification (Olympus Corporation) and migrating cells in five random microscopic fields were counted.

Western blot analysis. Western blotting was performed to detect MMP13 protein expression. Total protein was extracted from cells using RIPA lysate (cat. no. AR0105; Wuhan Boster Biological Technology, Ltd.). Following quantification of protein concentration using a BCA assay kit (cat. no. AR0146A; Wuhan Boster Biological Technology, Ltd.), a total of 50 µg total protein was subjected to 12% SDS-PAGE. Following transfer to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), the membranes were blocked with 5% non-fat milk at 37˚C for 2 h and probed with rabbit polyclonal anti-MMP13 (1:5,000; cat. no. ab39012; Abcam) and mouse monoclonal anti-GAPDH (1:1,000; cat. no. ab8245; Abcam) primary antibodies at 37˚C for 2 h. The membranes were washed twice for 5 min in tris-buffered saline containing 0.05% Tween-20, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:10,000; cat. nos. BA1054 and BA1050; Wuhan Boster Biological Technology, Ltd.) with gentle shaking at 37˚C for 25 min. The protein bands were visualized using the Beyo ECL Star kit (cat. no. P0018A; Beyotime Institute of Biotechnology, Jiangsu, China) and quantified by the Image Quant TL software (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. All experiments were repeated three times. The data are presented as the mean ± standard deviation.

Comparison of two means was performed using Student's t-test, whereas comparison of multiple means was conducted with analysis of variance followed by Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results
circRNA-CER expression is increased in breast cancer tissues. To determine the expression levels of circRNA-CER, MMP13 and miR-136 in breast cancer tissues, circRNA-CER and miR-136 were quantified by RT-qPCR, whereas MMP13 was detected by IHC. The results revealed that the expression
levels of circRNA-CER (Fig. 1A) and MMP13 (Fig. 1B) were significantly elevated in breast cancer tissues compared with in adjacent non-tumor tissues. Conversely, miR-136 expression was decreased in breast cancer tissues compared with in adjacent non-tumor tissues (Fig. 1C).

**circRNA-CER knockdown inhibits MCF-7 cell proliferation and migration.** To examine whether circRNA-CER mediates the malignant phenotype of breast cancer, its role in breast cancer cell proliferation and migration was investigated. RT-qPCR revealed that circRNA-CER expression was significantly higher in the MCF-7 cell line compared with in the ZR-75-30 cell line (Fig. 2A). Based on this observation, the MCF-7 cell line was selected for conducting silencing experiments, and the results demonstrated that silencing of circRNA-CER efficiently suppressed the expression of circRNA-CER (Fig. 2B). In addition, the results from the CCK-8 and Transwell assays indicated that knockdown of circRNA-CER significantly suppressed proliferation (Fig. 2C) and migration (Fig. 2D) of MCF-7 cells.

**Overexpression of miR-136 inhibits MCF-7 cell proliferation and migration.** The present study revealed that miR-136 expression levels were significantly lower in MCF-7 cells compared with in ZR-75-30 cells (Fig. 3A). Thus, miR-136 was overexpressed in MCF-7 cells to evaluate the potential effects of miR-136 on breast cancer (Fig. 3B). The results demonstrated that overexpression of miR-136 in MCF-7 cells inhibited both cell proliferation (Fig. 3C) and migration (Fig. 3D).

**circRNA-CER knockdown regulates the expression of miR-136 and its target gene MMP13.** It is accepted that MMP13 is a target of miR-136 in human cartilage degradation (12). In accordance with this notion, the present study demonstrated that transfection with miR-136 mimics suppressed MMP13 protein expression in MCF-7 breast cancer cells (Fig. 4A). In addition, the results demonstrated that silencing of circRNA-CER increased the expression levels of miR-136 (Fig. 4B) and inhibited the protein expression of MMP13 (Fig. 4C), suggesting that miR-136/MMP13 crosstalk is regulated by circRNA-CER.

**Knockdown of circRNA-CER suppresses MCF-7 cell proliferation and migration by targeting miR-136.** To further demonstrate the association between circRNA-CER and miR-136, the present study conducted transfection experiments using miR-136 inhibitors to downregulate miR-136 expression in MCF-7 cells. The results revealed that miR-136 expression levels were successfully decreased; however, the presence of circRNA-CER knockdown reversed this effect (Fig. 5A). In addition, downregulation of miR-136 expression promoted MCF-7 cell proliferation and migration, and these biological effects were abolished upon silencing of circRNA-CER (Fig. 5B and C).
Discussion

Non-coding RNAs (ncRNAs), which include miRNAs, long ncRNAs and circRNAs, are involved in the development of several diseases including pathological cardiac remodeling, nervous system diseases and several cancers (16-19). circRNAs are a novel type of ncRNA characterized by a stable structure and highly specific tissue expression. circRNAs are more stable than linear RNAs due to their covalently closed continuous loop without a free 3’ or 5’ end and their polyadenylated tail (11). Several studies have proposed that circRNAs mediate diverse biological processes, including miRNA ‘sponging’, splicing and transcription regulation, protein binding and RNA transport (13).

The present study revealed that the levels of circRNA-CER were higher in breast cancer tissues compared with adjacent non-tumor tissues. Consistent with our findings, a previous study identified that among 101 patients with non‑small cell lung cancer, the expression levels of circRNA‑CER were significantly elevated in cancer tissues, which was markedly associated with lymph node metastasis, overall survival and tumor stage (13). To understand whether circRNA-CER is involved in the malignant progression of breast cancer, the present study conducted CCK-8 and Transwell assays. The results demonstrated that silencing of circRNA-CER inhibited the proliferation and migration of MCF-7 cells. Furthermore, downregulation of circRNA-CER by transfection with siRNA reduced MMP13 expression and increased extracellular matrix formation (12). These findings suggested that there exists cross-talk between circRNA-CER and the miR-136/MMP13 axis. To further confirm this, the present study performed proliferation and migration assays to assess the relationship between circRNA-CER and miR-136. The results revealed that silencing of miR-136 using miR-136 inhibitors resulted in an increase in MCF-7 cell proliferation and migration. The biological effects caused by miR-136 inhibitors were reversed by co-transfection with circRNA-CER siRNA, suggesting that circRNA-CER serves as a ‘sponge’ and competitively prevents miR-136 activity.

miRNAs have been demonstrated to serve a crucial role in regulation of human tumor initiation, development and metastasis (20). Several miRNAs are recognized to serve as potential tumor suppressors and their low expression levels are associated with upregulation of oncogenic genes in several types of cancer (21). An miRNA microarray expression assay identified that miR-136 is upregulated in human and murine lung cancers (22), and miR-136 was able to target the tumor suppressor phosphatase and tensin homolog (23), suggesting a potential role of miR-136 in the development of cancer. However, it is now well accepted that miR-136 may also function as a tumor suppressor, since it promotes the apoptosis of glioma cells by targeting astrocyte elevated gene-1 and B-cell lymphoma 2 (24). In breast cancer, miR-136 is considered as an anti-invasive miRNA due to its suppressive role in the mesenchymal migration and metastasis of breast cancer cells. Furthermore, its expression is downregulated in breast cancer tissues and
is negatively correlated with World Health Organization grades (25). In accordance with the above findings on miR-136 expression in breast cancer, the present study observed that miR-136 was downregulated, whereas its downstream molecule MMP13 was upregulated, in breast cancer tissues. MMP13 is a member of the human matrix metalloproteinase family, which comprises of 26 zinc-dependent transmembrane and secreted neutral endopeptidases that contribute to the homeostasis of the extracellular matrix (26). MMP13 is considered a potential new tumor marker for breast cancer diagnosis (27), and its elevated expression is closely associated with decreased overall survival and lymph node metastasis in breast cancer (28). By contrast, a selective inhibitor targeting MMP13 delays tumor growth and reduces the severity of tumor-associated osteolytic lesions in experimental models of breast cancer, indicating a potential therapeutic role for MMP13-selective inhibitors in primary breast cancer and cancer-induced bone osteolysis (29). In conclusion, the present study demonstrated that circRNA-CER expression was upregulated in breast cancer tissues compared with adjacent non-tumor tissues, and provided insight into the potential mechanisms of circRNA-CER in regulating the activity of the miR-136/MMP13 axis. The results supported the value of circRNA-CER as a possible molecular target for the diagnosis and treatment of breast cancer.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

HS designed the study and contributed to revision of the manuscript. YQ and PD performed cellular and molecular biology experiments and wrote the manuscript. MH participated in sample collection and immunohistochemistry experiments. JX performed cellular experiments. WX participated in the molecular biology experiments and data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jiamusi University (approval no. JMSU-215) and all patients provided written informed consent prior to enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

References