CircRNA circ_0075796 is downregulated in breast cancer and suppresses cell proliferation, migration and invasion

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Primary research

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Abstract

Background

Emerging evidence shows that circular RNAs (circRNAs) play crucial parts in tumorigenesis and progression. In this work, the expression, clinical significance, function and potential mechanism of circ_0075796 in breast cancer were explored.

Methods

The expression of circ_0075796 in 189 pairs of breast cancer tissues and adjacent normal tissues was detected by quantitative real-time PCR (qRT-PCR). Cell Counting Kit-8 (CCK-8) assay, methyl thiazolyl tetrazolium (MTT) assay and colony formation assay were conducted for cell proliferation. Transwell assay and wound healing assay were used for cell migration and invasion. Flow cytometry analysis was adopted for cell cycle and cell apoptosis. The cellular localization of circ_0075796 was determined by fluorescence in situ hybridization (FISH). The circ_0075796/miR-452-3p/SAMD5 axis was screened out by bioinformatics analysis and verified by qRT-PCR. Methylated RNA Immunoprecipitation (MeRIP) was used to detect the N6-methyladenosine (m6A) modification levels of circ_0075796. QRT-PCR was used to detect the expression of RNA binding protein Quaking (QKI) in breast cancer tissues and adjacent normal tissues.

Results

circ_0075796 was downregulated in breast cancer tissues compared with adjacent normal tissues. In addition, circ_0075796 showed satisfactory diagnostic value to discriminate breast cancer and normal controls. Downregulated circ_0075796 expression was correlated with lymph node metastasis, HER2 expression, larger tumor size, high Ki-67 expression, advanced histological grade, aggressive molecular subtypes and advanced clinical stages. Overexpression of circ_0075796 inhibited cell proliferation, migration and invasion in vitro. FISH showed that circ_0075796 was localized in the cytoplasm and nucleus of breast cancer cells. Bioinformatics analysis and qRT-PCR revealed the potential circ_0075796/miR-452-3p/SAMD5 axis. Moreover, circ_0075796 showed lower m6A modification levels in breast cancer tissues compared to adjacent normal tissues. QKI was predicted to contain binding sites of circ_0075796 and was downregulated in breast cancer tissues compared to adjacent normal controls.

Conclusions

circ_0075796 was downregulated in breast cancer compared to normal controls, and showed potential diagnostic value for breast cancer. Downregulation of circ_0075796 was correlated with aggressive clinical features of breast cancer and overexpression of circ_0075796 inhibited the progression of breast
cancer \textit{in vitro}, indicating that circ\_0075796 may be related to tumorigenesis and development of breast cancer.

**Background**

Breast cancer is a serious threat to the health of women, which has become one of the most prevalent and invasive malignancies for female worldwide [1, 2]. According to public data, it is the second leading cause of cancer-related deaths in women [3]. Although there are various treatments for breast cancer (e.g. surgery, radiotherapy and chemotherapy), the incidence and prognosis remain pessimistic [4]. In order to improve the curative effect and prognosis of breast cancer, it is essential to discover new biomarkers and therapeutic targets. Therefore, detailed researches into the mechanisms of breast cancer are urgently needed.

Circular RNAs (circRNAs) is a novel class of endogenous non-coding RNAs (ncRNAs). In contrast to linear RNA, circRNAs are formed by covalently closed loop and have neither 5' to 3' end polarity nor polyadenylation tail [5, 6]. Studies have confirmed that circRNAs can play key roles in the progression of various diseases. For example, circular RNA 406961 can regulate the inflammatory response of human bronchial epithelial cells by activating the STAT3/JNK pathway [7]. Circular RNA-ZNF532 can regulate retinal pericyte degeneration and vascular dysfunction caused by diabetes [8]. Moreover, an increasing number of studies have shown that circRNAs are involved in tumorigenesis and progression, including breast cancer [9–11]. CircRNAs participate in a number of different biological processes in tumor cells, including cell growth, metastasis, cell cycle control, nuclear and cytoplasmic transport, cell differentiation, RNA decay, transcription, translation and so on [12]. Concretely, microRNA(miRNA) sponges are the most common roles of circRNAs in many different types of cancers [10, 13]. Many RNA transcripts share binding sites with miRNAs and compete with each other to act as competitive endogenous RNAs (ceRNAs) to further regulate the occurrence and progress of tumor [14]. Numerous studies have shown that circRNAs can play the role of ceRNAs by acting as miRNA sponges in various tumors [15]. For instance, Tang et al. revealed that circGFRA1 and GFRA1 played the role of ceRNAs in triple negative breast cancer by regulating miR-34a [16].

N6-methyladenosine (m6A) is one of the most abundant modification of eukaryotic mRNAs and ncRNAs, which can control many aspects of post-transcriptional regulation of mRNA, including splicing, export, stability, and translation [17, 18]. In recent years, m6A modification of circRNAs has been brought into the spotlight [19]. For example, Timoteo et al. have showed that the decrease in methylation could affect the conversion of the pre-mRNA into the circRNA and further influence circRNA levels [20]. Zhao et al. revealed that m6A modification was essential for the protein-coding ability of circE7 [21].

RNA binding proteins (RBPs) are reported to interact with circRNAs and influence their biological functions [22]. For instance, RBP SRSF10 affects the miR-526b-3p/MMP2 pathway by inhibiting the expression of circ-ATXN1, thus regulating glioma angiogenesis [23]. Among these RBPs, the interactions between Quaking (QKI) and circRNAs are more reported [24]. QKI belongs to STAR family which contains
SH2 and SH3 domains. There are three major QKI isoforms, named QKI-5, QKI-6, and QKI-7, showing different biological characteristics [25]. Studies have shown that QKI may be involved in pre-mRNA splicing [26], mRNA translation [25] and related to multiple diseases, such as schizophrenia [27], glioma [28] and breast cancer [29].

CircRNA circ_0075796 is located on chromosome chr6:18236683-18258636-, and its predicted length is 902nt. At present, there is no research report on circ_0075796 in human diseases. In this study, the expression, clinical significance, function in vitro and potential mechanism of circ_0075796 were explored.

Materials And Methods

Tissue specimens

Breast cancer tissues and adjacent normal tissues of 189 breast cancer patients who underwent surgical treatment at Qilu Hospital of Shandong University were collected from May 2017 to July 2021. Tissue specimens were frozen in liquid nitrogen immediately after surgery until further use. All patients signed informed consents.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA-easy™ Isolation Reagent (Vazyme, Nanjing, China) was used to extract total RNA from tissues. The quality and concentration of RNA were measured on a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies). For circ_0075796 and mRNAs, cDNA was synthesized using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). SYBR® Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) was used for qRT-PCR on a LightCycler480 machine (ROCHE, Basel, Switzerland). For miRNAs, reverse transcription was performed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). QRT-PCR was conducted using qPCR SYBR Green Master Mix (CloudSeq, Shanghai, China). GAPDH was used as an internal standard control for circ_0075796 and mRNAs, while U6 was used for miR-452-3p. Related primers were detailed in Supplementary Table S1. The relative expression of RNAs was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell culture

Breast cancer cell line MDA-MB-231 and BT474 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 were cultured in DMEM medium (FBS, Gibreast cancero, USA) containing 10% fetal bovine serum (FBS, Gibreast cancero, USA). BT474 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibreast cancero, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Plasmid construction and cell transfection
For the overexpression of circ_0075796, 902bp cDNA fragment was cloned into pLC5-ciR vector, which was then verified by PCR product sequencing analysis (Supplementary Fig. S1A). MDA-MB-231 cells and BT474 were seeded in 6-well culture plates and cultured to 70-90% confluence before transfection, respectively. According to the manufacturer's instructions, circ_0075796 overexpression plasmid and negative control (Geneseed Biotech Co., Ltd., Guangzhou, China) were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was detected by qRT-PCR analysis 48 hours after transfection.

**Methyl thiazolyl tetrazolium (MTT) assay**

MDA-MB-231 cells were seeded in 96-well plates at a density of 5000 cells per well after transfection. MTT solution (20µL) (Sigma, Saint Louis, MO, USA) was added to each well, and the OD value was measured after incubation for 4 hours. The OD values at 0, 24, 48, 72 and 96h after transfection were recorded for statistical analysis.

**Cell counting kit-8 (CCK-8) assay**

BT474 cells were seeded in 96-well plates at a density of 5000 cells per well after transfection. CCK-8 reagent (10µL) (Bestbio, Shanghai, China) was added to each well, and the OD value was measured after incubation for 2 hours. The OD values at 24, 48 and 72h after transfection were recorded for statistical analysis.

**Colony formation assay**

The transfected MDA-MB-231 and BT474 cells (700 cells/well) were plated into 6-well plates. The plate was placed in a 37°C incubator containing 5% CO₂ to induce colony growth. After cultivation for 10 days, cell surface was rinsed using cooled PBS. Cell colonies were fixed with 4% paraformaldehyde, stained with crystal violet and finally observed under a microscope.

**Transwell assay**

Transwell inserts precoated with or without Matrigel (BD, Biosciences, USA) were used for the determination of cell invasion and migration, respectively. Transfected cells (1×10⁵) were suspended in 100µL serum-free medium and inoculated into upper chamber of the transwell. For migration assays, the Matrigel (BD, Biosciences, USA) was not coated on the membrane of the upper chamber, while the Matrigel (BD, Biosciences, USA) was coated for invasion assays. Medium containing 10% FBS was placed in the bottom chamber as a chemoattractant. Transfected cells were incubated at 37°C with 5% CO₂ for 24h, then the cells on the lower surface were fixed with methanol, stained with crystal violet, and finally observed under a microscope.

**Wound healing assay**

The transfected MDA-MB-231 cells and BT474 cells were cultured in 6-well plates. The monolayer of cells was created with a scratch using a 200-µL pipette tip and then washed with PBS. Images of cell migration were captured at 0 and 24 h for MDA-MB-231 cells and BT474 cells, respectively.
Cell cycle and apoptosis assays

For cell cycle assays, cells were stained with propidium iodide using Cell Cycle Detection Kit (Bestbio, Shanghai, China) and detected by flow cytometry. The ratios of cells in the G1, S, G2 phases were counted and compared. To detect cell apoptosis, cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (Bestbio, Shanghai, China) and analyzed by flow cytometry. The ratio of normal cells, early apoptotic cells, late apoptotic cells and total apoptotic cells was compared with the value of the control group in each experiment.

Fluorescence in situ hybridization (FISH)

circ_0075796 specific probe was used for in situ hybridization. Nuclei were counter by staining with 4,6-diamidino-2-phenylindole (DAPI). All the procedures were conducted according to the manufactory’s instruction (Geneseed, Guangzhou, China).

The final specimen was analyzed on a laser confocal microscope, TCS SP2 AOBS (Leica, Wetzlar, Germany). The probe was labeled with digoxin and the sequence is 5’-ggatacattcttgcaaatc-3’.

Bioinformatics analysis

Starbase [30] was applied to predict the potential binding sites between QKI and circ_0075796. Miranda [31] and TargetScan [32] databases were used to predict the relationship between 836 disease-related miRNAs (from the Human microRNA Disease Database, version 2.0) and circ_0075796. The target genes of the candidate miRNAs and the potential binding sites between them were further predicted based on these two databases.

Methylated RNA immunoprecipitation (MeRIP)

In brief, total RNA was extracted from three pairs of breast cancer tissues and adjacent normal tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and then fragmented using 2µL fragmentation buffer later. The fragmented RNA was then incubated with anti-m6A onoclonal antibody and A/G magnetic beads in IP buffer at 4°C for 2 hours for immunoprecipitation. Next, the bound RNA was eluted from the beads in IP buffer. SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was applied for reverse transcription of the eluted RNA after purification. QRT-PCR was conducted using qPCR SYBR Green Master Mix (CloudSeq, Shanghai, China) in QuantStudio 5 real-time PCR System (Thermo Fisher, Waltham, MA, USA). The Ct difference between input and the immunoprecipitated RNA was identified, and the relative enrichment was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (SPSS, Chicago, IL, USA). Data differences between two groups were analyzed using the Student’s t test. One way analysis of variance (ANOVA) was used to analyze data differences between three or four groups. Receiver operating characteristic (ROC) curves were generated to evaluate the diagnostic value of
circ_0075796 for breast cancer. The correlations between circ_0075796 expression level and the clinicopathological parameters of breast cancer patients were analyzed using the chi-squared test. P < 0.05 was considered to be statistically significant.

Results

circ_0075796 was significantly downregulated in breast cancer tissues compared with adjacent normal tissues

To examine the expression levels of circ_0075796, qRT-PCR was performed in 189 pairs of breast cancer tissues and adjacent normal tissues. Results showed that circ_0075796 was prominently downregulated in breast cancer tissues compared to adjacent normal tissues (Fig. 1A, P = 0.0028). Additionally, the area under the receiver operating characteristic curve (AUC) of circ_0075796 was 0.8015 (Figure 1B, P < 0.0001), showed that circ_0075796 could well distinguish breast cancer from adjacent normal tissues.

Association of circ_0075796 expression with clinicopathological features

The relationship between the expression level of circ_0075796 and clinicopathological features of breast cancer patients was analyzed. The data implied that circ_0075796 was downregulated in patients with lymph node metastasis than those without lymph node metastasis (Fig. 1C, P = 0.0174). In HER2 positive breast cancer, circ_0075796 showed lower expression level than in HER2 negative breast cancer (Fig. 1D, P = 0.0182). The expression level of circ_0075796 were negatively correlated with HER2 expression (Table 1, P = 0.028). As for tumor size, results showed that circ_0075796 was downregulated in patients with tumor size ≥ 2cm than patients with tumor size ≤ 2cm (Fig. 1E, P = 0.0251). The expression level of circ_0075796 were negatively correlated with tumor size (Fig. 1F, Spearman correlation r = -0.2253, P = 0.0018; Table 1, P = 0.029). In addition, patients with higher expression of circ_0075796 had lower Ki-67 expression (Fig. 1G, P = 0.0320). And the expression level of circ_0075796 were negatively correlated with Ki-67 expression (Fig. 1H, Spearman correlation r = -0.1405, P = 0.0279). Meanwhile, the expression level of circ_0075796 showed progressive decrease with the increase of pathological N (pN) stages (Fig. 1I, P = 0.0368). In particular, patients with stage pN0 had higher expression level of circ_0075796 compared to patients with stage pN1 and patients with stage pN2-3, respectively (Fig. 1I, pN0 vs pN1, P = 0.0338; pN0 vs pN2-3, P = 0.0489). As for histological grade, decrease of circ_0075796 expression was found from Grade III to Grade I (Fig. 1J, P = 0.0118). Specifically, circ_0075796 showed higher expression in patients with Grade I than patients with Grade II and Grade III, separately (Fig. 1J, Grade I vs Grade II, P = 0.0355; Grade I vs Grade III, P = 0.0038). The negative correlation between circ_0075796 expression and histological grade was also discovered (Table 1, P = 0.006). Moreover, circ_0075796 represented differential expression levels in different molecular subtypes of breast cancer. circ_0075796 showed higher expression level in Luminal A breast cancer compared with HER2 enriched breast cancer (Fig. 1K, Luminal A breast cancer vs HER2 enriched breast cancer, P = 0.0252). As for clinical stages of breast
cancer, circ_0075796 was downregulated in patients with advanced clinical stages than patients with early clinical stages (Fig. 1L, P = 0.0480). The expression level of circ_0075796 was not correlated with estrogen receptor (ER) and progesterone receptor (PR) (Table 1).
Table 1
Association between circ_0075796 expression and clinicopathological features in breast cancer

| Variables                | Cases | circ_0075796 expression | P-value |
|--------------------------|-------|-------------------------|---------|
|                          |       | Low | High |       |
| **Age (y)**              |       |     |      |       |
| ≤ 50                     | 80    | 45 | 35  |       |
| > 50                     | 109   | 50 | 59  | 0.186 |
| **Tumor size (cm)**      |       |     |      |       |
| ≤ 2 (Tis-T1)             | 87    | 36 | 51  |       |
| > 2 (T2-T3)              | 102   | 59 | 43  | 0.029 |
| **Histological grade**   |       |     |      |       |
| I                        | 11    | 1  | 10  |       |
| II                       | 97    | 48 | 49  |       |
| III                      | 68    | 41 | 27  | 0.006 |
| Unknown                  | 13    | 13 |     |       |
| **Lymph node metastasis**|       |     |      |       |
| Negative                 | 104   | 46 | 58  |       |
| Positive                 | 82    | 47 | 35  | 0.104 |
| Unknown                  | 3     | 3  |     |       |
| **ER**                   |       |     |      |       |
| Negative                 | 46    | 26 | 20  |       |
| Positive                 | 143   | 69 | 74  | 0.397 |
| **PR**                   |       |     |      |       |
| Negative                 | 47    | 28 | 19  |       |
| Positive                 | 142   | 67 | 75  | 0.178 |
| **HER2**                 |       |     |      |       |
| Negative                 | 132   | 60 | 72  |       |
| Positive                 | 53    | 33 | 20  | 0.028 |
| Unknown                  | 4     | 4  |     |       |
| Variables | Cases | circ_0075796 expression | P-value |
|-----------|-------|-------------------------|---------|
|           |       | Low | High |       |
| **Ki-67** |       |     |      |       |
| < 30%     | 83    | 36 | 47  |       |
| ≥ 30%     | 106   | 59 | 47  | 0.108 |
| **Molecular Subtypes** |       |     |      |       |
| Luminal A | 32    | 13 | 19  |       |
| Luminal B | 91    | 43 | 48  |       |
| HER2 enriched | 49  | 29 | 20  |       |
| Triple Negative | 13 | 8  | 5   | 0.291 |
| Unknown   | 4     | 4  |     |       |
| **pN stage** |       |     |      |       |
| pN0       | 105   | 46 | 59  |       |
| pN1       | 45    | 26 | 19  |       |
| pN2-N3    | 36    | 21 | 15  | 0.157 |
| Unknown   | 3     | 3  |     |       |
| **Tumor stage** |       |     |      |       |
| 0-II      | 147   | 70 | 77  |       |
| 0-III     | 39    | 23 | 16  | 0.28  |
| Unknown   | 3     | 3  |     |       |

circ_0075796 promoted the progression of breast cancer in vitro

To further investigate the role of circ_0075796 in the tumorigenesis and development of breast cancer, cell function experiments were performed. Since the expression of circ_0075796 was down-regulated in breast cancer tissues, the overexpression vector of circ_0075796 was constructed to transfect the breast cancer cell line MDA-MB-231 and BT474. After transfection, the expression of circ_0075796 was significantly up-regulated compared to the controls (Fig. 2A; Supplementary Fig. S1B). In cellular experiments, MTT (Fig. 2B), CCK-8 (Supplementary Fig. S1C) and colony formation assays (Supplementary Fig. S1D) indicated that overexpression of circ_0075796 could significantly promote cell proliferation. Transwell (Fig. 2C-D) and wound healing assays (Supplementary Fig. S1E-F) showed that the migration and invasion abilities of breast cancer cell were facilitated by circ_0075796. Flow cytometry
analysis was conducted to detect the influence of circ_0075796 on cell cycle and apoptosis. According to the consequence, there was no significant difference in cell cycle distribution (Fig. 2E-F; Supplementary Fig. S1G) and apoptosis rates (Fig. 2G-H; Supplementary Fig. S1H) between circ_0075796 overexpression group and the control group. These results revealed that circ_0075796 could promote the progression of breast cancer in vitro.

**Bioinformatics analysis and preliminary verification of circ_0075796/miR-452-3p/SAMD5 axis**

Numerous studies have reported that circRNAs can serve as miRNA sponges in cancers and further regulate gene expression [33]. In this work, FISH assays confirmed that circ_0075796 was localized both in the cytoplasm and nucleus of breast cancer cells (Fig. 3A), suggesting its potential of miRNA sponge in breast cancer. To further study the miRNAs related to circ_0075796, Miranda and Targetscan databases were used to predict candidate miRNAs. There were 90 miRNAs with potential binding sites for circ_0075796 (Supplementary Table S2). According to the combination of the number of binding sites, the context score from TargetScan and the thermodynamic properties of the binding site from miRanda, miR-452-3p was predicted to have the highest degree of association with circ_0075796. In addition, the possible target genes of candidate miRNAs were predicted on the basis of the two databases. Among the predicted circ_0075796/miRNA/mRNA interactions, circ_0075796/miR-452-3p/SAMD5 axis is of great interest since both miR-452-3p and SAMD5 have been reported to be related to cancer development and progression [34, 35].

Sequence comparison revealed the potential binding sites of miR-452-3p and SAMD5, circ_0075796 and miR-452-3p (Fig. 3B-C). QRT-PCR confirmed that miR-452-3p expression was upregulated in breast cancer tissues than adjacent normal tissues (Fig. 3D, $P < 0.0001$), whereas SAMD5 expression was downregulated in breast cancer tissues than adjacent normal tissues (Fig. 3E, $P = 0.0002$). Besides, correlation analysis of expression levels in tissues showed that circ_0075796 was negatively correlated with miR-452-3p (Fig. 3F, Spearman correlation $r = -0.4297$, $P = 0.0459$) and positively correlated with SAMD5 (Fig. 3G, Spearman correlation $r = 0.3698$, $P = 0.0451$), while miR-452-3p was negatively correlated with SAMD5 (Fig. 3H, Spearman correlation $r = -0.7414$, $P < 0.0001$). These results were consistent with the regulation mode of circ_0075796/miR-452-3p/SAMD5 axis.

**circ_0075796 showed different m6A modification level in breast cancer tissues and normal controls**

M6A modification of circ_0075796 was predicted by SRAMP website (http://www.cuilab.cn/sramp/). Results showed that there were multiple m6A modification sites in circ_0075796 (Fig. 4A). Furthermore, methylated RNA immunoprecipitation (MeRIP) showed that m6A modification of circ_0075796 existed in both breast cancer tissues and adjacent normal tissues (Fig. 4B). Compared with normal controls, breast cancer tissues showed lower m6A modification levels (Fig. 4B). The mRNA levels of m6A-related enzymes in breast cancer tissues and adjacent normal tissues were also monitored. As expected,
YTHDF2 and IGF2BP2, known as m6A readers [36], were downregulated in breast cancer tissues contrast to normal controls (Fig. 4C-D, P < 0.05).

**QKI was downregulated in breast cancer tissues contrast to normal controls**

Given that QKI can be involved in the formation of circRNAs and tumor progression, Starbase was applied to further explore the interaction of QKI and circ_0075796. The predictions showed that there were several potential binding sites between QKI and circ_0075796 (Fig. 5A-B). The expression levels of QKI were detected by qRT-PCR in 11 pairs of breast cancer tissues and adjacent normal tissues. Results indicated that QKI was significantly downregulated in breast cancer tissues (Fig. 5C, P = 0.0437), consistent with the expression trend of circ_0075796.

**Discussion**

Increasing studies have revealed the potential of circRNAs in biomarkers and molecular targets for varieties of cancers. For example, Melika et al. revealed the potential of hsa_circ_0005046 and hsa_circ_0001791 as diagnostic biomarkers for breast cancer [37]. Fu et al. provided a circRNA profile as potential biomarkers and therapeutic targets for breast cancer brain metastasis [38]. In this study, qRT-PCR was used to analyze circ_0075796 expression in 189 pairs of breast cancer and adjacent normal tissues. Results showed that circ_0075796 was significantly downregulated in breast cancer tissues, which suggests the potential tumor suppressor role of circ_0075796. In addition, the ROC curve of circ_0075796 demonstrates that circ_0075796 has a certain significance in the diagnosis of breast cancer. Furthermore, the analysis of the clinicopathological features indicated that circ_0075796 showed down-expression in patients with larger tumor size, positive HER2 expression, higher Ki-67 expression and advanced histological grade, emphasizing the involvement of circ_0075796 in the tumorigenesis and development of breast cancer. circ_0075796 expression level was also relevant to lymph node involvement and different molecular subtypes, which implies that circ_0075796 may be associated with metastasis and prognosis of breast cancer.

In addition, circRNAs are gaining increasing attention in special regulatory functions in various biological processes of multiple cancers including breast cancer. For example, circ_0069718 was demonstrated to promote the progression of breast cancer by up-regulating NFIB through sequestering miR-590-5p [39]. Shi et al. revealed that hsa_circ_0006220 remarkably inhibited the proliferation, migration, and invasion of TNBC cells, playing an inhibitory role in TNBC progression [40]. Therefore, in-depth researches of the functions and regulatory mechanisms of circRNAs could provide novel insights into the molecular mechanisms of breast cancer progression. In this study, we recognized that circ_0075796 overexpression statistically inhibited cell proliferation, migration, and invasion, which was consist with the expression of circ_0075796 in breast cancer tissues. These results convincingly suggested the tumor suppressor role of circ_0075796 in breast cancer.
Researches have indicated that circRNAs can serve as ceRNAs to accommodate the expression of miRNAs and further influence the expression of mRNAs to affect tumor progression [10, 41, 42]. To further explore the mechanism of circ_0075796 as ceRNA in breast cancer, we obtained the potential circ_0075796/miR-452-3p/SAMD5 axis by bioinformatics analysis. QRT-PCR analysis preliminarily verified that circ_0075796 may serve as ceRNA to eliminate the inhibitory effect of miR-452-3p on SAMD5. Tang et al. have disclosed that miR-452-3p can promote the proliferation and migration of liver cancer cells by directly targeting the CPEB3/EGFR axis [34]. And Li et al. indicated that miR-452-mediated miR-452-GSK3β-LEF1/TCF4 loop could induce colorectal cancer proliferation and migration [43]. In this study, the proposal of potential circ_0075796/miR-452-3p/SAMD5 axis and the verification of the upregulation of miR-452-3p in breast cancer tissues revealed the role of miR-452-3p in breast cancer. SAMD5 is a protein containing SAM domain, which is distributed on about 70 residues and has different effects on cellular processes through polymerization [44]. It has been reported that knockdown of SAMD5 can inhibit cell proliferation in small cell lung cancer [45]. Inversely, Tomoki Yagai et al. demonstrated that knockdown and overexpression of SAMD5 resulted in promotion and inhibition of cell proliferation in cholangiocarcinoma respectively [35]. In this work, the potential circ_0075796/miR-452-3p/SAMD5 axis and the correlation of the expression levels of circ_0075796, miR-452-3p and SAMD5 indicated the role of circ_0075796 in breast cancer.

According to the prediction of m6A modification sites of circ_0075796 based on the SRAMP website, we detected the level of m6A modification of circ_0075796 and the mRNA expression of m6A-related enzymes in breast cancer tissues and normal controls. The reduced expression levels of m6A readers YTHDF2 and IGF2BP2 is consist with the lower m6A modification level in breast cancer tissues contrast to normal tissues. There are numerous researches related to the effect of m6A modification on the expression and biological functions of circRNAs. For example, Zhou et al. have reported that m6A levels were related to expression levels of circRNAs and showed different patterns in different cell lines [46]. Park et al. found the role of m6A modification in circRNA degradation [47]. Additionally, extensive translation of circRNAs driven by m6A modification also has been reported [48]. These findings revealed the close correlation between m6A modification and biological behaviors of circRNAs. In the present study, the decreased m6A modification degree of circ_0075796 may be concerned with the differential expression of circ_0075796 in breast cancer tissues and adjacent normal tissues.

Over the years, more studies have documented that QKI can regulate the formation of circRNAs and thus affects the progression of diseases [49]. For example, circNRIP1 was upregulated on account of the promotion of QKI in gastric cancer tissues [50]. In prostate cancer, QKI can increase circZEB1 levels [51]. In non-small cell lung cancer, QKI promoted the information of circ-SLC7A6 and facilitated the suppression of tumor progression [52]. In addition, Cao et al. found that QKI could suppress breast cancer via RASA1/MAPK signaling pathway [53]. Our study predicted the potential binding sites of QKI and circ_0075796 and revealed the downregulation of QKI in breast cancer tissue. Decreased expression levels of QKI may be involved in the downregulation of circ_0075796 and then affect the progression of breast cancer, which requires continually further researches.
Conclusions

In summary, this study explored the expression, roles, functions and potential mechanisms of circ_0075796 in breast cancer. The results disclosed the downregulation of circ_0075796 in breast cancer tissues and its diagnostic value for breast cancer. Besides, the close association of circ_0075796 expression and clinicopathological features revealed the possible role of circ_0075796 in the occurrence, progression, metastasis and prognosis of breast cancer. The cell experiments uncovered the potential functions of circ_0075796 in the progression of breast cancer. In addition, the potential circ_0075796/miR-452-3p/SAMD5 axis was revealed. The specific mechanisms of the ceRNA axis and the roles of circ_0075796 in the occurrence and development of breast cancer remain to be further studied.

Abbreviations

CircRNAs, circular RNAs; qRT-PCR, quantitative real-time PCR; CCK-8, cell counting kit-8; MTT, methyl thiazolyl tetrazolium; FISH, fluorescence in situ hybridization; MeRIP, methylated RNA immunoprecipitation; m6A, N6-methyladenosine; QKI, quaking; ncRNAs, endogenous non-coding RNAs; miRNA, microRNA; ceRNAs, competitive endogenous RNAs; RBPs, RNA binding proteins; DAPI, 4,6-diamidino-2-phenylindole; AUC, area under the receiver operating characteristic curve; pN, pathological N; ER, estrogen receptor; PR, progesterone receptor.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Qilu Hospital of Shandong University and was carried out according to the World Medical Association Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KZ conceived and designed the experiments. KZ, YX, XC, YW, YD, ZQ, CL, HD and RM collected samples. YX, YD, CL, BY, ZQ and YR conducted the experiments. YX and YD analyzed the data. KZ and YW supervised the whole work and revised the manuscript. YX wrote the paper. All authors read and approved the final manuscript.

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Figures
Figure 1

Expression and clinical significance of circ_0075796 in breast cancer. (A) circ_0075796 was downregulated in breast cancer tissues compared with normal controls (P = 0.0028). (B) ROC curve analysis showed that the AUC of circ_0075796 was 0.8015 (P < 0.0001). (C) In breast cancer tissues, circ_0075796 was downregulated in lymph node metastasis (LNM)-positive group than LNM-negative group (P = 0.0174). (D) circ_0075796 was downregulated in HER2 positive group than HER2 negative group (P = 0.0182). (E) circ_0075796 was downregulated in patients with tumor size ≤ 2cm than patients with tumor size > 2cm (P = 0.0251). (F) circ_0075796 was negatively correlated with tumor size (Spearman correlation r = -0.2253, P = 0.0018). (G) circ_0075796 was downregulated in patients with Ki-67 ≥ 30% than patients with Ki-67 < 30% (P = 0.0320). (H) circ_0075796 was negatively correlated with Ki-67 expression (Spearman correlation r = -0.1405, P = 0.0279). (I) circ_0075796 showed progressive decrease with the increase of pathological N (pN) stages (P = 0.0368). Patients with stage pN0 had higher expression level of circ_0075796 compared to patients with stage pN1 and patients with stage pN2-3, respectively (pN0 vs pN1, P = 0.0338; pN0 vs pN2-3, P = 0.0489). (J) circ_0075796 showed progressive decrease from Grade I, Grade II to Grade III (P = 0.0118). circ_0075796 showed higher expression in patients with Grade I than patients with Grade II and Grade III separately (Grade I vs Grade III).
II, P = 0.0355; Grade I vs Grade III, P = 0.0038). (K) circ_0075796 was upregulated in Luminal A breast cancer compared with HER2 enriched breast cancer (Luminal A breast cancer vs HER2 enriched breast cancer, P = 0.0252). (L) circ_0075796 was downregulated in patients with clinical stage 0-II than patients with clinical stage III- (P = 0.0480).

Figure 2
circ_0075796 promoted the progression of breast cancer in MDA-MB-231 cells. (A) After transfection of overexpression vector of circ_0075796, the expression of circ_0075796 in MDA-MB-231 cells was upregulated compared to the controls. (B) circ_0075796 inhibited cell proliferation by MTT assays. (C-D) circ_0075796 inhibited cell migration and invasion abilities by Transwell assays. (E-F) Flow cytometry analysis showed that circ_0075796 had no effect on cell cycle. (G-H) Flow cytometry analysis showed that circ_0075796 had no effect on cell apoptosis rates. *P < 0.05, ***P < 0.001.

Figure 3

Bioinformatics analysis and related verification of circ_0075796/miR-452-3p/SAMD5 axis. (A) FISH assays showed that circ_0075796 was localized both in the cytoplasm and nucleus of breast cancer MDA-MB-231 cells. (B-C) Potential binding sites of miR-452-3p and SAMD5, circ_0075796 and miR-452-3p revealed by sequence comparison. (D-E) QRT-PCR for breast cancer tissues (n =11) and adjacent
normal tissues (n = 11) demonstrated that miR-452-3p was upregulated in breast cancer tissues, while SAMD5 was downregulated (All P < 0.0001). (F-G) circ_0075796 was negatively correlated with miR-452-3p (Spearman correlation r = -0.4297, P = 0.0459) and positively correlated with SAMD5 (Spearman correlation r = 0.3698, P = 0.0451). (H) miR-452-3p was negatively correlated with SAMD5 (Spearman correlation r = -0.7414, P < 0.0001).

| # | Position | Sequence context | Structural context | Local structure visualization | Score(binary) | Score(kmer) | Score(spectrum) | Score(combined) | Decision |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 170 | UAAUG | GUAAAU | GAAGGA | UGAAA | A | PPPPP IIII I PPPHH | 0.611 | 0.452 | 0.593 | 0.591 | m^6A site (Moderate confidence) |
| 2 | 188 | AACUA | GAGCA | GCACU | ACGGA | C | PPPPP IIII I PPPHH | 0.576 | 0.44 | 0.615 | 0.586 | m^6A site (Moderate confidence) |
| 3 | 277 | GAGGA | UCGAG | GUGGA | AGAGA | C | PPPPP IIII I PPPHH | 0.526 | 0.274 | 0.562 | 0.529 | m^6A site (Low confidence) |
| 4 | 430 | CCAAU | UGCGG | AAUAC | UARAC | A | PPPPP IIII I PPPHH | 0.580 | 0.512 | 0.652 | 0.611 | m^6A site (High confidence) |
| 5 | 481 | AAGGA | CAGUA | AAAAG | AAACU | A | PPPPP PPPPP PPPPP | 0.506 | 0.627 | 0.640 | 0.569 | m^6A site (Moderate confidence) |
| 6 | 620 | UAGAAG | AGGGG | CCACC | GACAC | A | PPPPP PPPPP PPPPP | 0.567 | 0.626 | 0.572 | 0.576 | m^6A site (Moderate confidence) |
| 7 | 785 | CCAAC | GGGAC | UUAGA | AGAGA | G | PPPPP PPPPP PPPPP | 0.447 | 0.628 | 0.651 | 0.528 | m^6A site (Low confidence) |

**Figure 4**

M6A modification level of circ_0075796 in breast cancer tissues and normal controls. (A) M6A modification sites in circ_0075796 was predicted by SRAMP website. (B) Breast cancer tissues showed lower m6A modification levels compared to normal controls. (C-D) The m6A-related enzymes, YTHDF2 (P = 0.0305) and IGF2BP2 (P =0.0308), were downregulated in breast cancer tissues contrast to normal controls.
Figure 5

circ_0075796 was predicted to contain potential binding sites of QKI based on Starbase (A-B). QKI was downregulated in breast cancer tissues compared with normal controls (C, P = 0.0327).

Supplementary Files

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