Circular RNA-0001283 Suppresses Breast Cancer Proliferation and Invasion via MiR-187/HIPK3 Axis

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Background:  
Circular RNAs (circRNAs) are key regulators that take part in the carcinogenesis and development of breast cancer. The current study aimed to identify the expression of and explored the function of circRNA-0001283 in breast cancer.

Material/Methods:  
Breast cancer tissue samples were tested using high-throughput sequencing to identify the levels of relative genes; and proteins were addressed by using quantitative real-time polymerase chain reaction (qRT-PCR) and western-blot. Cell ability and cell apoptosis were investigated by Cell Counting Kit-8 (CCK-8) and flow cytometry. Invasion was detected by Transwell invasion assay. The identification of target genes was analyzed by dual-luciferase reporter assay.

Result:  
Downregulation of circRNA-0001283 expression was observed in breast cancer tissue samples. Ectopic expression of circRNA-0001283 remarkably suppressed cell viability and invasion, and induced apoptosis in breast cancer cells. Furthermore, circRNA-0001283 bound to miR-187 and decreased the expression of miR-187, which resulted in inhibition in cell growth and invasion. Finally, we showed that circRNA-0001283 positively regulated HIPK3 expression by sponging miR-187.

Conclusions:  
The results reveal a new functional circRNA-0001283 in breast cancer and may provide targets for developing novel therapeutic strategies for breast cancer.

MeSH Keywords:  
Cell Proliferation • DNA, Circular • Neoplasm Invasiveness

Abbreviation:  
circRNA – circular RNA; qRT-PCR – quantitative real-time reverse transcription PCR; ceRNA – competing endogenous RNA; NSCLC – non-small cell lung cancer; miRNA – microRNA; IncRNA – long non-coding RNA

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Background
Breast cancer is a common malignant tumor [1]. Although the cure rate has increased, the mortality of this disease remains high in women with breast cancer [2]. Therefore, gaining more insights into the molecular mechanisms that promote breast cancer progression, and developing novel therapies, are still urgently needed.

Breast carcinogenesis is a complex process involving a number of modulators and pathways. Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are important regulators implicated in the formation and development of breast cancer [3]. Circular RNAs (circRNAs) are non-coding RNAs discovered recently, which form a continuous cycle of covalent closures [4]. CircRNAs play critical functions in a number of biological processes [5]. Emerging evidence shows that circRNAs take part in various diseases, including cardiovascular disease, neurological disorders, diabetes mellitus, and osteoarthritis [6–9]. Recently, the critical role of circRNA in cancer has been also revealed [10]. Ablation expression of circRNAs was related to prevention, prognosis, and drug resistance of cancers [11–13]. But, the working mechanism of circRNAs is not fully understood in breast cancer.

In the present study, high-throughput sequencing was utilized to investigate the expression profile of circRNA in breast cancer. A significantly downregulated circRNA, circRNA-0001283 was discovered. Overexpression of circRNA-0001283 suppressed cell proliferation and invasion, and enhanced apoptosis in breast cancer cells. Moreover, our data demonstrated that circRNA-0001283 functions via miR-187/HIPK3 axis. Our findings might represent novel targets for the treatment of breast cancer.

Material and Methods

Clinical specimens
Breast cancer samples were obtained between 2016 and 2017 from the First Affiliated Hospital of Gannan, Medical University. Informed consents from all patients were obtained.

Cell culture
MCF-7, MDA-MB-231, MDA-MB-468 MDA-MB-453, and MCF-10A was purchased from the American Type Culture Collection (ATCC, USA). Cells were incubated in Dulbecco’s Modified Eagle’s Medium (Invitrogen, USA) or RPMI-1640 medium (Gibco, USA) with addition of 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin and streptomycin. All cells were cultured and incubated in a 5% CO₂ atmosphere at 37°C.

CircRNA analysis
Total RNA was isolated from 10 pairs of breast cancer tissues using TRIzol reagent (Invitrogen, USA). CircRNA was enriched and analyzed by high-throughput sequencing.

Quantitative real-time reverse transcription PCR (qRT-PCR)
Total RNA was purified from the tissue and cells by TRIzol reagent (Invitrogen, USA). RNA was retrieved into cDNA (complementary DNA) by PrimeScript RT reagent kit (Takara, Shiga, Japan) as the template of following amplification experiment by using the SYBR Premix Ex TaqII (TliRNaseHPlus) kit (Takara). Samples were normalized to the internal control. The values were calculated using 2–ΔΔCT method.

Luciferase reporter gene assay
The circRNA target genes were predicted by starBase, wild type and mutant circRNA-0001283 (with mutated miR-187 binding sites) were constructed using luciferase reporter plasmids. HEK293T cells were co-transfected with plasmids and miR-187 mimics using Lipofectamine 2000. Similarly, wild type and mutant HIPK3 3’UTR (with mutated miR-187 binding sites) were constructed using dual luciferase reporter plasmids. HEK293T cells were co-transfected with plasmids and miR-187 mimics using Lipofectamine 2000. After incubated for 48 hours, luciferase activity was measured and analyzed by a multifunctional fluorescent enzyme marker (Tecan, Switzerland).

Cell proliferation and apoptosis analysis
Cell proliferation was detected by a Cell Counting Kit-8 (CCK-8) according to the manufacturer’s instructions (Beyotime, China). A FITC Annexin V apoptosis detection kit (BD Biosciences, USA) was used to detect the apoptosis: 1×10⁶ cells were stained with Annexin V/propidium iodide (PI) at 4°C for 15 minutes. Cells were then washed by precooled phosphate-buffered saline (PBS) and suspended in buffer for the next analysis.

Cell invasion assay
Treated cells were cultured in a Transwell chamber with Matrigel-coated membrane (BD Biosciences, Bedford, MA, USA). After culture for 24 hours, cells were fixed with 4% paraformaldehyde. After that, cells were incubated with crystal violet. Five visual fields were selected for counting under the microscope.

Statistical analysis
Data were expressed as mean±standard deviation (SD). Significance was analyzed using SPSS 18.0. Student’s t-test and one-way ANOVA were used for analysis. Significance was
considered as the $P$ value <0.05. All experiments were conducted in 3 times.

**Results**

**CircRNA-0001283 is downregulated in breast cancer tissues**

To screen dysregulated circRNAs, microarray analysis was performed with 10 pairs of cancer and adjacent non-cancer tissues. A heat map showed the 23 most downregulated circRNAs in 3 paired samples of tumor tissues (Tumor) and corresponding normal tissues (Normal) by microarray analysis. The subcellular localization of circRNA-0001283 was determined by RNA fluorescence in situ hybridization. Expression levels of circRNA-0001283 in 10 paired samples of breast cancer were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Expression levels of circRNA-0001283 in cell lines were examined by qRT-PCR. * $P<0.05$, ** $P<0.01$.

**CircRNA-0001283 represses cell growth and invasion and enhances apoptosis**

To further explored the effects of circRNA-0001283 on cancer cells, we carried out CCK-8 assay, and invasion and apoptosis analysis. Overexpression of circRNA-0001283 highly inhibited the growth of MCF7 and MDA-MB-231 cells (Figure 2A). Invasion assay revealed that ectopic expression of circRNA-0001283 remarkably inhibited the invasion abilities of MCF7 and MDA-MB-231 cells (Figure 2B). Moreover, overexpression of circRNA-0001283 markedly enhanced the apoptotic rate of breast cancer cells (Figure 2C). Consistent with these results, overexpression of circRNA-0001283 augmented the level of Bax and downregulated anti-apoptotic protein Bcl2 (Figure 2D). Together, these results suggest that circRNA-0001283 has a tumor suppressive function in breast cancer.

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**Figure 1.** CircRNA-0001283 is downregulated in breast cancer samples and cell lines. (A) A heatmap shows most 23 downregulated circRNAs in 3 paired samples of tumor issues (Tumor) and corresponding normal tissues (Normal) by microarray analysis. (B) The subcellular localization of circRNA-0001283 was determined by RNA fluorescence in situ hybridization. (C) Expression levels of circRNA-0001283 in 10 paired samples of breast cancer were determined by quantitative real-time polymerase chain reaction (qRT-PCR). (D) Expression levels of circRNA-0001283 in cell lines were examined by qRT-PCR. * $P<0.05$, ** $P<0.01$. 

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CircRNA-0001283 sponges miR-187

CircRNAs normally act as sponges to exert their function. Bioinformatics prediction suggested miR-187 to be a candidate target of circRNA-0001283. MiR-187 was shown to be upregulated in breast cancer cells compared with normal epithelial breast cells MCF-10A (Figure 3A). Overexpression of circRNA-0001283 markedly decreased the expression miR-187 (Figure 3B). To determine the direct interaction between circRNA-0001283 and miR-187, we performed luciferase reporter assay. The results showed that miR-187 remarkably inhibited the activity of wild type circRNA-0001283, however, it showed no obvious effect on the circRNA-0001283 mutant (Figure 3C). This result indicated that there is a direct interaction between circRNA-0001283 and miR-187. Moreover, ectopic expression of miR-187 remarkably increased the viability of MCF-7/MDA-MB-231 cells, and this effect of miR-187 was abolished by overexpression of circRNA-0001283 (Figure 3D). Together, these results suggest that miR-187 contributes to circRNA-0001283-mediated inhibition in the growth and invasion of breast cancer cells.

MiR-187 targets HIPK3

MiRNAs mediate their roles via regulating target genes. Targetscan and microRNA.org were used to explore the possible downstream targets of miR-187, and HIPK3 was predicted to be a potential target (Figure 4A). To confirm the direct
We carried out the luciferase reporter gene assay. As expected, the luciferase activity of the wild type HIPK3 3'UTR was markedly reduced upon transfection with miR-187, while no notable changes shown in the HIPK3 3'UTR mutant (Figure 4A). Furthermore, a remarkable decrease of the mRNA and protein expression of HIPK3 were detected in breast cancer cells (Figure 4B, 4C). Then, we determined the influence of miR-187 on circRNA-0001283-induced HIPK3 expression. MiR-187 mimics abolished the enhanced effect of circRNA-0001283 on HIPK3 expression (Supplementary Figure 1B). Moreover, the functional activity of HIPK3 was detected in MCF-7 cells. The result showed that overexpression of HIPK3 significantly inhibited the migration of MCF-7 cells.

Moreover, ectopic expression of HIPK3 reduced cell migration increased by miR-187 (Supplementary Figure 1A).

**CircRNA-0001283 regulates NF-κB signaling via miR-187/HIPK3 axis**

The mRNA and protein levels of HIPK3 were markedly enhanced in cells with circRNA-0001283 overexpression (Figure 5A, 5B). On the contrary, miR-187 mimic obviously inhibited HIPK3 expression (Figure 5C, 5D). Moreover, ectopic expression with circRNA-0001283 reduced the levels of p65 and p50 (Figure 5E), while transfection of miR-187 highly increased the levels of p65 and p50 (Figure 5F). Collectively, these results
suggest that circRNA-0001283 may regulate NF-κB signaling via miR-187/HIPK3 cascade.

**Discussion**

Exploration of circRNA expression profile is an effective way for identifying novel regulators in breast cancer. Here, we showed that circRNA-0001283 was remarkably decreased in breast cancer tissues and cells. Our results demonstrated that circRNA-0001283 was related to cell growth, invasion, and apoptosis. CircRNA-0001283 exerted its function as a ceRNA (competing endogenous RNA) that bound to miR-187 and blocked the suppressive effect of miR-187 on HIPK3 expression.

Previous studies reported that circRNAs can act as either oncogenic or suppressive factors in breast cancer. For example, upregulated circ-UBE2D2 is associated with poor prognosis and enhances the progression of breast cancer [14]. Zhang et al. showed that in triple-negative breast cancer, circRNA_069718 promotes cell viability and invasion [15]. Yang et al. reported that circ_0103552 facilitates breast cancer cell proliferation and decreases apoptotic cells [16]. CircRNA_0025202 inhibits colony formation and migration, and it promotes cell apoptosis in breast cancer [17]. CircTADA2As is demonstrated to be a negative regulator for breast cancer progression and metastasis [18]. Our data showed that circRNA-0001283 was decreased in the tissues of breast cancer. Overexpression of circRNA-0001283 inhibited cell proliferation and invasion and induced apoptosis, indicating a suppressive role of circRNA-0001283 in breast cancer.

Numbers of studies have revealed that circRNAs function in cancer via diverse mechanisms, such as acting as scaffolds of protein complexes, regulating protein subcellular localization, controlling gene expression, sequestering RNA-binding proteins, and functioning as competing endogenous RNA (ceRNA) [19,20]. MiRNAs are supposed to be important modulators in cancer [21,22]. Multiple circRNAs have been revealed to exert their function via sponging miRNAs [23–28]. It has been reported that miR-187 enhances cell growth and migration and represses the apoptosis of bladder cancer cells. High miR-187 expression is related to the advanced oral carcinoma [29]. In breast cancer, miR-187 is suggested to be a prognostic factor and facilitates aggressive invasion [30]. Here, we demonstrated that circRNA-0001283 suppresses breast cancer cell proliferation and invasion via regulating miR-187 expression [31].

In our further exploration of the mechanism of circRNA-0001283-mediated inhibition in breast cancer, we identified that HIPK3 was a downstream of circRNA-0001283. HIPK3 is a member of HIPK family [32]. The role of HIPK3 in cancer has been revealed previously. For instance, Curtin et al. reported that JNK modulates HIPK3 expression to promote resistance...
to Fas-mediated apoptosis in prostate cancer cells [33]. HIPK3 level is decreased in non-small cell lung cancer (NSCLC). Lower HIPK3 expression was associated with poorer survival in patients with NSCLC [34]. Here, our data showed that circRNA-0001283 increased the level of HIPK3 via downregulation of miR-187 expression.

Our data revealed that circRNA-0001283 was downregulated in breast cancer tissues. Overexpression of circRNA-0001283 repressed cell proliferation and invasion, and elevated apoptosis in breast cancer cells. Mechanically, we found that circRNA_069718 downregulated HIPK3 expression by sponging miR-187. These findings may support circRNA-0001283/miR-187/HIPK3 as potential targets for the treatment of breast cancer.

**Conclusions**

The results of our study revealed a new functional circRNA-0001283 in breast cancer progression and may provide novel targets for the treatment of breast cancer.

**Availability of data and materials**

Data used to support our findings in this study are available from the corresponding author upon request.

**Conflict of interest**

None.
Supplementary Data

Supplementary Figure 1. The influence of miR-187 on circRNA-001283-induced HIPK3 expression. (A) The effect of overexpression of miR-187 mimics on breast cancer cell invasion was determined by Transwell invasion assay. (B) MiR-187 mimics abolished the enhanced effect of circRNA-001283 on HIPK3 expression.

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