Hsa_circ_0005273 regulates YAP1-Hippo signaling pathway by targeting miR-200a-3p to promote breast cancer progression.

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Research

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Abstract

Background : Hsa_circ_0005273 is a circRNA generated from several exons of PTK2. However, the potential functions of hsa_circ_0005273 in breast cancer remains unknown. Here we aim to explore the role of hsa_circ_0005273 in BC.

Methods : We chose miR-200a-3p as the potential target of hsa_circ_0005273. The expression levels of hsa_circ_0005273 and miR-200a-3p were examined in BC tissues compared with adjacent normal tissues by qRT-PCR. To characterize the function of hsa_circ_0005273, experiments of cell proliferation and migration were performed in BC cell lines infected with lentivirus targeting hsa_circ_0005273. In addition, xenografts experiment in nude mice were performed to evaluate the effect of hsa_circ_0005273 in BC. Luciferase reporter assay was conducted to confirm the relationship between hsa_circ_0005273 and miR-200a-3p as well as miR-200a-3p and YAP1.

Results : Hsa_circ_0005273 is over-expressed in BC tissues and cell lines, whereas miR-200a-3p expression is repressed. Depletion of has_circ_0005273 or upregulation of miR-200a-3p inhibited the proliferation and migration of BC cells in vitro and vivo. Mechanistically, hsa_circ_0005273 upregulated YAP1 by targeting miR-200a-3p and activated Hippo signaling pathway to promote BC progression.

Conclusions : Hsa_circ_0005273 regulates the miR-200a-3p/YAP1 axis and activates Hippo signaling pathway to promote BC progression, and it may serve as a potential biomarker and therapeutic target.

Keywords : breast cancer, hsa_circ_0005273, miR-200a-3p, YAP1, progression

Background

Breast cancer(BC), the most common diagnosed malignant cancer and the leading cause of cancer-related death in women worldwide, with more about 1.7 million new cases worldwide diagnosed each year[1]. Though outstanding advances in surgical and medical management for breast cancer have been seen, the morbidity and mortality seems to be increasing[2]. High frequency of metastasis, recrudescence and drug resistance are significant reasons of cancer-associated deaths among breast cancer patients. It therefore comes as no surprise that further investigating the molecular mechanism of breast cancer remains a urgent and tough task.

As a novel class of endogenous non-coding RNAs (ncRNAs), circular RNAs(circRNAs) are formed from exons or introns through special selective shearing[3]. Unlike linear RNAs that are terminated with 5'caps and 3'tails, circRNAs are single-stranded covalently closed circular transcripts[4, 5], which gives them higher stability compared to linear types[6]. With the further study of ncRNAs, circRNA is now regarded as an emerging key player in the RNA world. Emerging evidence suggests that various physiological functions of circRNAs have been found, including serving as ceRNAs or miRNA sponges[7, 8], interacting with proteins[9–11], regulating gene transcription[12, 13] and translation[14–17]. A large number of circRNAs have been successfully suggested play significant roles in tumorigenesis, metastasis, and

therapy resistance and aberrant expression of circRNAs were correlated with progression and prognosis of various cancers [18]. Furthermore, circRNAs could function as diagnostic biomarkers of hepatoblastoma [19] and bladder cancer[20] in blood or plasma in according to their stability and tissue specificity [21, 22]. Nevertheless, the potential function and molecular mechanisms of circRNAs in breast cancer warrant further investigation.

Hippo signaling pathway, a highly conserved pathway, performs an important role in governing cell proliferation and apoptosis of various cancer types, including breast cancer. YAP1 (yes-associated protein), as the primary transcription co-activators of the Hippo signaling pathway, discovered function as an oncogene in breast cancer[23, 24]. Treatment with inhibiting the activity of YAP1 could inhibit vascular invasiveness of breast cancer cells[25]. Considering the mutation, activation and overexpression of YAP1 are related to the occurrence and development of breast cancer, it's significant to find new molecules that can suppress the expression of YAP1.

In the present research, we analyzed the has_circ_0005273, a circRNA produced at the PTK2 gene. Hsa_circ_0005273 was significantly highly expressed in BC tissues than in normal tissues and exerted its oncogenic role in BC cells via acting as a sponge of tumor suppressor miR-200a-3p to inactivate YAP1-Hippo signaling pathway. Our findings provided a new insights into the treatment and diagnosis of BC.

Materials And Methods

Tissue samples

Tumor tissues and their adjacent normal tissues from 34 BC patients were collected from the Department of Breast and Thyroid Surgery of Shanghai Tenth People's Hospital of Tongji University (Shanghai, China). None of the patients received any local or systemic treatment before surgery and all tissue specimens were immediately snap-frozen in liquid nitrogen until further use. Our study protocols were approved by Institutional Ethics Committees of Shanghai Tenth People's Hospital and written informed consent was obtained from all patients or their relatives. The methodology of this study adhered to the standards outlined in the Declaration of Helsinki.

Cell Culture And Transfection

The human BC cell lines MDA-MB-231, MCF-7, HCC-1937, SKBR3 and normal breast epithelial cell line MCF-10A were purchased from Chinese Academy of Sciences (Shanghai). MDA-MB-231, MCF-7, HCC-1937 and SKBR3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) with 10% Fetal Bovine Serum (FBS) (Gibco), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Enpromise, China). MCF-10A cells were cultured in Mammary Epithelial Basal Medium (MEBM) (Cambrex). All these cells were cultured at 37 °C with 5% CO₂. Small, interfering, specifically targeting human hsa_circ_0005273 (si-circ_0005273), non-specific negative control oligos (si-NC) and has_circ_0005273 overexpression(circ_0005273), were purchased from IBSBio(Shanghai, China).

Human miR-200a-3p-mimics and the corresponding negative control mimics (miR-200a-3p-NC) and miR-200a-3p inhibitor were purchased from RiboBio(Guangzhou, China). MDA-MB-231 and MCF-7 cells were cultured and transfected with reagents above using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions for 6 h at 37 °C for transient transfection. We used DNA Midiprep Kits (Qiagen, Hilden, Germany) to prepare plasmid vectors. A lentivirus carrying si-circ_0005273 was constructed by ZORIN(Shanghai, China) and transfection procedures were performed according to the manufacturer's instructions.

RNA extraction and qRT-PCR

Total RNA was extracted from frozen tissues and cultured cells by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration and purity of RNA samples was assessed with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized by a commercial cDNA synthesis kit (Takara Biotechnology, Dalian, China). We conducted quantitative real-time polymerase chain reaction (qRT-PCR) by using the SYBR Green PCR Kit (Takara Biotechnology, Dalian, China) and primer sequences were designed and synthesized by RiboBio (Guangzhou, China). Expression of circRNA, miRNA and mRNA were assessed by handling threshold cycle (CT) values and analysed using the $2^{-\Delta\Delta Ct}$ method.

RNase R Resistance Analysis Of circRNAs

The has_circ_05273 from MDA-MB-231 and MCF-7 cell lines were treated with RNase R (4 U/mg, Epicenter) and incubated for 30 min at 37 °C. Then, the treated RNAs were reverse transcribed with specific primers and detected by qRT-PCR assay.

MTT Assay

For MTT assay, a density of 2000 cells per well were placed into 96-well plates. The cells were detected in accordance with the manufacturer's instructions using MTT assay kit (Sigma, Santa Clara, CA, USA). The 490 nm optical density was detected by a microplate reader respectively at 24, 48, 72 and 96 h.

Colony Formation Assay

A density of 500 cells per well were transferred into six-well plates. Cell colonies were washed twice by using cold phosphate buffered saline (PBS), fixed with 75% ethanol and stained with 0.1% crystalline purple until the colonies were visible. Then colonies were counted and photographed.

Wound Healing Assay

MDA-MB-231 and MCF-7 cells were transfected with a range of constructs as indicated in 6-well plates. When the treated cells reached about 80% confluent, a scratch was produced in the cell monolayer by drawing a 200-μl-pipette tip over the surface of each well, holding the tip perpendicular to the plate. The monolayers were washed twice with 1x PBS and cultured with DMEM medium with 2%FBS. Wound

healing was observed under a light microscope and pictures were taken at 0 h, 24 h and 48 h at the same position to observe cell movement.

Migration Assays

Transwell chambers (Corning, Inc., Lowell, MA, USA) were used to measure the migration ability of the cells in 24-well plates. Cells were transferred into the upper chamber with 200 μ l serum-free medium and medium with 10% FBS was added to the lower chamber. 24 h later, cells in the upper chamber were carefully removed by a cotton swab. Then, the cells on the opposite side of the filter were fixed with 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. Representative pictures were taken with a microscope (Leica Microsystems, Mannheim, Germany) and migrated cells were counted in five random fields.

Dual-luciferase Reporter Assay

To confirm that miR-200a-3p directly targets has_circ_0005273 and YAP1 3'-UTR, Wild and mutant reporter plasmids of has_circ_0005273 and LATS2 were individually designed and synthesized by IBSBio (Shanghai, China). 293T cells were co-transfected with the constructed reporter plasmids, together with miR-200a-3p mimics or miR-200a-3p-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). 24 hours later, luciferase activities were measured by the Dual-Luciferase® Reporter Assay kit (Promega, Madison, USA). Then firefly to Renilla luciferase ratios were calculated.

Western Blot Analysis

Proteins were extracted by using RIPA lysis buffer (Beyotime, Jiangsu, China) and the concentrations were detected by using a protein assay kit (Beyotime). Protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membrane (Beyotime). The membrane was incubated with 5% non-fat milk for 1 h and immunoblotted at 4 °C overnight with primary antibodies. The next day, we incubated the membranes in secondary antibodies for 1 h at room temperature. Signals of protein bands were scanned by Odyssey Infrared scanning system (Li-Cor, Lincoln, NE, USA).

FISH Assay

Ribo™ Fluorescent In Situ Hybridization Kit (Ribo, China) was used in FISH assay and specific probes for the hsa_circ_0005273 were designed and synthesized by RiboBio (Guangzhou, China). 4',6-Diamidino-2-Phenylindole (DAPI) was used to stain cell nuclei. Fluorescence microscope (Olympus BX53 Biological Microscope) was used to capture the images of cells.

Xenograft Tumor Assay

Athymic nude mice (age, 4–6 weeks; weight, 18–22 g, 4 mice per group) were ordered from the laboratory animal center of Shanghai. Approximately 1×10^6 MDA-MB-231 cells with stable expression of si-circ_0005273 or si-NC were injected into the second mammary fat of the mice (n = 4, each group). Then,

Tumor size was measured and calculated every week using the following formula: Volume (mm³) = 0.5 * width² * length. After 5 weeks, the mice were killed by cervical dislocation and the tumors were collected.

Statistical analysis

The significance of differences between groups was assessed by GraphPad Prism V8.0 (GraphPad, CA, USA) and SPSS 20.0 (IBM, SPSS, IL, USA). All experiments were repeated for three times. Data were obtained from three independent experiments which are presented as the means ± standard deviation (SD) and a P-value < 0.05 was considered significant.

Results

The characteristic of hsa_circ_0005273 in BC cells

We analyzed the gene chip GSE113230 in GEO (<https://www.ncbi.nlm.nih.gov/geo/>) and found that the expression of a novel circRNA, termed hsa_circ_0005273, was higher in BC tissues than in adjacent normal tissues. According to the UCSC Genome Browser Home (<http://genome.ucsc.edu/>), the 357-bp hsa_circ_0005273 is formed by circularization of exon 27–29 of the gene PTK2, which is located at chromosome 8:141710989–141716304 (Fig. 1a). Since circRNAs have no 3' poly-A tail due to the closed loop structures, we used both random hexamer or oligo (dT)18 primers in MDA-MB-231 and MCF-7 cell lines. The results showed that the relative expression of hsa_circ_0005273 was significantly downregulated when oligo (dT)18 primers were used compared with random hexamer primers, proving that hsa_circ_0005273 has no 3' poly-A tail (Fig. 1b). Furthermore, RNase R exonuclease was used to further validate hsa_circ_0005273 in MDA-MB-231 and MCF-7 cells. Resistance to RNase R exonuclease confirmed that hsa_circ_0005273 was indeed circular (Fig. 1c-d). To explore the cellular distribution of hsa_circ_0005273, the expression levels of cytoplasmic control transcripts GAPDH, nuclear control transcript U6 and hsa_circ_0005273 were examined by qRT-PCR in the cytoplasmic and nuclear fractions of MDA-MB-231 and MCF-7 cell lines. Subcellular fractionation demonstrated quantitatively that hsa_circ_0005273 was enriched in the cytoplasm of two cell lines above (Fig. 1e-f). In addition, FISH analysis also revealed that hsa_circ_0005273 was mostly stained in cytoplasm of BC cell lines (Fig. 1g).

Hsa_circ_0005273 is highly expressed in BC and exerts oncogenic effects in BC cells

The expression of hsa_circ_0005273 was assessed by qRT-PCR in 34 pairs of BC tissues and adjacent normal tissues, and our results showed that hsa_circ_0005273 was significantly elevated in BC tissues (28/34, 82.4%) compared with the matched adjacent normal tissues (Fig. 2a). In addition, the expression of hsa_circ_0005273 was significantly increased in 4 BC cell lines (MDA-MB-231, MCF-7, HCC-1937 and SKBR3) compared to the normal breast epithelial cell line MCF-10A (Fig. 2b). Elevated expression of hsa_circ_0005273 in BC tissues reflected its tumor-promoting role in BC. To investigate the function of hsa_circ_0005273 in BC, we depleted the expression of hsa_circ_0005273 in the MDA-MB-231 and MCF-7 cell lines using a specific siRNA (si-circ_0005273) and took si-NC as a control. The transfection efficiency was verified by qRT-PCR (Fig. 2c). As expected, MTT assays and colony formation

assays demonstrated that hsa_circ_0005273 depletion led to decreased cell proliferation in MDA-MB-231 and MCF-7 cells(Fig. 2d-g). Results from western blotting analysis demonstrated expression of proliferation marker PCNA was inhibited by hsa_circ_0005273 siRNAs(Fig. 2h-i). Moreover, limited migration was seen in the hsa_circ_0005273 low-expression group compared to the controls in wound healing after 48 hours(Fig. 2j-l). Consistently, transwell migration assays hsa_circ_0005273 depletion led to decreased cell migration in MDA-MB-231(Fig. 2m-n). For further verification, we stably overexpressed more than 30-fold hsa_circ_0005273 in MDA-MB-231 and MCF-7 cell lines(Fig. 2o). Then, MTT assays and colony formation assays were performed to examine the effect of hsa_circ_0005273 overexpression, showing that upregulation of hsa_circ_0005273 significantly promoted BC cells proliferation(Fig. 2p-s). In addition, we analysed the relationship between the expression of hsa_circ_0005273 and various clinic pathological variables in 34 patients with BC. We found that the hsa_circ_0005273 high expression was positively associated with TNM stage, lymph node metastasis, tumor size and recrudescence, but had no correlation with age (Table 1).

Table 1

The relationship between the expression of has_circ_0005273 and various clinicopathological variables.

Patients Characteristics	Total	hsa_circ_0005273 expression		
		High (N = 28)	Low (N = 6)	P value*
Age				0.6602
< 60	18	14	4	
≥ 60	16	14	2	
TNM stage				0.0214*
I and II	13	8	5	
III and IV	21	20	1	
Lymph node metastasis				0.0180*
negative	8	4	4	
positive	26	24	2	
Tumor size(cm)				0.0013**
≤ 2	13	7	6	
>2	21	21	0	
Recrudescence				0.0311*
No	20	14	6	
Yes	14	14	0	

Hsa_circ_0005273 Serves As A Sponge For miR-200a-3p

Considering circRNAs have been reported to function as miRNA sponges and that hsa_circ_0005273 was mainly located in the cytoplasm of BC cells, we hypothesized that hsa_circ_0005273 exert oncogenic function through acting as a ceRNA in BC cells. Starbase (<http://starbase.sysu.edu.cn/index.php>), CircInteractome (<https://circinteractome.nia.nih.gov/>), miranda and RNAhybird were employed to predict potential miRNA binding partners of hsa_circ_0005273. With the help of Venn diagram, we chose 5 miRNAs(miR-200a-3p, miR-141-3p, miR-188-5p, miR-198, miR-619-3p) (Fig. 3a-b).The results of qRT-PCR showed that only the expression of miR-200a-3p performed negative correlation with hsa_circ_0005273 expression in MDA-MB-231 and MCF-7 cells, suggesting that hsa_circ_0005273 could bind to miR-200a-3p(Fig. 3c-d). As predicted in starbase,hsa_circ_0005273 and miR-200a-3p had one complementary base sequences(Fig. 3e). To validate whether miR-200a-3p could directly target hsa_circ_0005273, we performed dual luciferase assays. Luciferase reporters indicated that miR-200a-3p significantly inhibited

the luciferase reporter activity compared with the miRNA mimic negative control, suggesting that hsa_circ_0005273 could bind to miR-200a-3p(Fig. 3f).

MiR-200a-3p is expressed at low levels and acts as a tumor suppressor in BC cells

To further explore the role of miR-200a-3p in BC, we detected the expression level of it. The results of qRT-PCR demonstrated that miR-200a-3p performed significantly low expression in BC patient tissues and BC cell lines (Fig. 4a-b). Negative correlations between the expression of hsa_circ_0005273 and miR-200a-3p were found with Pearson's correlation analysis in 34 BC tissue samples(Fig. 4c). The effects of miR-200a-3p on the proliferation ability of BC cells were estimated in MTT assays, colony formation assays and western blotting (Fig. 4d-k). The effects of miR-200a-3p on the migration ability of BC cells were estimated in Wound healing assays and Transwell chambers(Fig. 4l-o). Results above suggested that miR-200a-3p could suppress the proliferation and migration ability of BC cells.

MiR-200a-3p Directly Target YAP1

In accordance with the prediction of TargetScan (http://www.targetscan.org/vert_71/), YAP1 was found to be the potential target of miR-200a-3p(Fig. 5a). By constructing plasmid and mutant vectors containing 3'-UTRs with wild-type and mutant sequences, a dual-fluorescein reporter assay confirmed that YAP1 was the direct target of miR-200a-3p(Fig. 5b). Then, the expression levels of YAP1 were tested in both BC tissues and cell lines through qRT-PCR and the results showed high expression levels of YAP1 in BC(Fig. 5c-d). Furthermore, the negative relevance between miR-200a-3p and YAP1 as well as the positive relevance between has_circ_0005273 and YAP1 were analyzed with Pearson's correlation analysis(Fig. 5e-f).

Hsa_circ_0005273 Upregulated YAP1 Through Targeting miR-200a-3p

Considering the interaction between hsa_circ_0005273 and miR-200a-3p as well as miR-200a-3p and YAP1, we wanted to make a thorough inquiry about whether hsa_circ_0005273 regulates the expression of YAP1. Firstly, we used qRT-PCR to detect the expression of miR-200a-3p and YAP1 in MDA-MB-231 and MCF-7 cells respectively infected with si-circ_0005273 or LV-circ_0005273(Fig. 6a-b). Results of qRT-PCR indicated that the expression of miR-200a-3p increased and the expression of YAP1 decreased after hsa_circ_0005273 was downregulated. Consistently, the expression of miR-200a-3p decreased and the expression of YAP1 increased after hsa_circ_0005273 was upregulated. Secondly, the results of western blotting revealed that the protein levels of YAP1 to be decreased when transfected with si-circ_0005273 and to be increased after transfected with miR-200a-3p-inhibitor in the MDA-MB-231 and MCF-7 cell lines(Fig. 6c-f). To determine whether has_circ_0005273 affects YAP1 via miR-200a-3p and the biological function of has_circ_0005273-miR-200a-3p-YAP1 axis in BC progression, we designed rescue assays in MDA-MB-231 and MCF-7 cell lines. We transfected a combination of both si-circ_0005273 and miR-200a-3p inhibitor to further detect the expression of YAP1 and BC cells proliferation rate. According to the results of MTT assays, decreased cell proliferation induced by the downregulated of has_circ_0005273 was rescued by miR-200a-3p inhibitor, which was consistent with the results of the PCNA(Fig. 6g-h).

Moreover, according to the results of western blotting, the decreased expression of YAP1 induced by si-circ_0005273 were recovered by miR-200a-3p inhibitor(Fig. 6i-l). Based on the results above, we confirmed that hsa_circ_0005273 acted as a oncogene in BC cells via miR-200a-3p/YAP1 axis.

Hsa_circ_0005273 Promotes BC Tumor Growth In Vivo

To further examine the role of hsa_circ_0005273 in BC tumorigenesis, we carried out a xenograft tumor assay in MDA-MB-231 cells stably infected by lentiviral, which expressed lv-sh_circ_0005273 or lv-sh_NC(Fig. 7a). Then, the cells were injected into nude mice(Fig. 7b). The mice tumors were photographed, measured and weighted, indicating that knockdown of hsa_circ_0005273 markedly decreased the tumor volume and weight compared with the negative controls(Fig. 7c-e). The results of xenograft tumor assay further support the oncogenic role of hsa_circ_0005273 in BC tumorigenesis. Next, we extracted protein from the xenograft tumors and expression of YAP1 was detected by western blotting(Fig. 7f). According to the results of western blotting, the expression of YAP1 was decreased in the group with lower expression of has_circ_0005273.

Has_circ_0005273 Inactivated Hippo Signaling Pathway

Considering has_circ_0005273 could play a oncogene role in BC via has_circ_0005273/miR-200a-3p/YAP1 axis and previous studies have reported that inactivation of Hippo pathway led to downregulation of LATS2 and upregulation of YAP1. We conjectured that whether has_circ_0005273 could inactivate Hippo signaling pathway. The downregulated expression level of LATS2 was examined in BC tissues and the negative correlation between LATS2 and has_circ_0005273 was analyzed (Fig. 8a-b). Protein levels and mRNA levels of LATS2 were tested in MDA-MB-231 and MCF-7 cells transfected with si-circ_0005273, showing that LATS2 was upregulated while YAP1 was downregulated(Fig. 8c-e). Taken these together, we confirmed that has_circ_0005273 inactivated Hippo pathway in BC. The mechanism diagram illustrating the mechanism of has_circ_0005273-miR-200a-3p-YAP1-Hippo pathway in BC was generated.(Fig. 8f).

Discussion

CircRNA has a covalently closed ring structure with no 5' caps and 3' ploy-A tails, thus is stable and insensitive to RNase R[26]. Significant proportions of circRNAs are located in the cytoplasm and few are found in the nucleus [26]. The past few decades have witnessed the rapid research progress of circRNAs and a number of circRNAs produced at various genes have been confirmed participate in the development of many diseases, especially in cancers. Considering the stability, long half-life as well as cell type-specific and tissue-specific expression, circRNAs have distinct advantages to act as potential biomarkers of cancer diagnosis and prognosis[27]. Based on the previous studies, circRNAs have been revealed perform in regulating gene expression by sponging miRNAs, interacting with RNA-binding proteins as well as regulating transcription factors. Among which the interaction between circRNAs and miRNAs is the most well-known function pattern. CircRNAs function as miRNA sponges to negatively regulate miRNA, resulting in a reduction in the expression and function of miRNA, further influencing the

expression of the target gene[28]. For example, circSLC8A1 acts as a sponge of miR-130b/miR-494 in suppressing bladder cancer progression via regulating PTEN [29], CircPSMC3 suppresses the proliferation and metastasis of gastric cancer by acting as a competitive endogenous RNA through sponging miR-296-5p [30]. More importantly, studies focused on the relationship between BC and circRNAs also achieved attention. For instance, circRNA_0025202 regulates tamoxifen sensitivity and tumor progression via regulating the miR-182-5p in Breast Cancer[31]. CircTADA2As suppress BC progression and metastasis via targeting miR-203a-3p[32].

This study is the first to report the mechanism and clinical significance of hsa_circ_0005273 in BC. In the present research, we analysed the gene chip GSE113230 in GEO (<https://www.ncbi.nlm.nih.gov/geo/>) and find that the expression of a novel circRNA, termed hsa_circ_0005273, was higher in BC tissues than in adjacent normal tissues. Then we proved its high expression in BC and found it significantly upregulated in human BC and associated with tumor size, TNM stage and recrudescence. Functionally, the knockdown of hsa_circ_0005273 could inhibit the proliferation and migration of BC cells as well as the growth of tumors in vivo. Mechanistically, we demonstrated the hsa_circ_0005273 target to miR-200a-3p, as indicated by the results of the dual luciferase reporter assays. Results from functional experiments and Western blotting showed the correlations between hsa_circ_0005273 and miR-200a-3p as well as miR-200a-3p and YAP1. Rescue assays demonstrated that hsa_circ_0005273 regulated the expression of YAP1 by acting as a sponge for miR-200a-3p. Our findings suggested that the hsa_circ_0005273/miR-200a-3p/YAP1 axis may play a key role in the development of BC and can provide a novel strategy for inhibiting YAP1. YAP1, famous as the downstream gene of Hippo signaling pathway, was demonstrated an oncogene in a considerable number types of cancers. Therefore, we hypothesized that hsa_circ_0005273 could inactivate Hippo signaling pathway. Results showed that the mRNA level and protein level of LATS2 was negatively regulated by hsa_circ_0005273 in BC cells, demonstrating that hsa_circ_0005273 could activate Hippo pathway to function as a cancer-driver in the development of BC. Therefore, the discovery and production of YAP1 inhibitors may be beneficial for BC patients.

Conclusion

In summary, we found that hsa_circ_0005273 is highly expressed and acts as an oncogene in BC. Moreover, hsa_circ_0005273 regulates YAP1 via serving as a sponge of miR-200a-3p, thereby regulating Hippo signaling pathway. Our experimental findings might provide a potential novel biomarker and therapeutic target for BC.

Abbreviations

circRNA: Circular RNA; ncRNA: Non-coding RNA; GEO: Gene Expression Omnibus; YAP1: yes-associated protein; LATS2: Large tumor suppressor 2; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; qRT-PCR: Quantitative real-time polymerase chain reaction;

Declarations

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Availability of data and materials

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

Authors' contributions

XW and LF designed the research. XW, CJ and QL performed the research and analyzed results. XW wrote the paper. JH, XD, WZ, YY, KH, XZ and JL edited the manuscript and provided critical comments.

All authors read and approved the final manuscript.

Ethics approval

Animal experiments were conducted in mice using protocols approved by the Ethics Committee of Shanghai Tenth People's Hospital of Tongji University, and written informed consent was obtained from all patients or their relatives.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 2

The characteristic of hsa_circ_0005273 in BC cells. a Hsa_circ_0005273 is formed by circularization of exon 27-29 of the gene PTK2. b Expression of hsa_circ_0005273 in MDA-MB-231 and MCF-7 cells with random hexamer or oligo (dT)18 primers (**** $p < 0.0001$). c-d RT-PCR analysis of hsa_circ_0005273, linear PTK2 and β-actin in MDA-MB-231 and MCF-7 cells treated with RNase R. e-f Expression levels of cytoplasmic control transcripts (GAPDH), the nuclear control transcript (U6), and hsa_circ_0005273 were determined by qRT-PCR in the cytoplasmic and nuclear fractions of MDA-MB-231 and MCF-7 cells. g RNA FISH for hsa_circ_0005273 and nuclei was stained with DAPI.

Figure 4

Hsa_circ_0005273 is highly expressed in BC and exerts oncogenic effects in BC cells. a Hsa_circ_0005273 was highly expressed in tumor tissues compared with adjacent normal tissues (****p < 0.0001). b Relative expression of hsa_circ_0005273 in MDA-MB-231, MCF-7, HCC-1937 and SKBR3 cell lines (****p < 0.0001, ****p < 0.0001, *p < 0.05, ***p < 0.001). c Expression of hsa_circ_0005273 was confirmed by qRT-PCR in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0005273 (**p <

0.01, ****p < 0.0001). d-g Effect of si-circ_0005273 on proliferation in MDA-MB-231 and MCF-7 cell lines by MTT assay and colony formation assays(****p < 0.0001, ****p < 0.0001, ** p < 0.01, ****p < 0.0001). h-i Effect of si-circ_0005273 on proliferation in MDA-MB-231 and MCF-7 cells by western blotting. j-l Wound healing assays were performed in MDA-MB-231 and MCF-7 cells treated with si-circ_0005273(***p < 0.001, *p < 0.05). m-n Cell migration assays were performed in MDA-MB-231 and MCF-7 cells using Transwell chambers (**p < 0.01). o Expression of hsa_circ_0005273 was confirmed by qRT-PCR in MDA-MB-231 and MCF-7 transfected with LV-vtctor or LV-circ_0005273 (****p < 0.0001, ****p < 0.0001). p-s Effect of LV-circ_0005273 on proliferation in MDA-MB-231 and MCF-7 cell lines by MTT assay and colony formation assays(****p < 0.0001, ****p < 0.0001, ***p < 0.001, * p < 0.05).

Figure 5

Hsa_circ_0005273 serves as a sponge for miR-200a-3p. a-b Venn diagram showing the potential target miRNAs(miR-200a-3p, miR-141-3p, miR-188-5p, miR-198, miR-619-3p) of hsa_circ_0005273. c-d Expression of miRNAs in MDA-MB-231 and MCF-7 cells transfected with si-circ_0005273 or LV-circ_0005273. e Putative complementary sites within miR-200a-3p and hsa_circ_0005273 predicted by bioinformatics analysis (starbase). f Dual luciferase reporter assays demonstrated that miR-200a-3p is a direct target of hsa_circ_0005273 (*p < 0.05).

Figure 7

MiR-200a-3p is expressed at low levels and acts as a tumor suppressor in BC cells. a MiR-200a-3p had low expression in BC tissues compared with adjacent normal tissues (** $p < 0.001$). b MiR-200a-3p had low expression in BC cell lines (** $p < 0.01$, *** $p < 0.001$, *** $p < 0.001$, **** $p < 0.0001$). c Correlations between the expression of hsa_circ_0005273 and miR-200a-3p were found with Pearson's correlation analysis in BC tissue samples ($n = 34$). d-i Effect of miR-200a-3p-inhibitor and miR-200a-3p-mimics on

proliferation in MDA-MB-231 and MCF-7 cell lines by MTT assay and colony formation assays(****p < 0.0001, ****p < 0.0001, ****p < 0.0001, ** p < 0.01, ****p < 0.0001, ****p < 0.0001, ***p < 0.001, ***p < 0.001). j-k Effect of miR-200a-3p-inhibitor on proliferation in MDA-MB-231 and MCF-7 cells by western blotting(**p < 0.01, **p < 0.01). l-m Wound healing assays were performed in MDA-MB-231 cell treated with miR-200a-3p-inhibitor (**p < 0.01). n-o Cell migration assays were performed in MDA-MB-231 cells using Transwell chambers (**p < 0.01)

Figure 9

MiR-200a-3p directly target YAP1. a Putative complementary sites within miR-200a-3p and YAP1 predicted by bioinformatics analysis (TargetScan). b Dual luciferase reporter assays demonstrated that YAP1 is a direct target of miR-200a-3p (****p < 0.0001). c YAP1 had high expression in BC tissues compared with adjacent normal tissues (***p < 0.001). d YAP1 had high expression in BC cell lines (****p < 0.0001, ****p < 0.0001, *p < 0.05, ***p < 0.001). e Positive correlations between the expression of hsa_circ_0005273 and YAP1 were found with Pearson's correlation analysis in BC tissue samples (n = 34). f Negative correlations between the expression of hsa_circ_0005273 and YAP1 were found with Pearson's correlation analysis in BC tissue samples (n = 34).

Figure 12

Hsa_circ_0005273 upregulated YAP1 through targeting miR-200a-3p. a-b the expression of miR-200a-3p increased and the expression of YAP1 decreased after after hsa_circ_0005273 was downregulated by qRT-PCR in MDA-MB-231 and MCF-7 cells. c and e The protein levels of YAP1 to be decreased after transfection of si-circ_0005273 in MDA-MB-231 and MCF-7 cells by Western blot. d and f The protein levels of YAP1 to be increased after transfection of miR-200a-3p inhibitor in MDA-MB-231 and MCF-7

cells by Western blot. g-h MiR-200a-3p-inhibitor rescued the promotive effects of hsa_circ_0005273 in MDA-MB-231 and MCF-7 cells by MTT assay. i-l Western blot showed that miR-200a-3p-inhibitor can partly rescue the low expression of YAP1 and PCNA caused by si-circ_0005273in MDA-MB-231 and MCF-7 cells.

Figure 14

Hsa_circ_0005273 promotes BC tumor growth in vitro. a Hsa_circ_0005273 knockdown efficiency using shRNAs in MDA-MB-231 cells was verified by qRT-PCR. b Representative images of nude mice injected with MDA-MB-231 cells(4 mice per group). c Representative images of xenograft tumors in nude mice. d The growth curves of xenografts (**p < 0.001). e Average tumor weight of nude mice(**p<0.01). f Extract protein from tumors and measuring the expression of YAP1 by Western blot.

Figure 16

Hsa_circ_0005273 inactivated Hippo signaling pathway. a LATS2 had low expression in BC tissues compared with adjacent normal tissues ($**p < 0.01$). b Negative correlations between the expression of hsa_circ_0005273 and LATS2 were found with Pearson's correlation analysis in BC tissue samples ($n = 34$). c-d mRNA levels of LATS2 and YAP1 were tested in MDA-MB-231 and MCF-7 cells transfected with si-circ_0005273 ($****p < 0.0001$, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$). e protein levels of LATS2 and YAP1

were tested in MDA-MB-231 and MCF-7 cells transfected with si-circ_0005273. f The mechanism diagram was generated to illustrate the mechanism of has_circ_0005273-miR-200a-3p-YAP1-Hippo pathway in BC.

Supplementary Files

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