Circ-ABCC1 enhances radioresistance of breast cancer cells via miR-627-5p/ABCC1 axis

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ABSTRACT
Adjuvant radiotherapy represents the standard of care for breast cancer (BC) following breast-conserving surgery. Tumor recurrence after radiotherapy, attributed to acquired radioresistance, has been a haunting and intractable problem. Therefore, preventions from tumor recurrence are vital for improving survival. Recent evidence has suggested circular RNAs (circRNAs) play a part in regulating the radioresistance of varied cancers, including BC. This research concentrated on a novel circRNA hsa_circ_0003427 (termed as circ-ABCC1), probing its influence on the radioresistance of BC cells, together with the latent molecular mechanism. For this aim, CCK-8 and colony formation assays monitored the changes in viability and proliferation of radio-resistant BC cells. Caspase-3 activity was examined to evaluate cell apoptosis. Bioinformatics prediction and mechanistic assays were involved to determine RNA interactions. Results showed that Circ-ABCC1 was found to be significantly up-regulated in radio-resistant BC cells, in comparison with the corresponding parental BC cells. As to molecular mechanism, circ-ABCC1 served as the miR-627-5p decoy, consequently increasing ABCC1 expression. Rescue assays uncovered that the suppressive impact of circ-ABCC1 silence on BC cell radioresistance was allowed to be antagonized by miR-627-5p inhibition or ABCC1 up-regulation. In conclusion, Circ-ABCC1 aggravates the radioresistance of BC cells by targeting the miR-627-5p/ABCC1 axis.

Introduction
As the most commonly diagnosed malignancy, breast cancer (BC) ranks second triggering cancer-related death among women (1). Contemporarily, breast-conserving therapy represents the solid achievement of modern cancer care. For patients with early-stage BC, adjuvant radiotherapy is provided as the standard of care following breast-conserving surgery. Recent evidence has also indicated an increasing role of regional nodal irradiation in BC patients at more advanced stages (2, 3). Nevertheless, BC patients who have completed a course of radiotherapy are peculiarly prone to locoregional recurrence and distant metastasis on account of cellular radioresistance (4). A noting proportion of BC patients (approximately 33.8% in 1-3 node-positive BC patients) relapse within 10 years following radiotherapy because of radioresistance, which results in an unfavorable overall survival (5). Existing research work has indicated that the development of radioresistance is a process of enormous complexity involving multiple factors, for instance, the alterations in oncogenes or tumor suppressor gene expression (6). Given that, it is a prime target to understand the underlying mechanism responsible for radioresistance, which will benefit in improving BC patients survival.

Recently, circular RNAs (circRNAs) have been gradually recognized to play an essential part in cancer progression (7). For instance, circ_0011058 has been corroborated to contribute to the proliferation, angiogenesis as well as radioresistance of papillary thyroid cancer cells, which enlarges the knowledge of circRNA-regulated radioresistance (8). As reported by Sang et al., circRNA_0025202 modulates Tamoxifen sensitivity and tumor progression in BC (9). CircRNF20 has also been identified to induce BC tumorigenesis and the Warburg effect (10). Based on previous literature, the diverse biological effects of circRNAs, along with their molecular mechanisms, especially regarding radioresistance, have not been fully understood in BC. Accordingly, this study concentrated on a novel circRNA hsa_circ_0003427 whose biological influences on BC cell growth and radioresistance remained nebulous.

CircRNAs possess covalently closed structures, which are generated through precursor messenger RNAs (mRNAs) through back-splicing of exons (11). Current evidence has revealed the regulatory relationship between circRNAs and their host genes in varied cancer progression. For example, circAMOTL1 has been verified to elevate the expression of its host gene AMOTL1 via functioning as a competing endogenous RNA (ceRNA) in cervical cancer growth (12). CircRNAs have also been widely approved to contribute to BC progression through the ceRNA regulation network, in which circRNAs bind with microRNAs...
(miRNAs) to indirectly up-regulate downstream mRNAs (13-15).

In brief, this research aimed to unveil the biological impact of hsa_circ_0003427 (derived from its host gene ABCC1 and hereinafter called circ-ABCC1) on the radioresistance of BC cells and to further probe whether there existed a ceRNA regulatory mechanism involving circ-ABCC1 and its host gene ABCC1. Our findings are expected to enrich the understanding of the molecular mechanism underlying radioresistance in BC and provide novel promising biomarkers for improving BC treatment.

Materials and Methods

Cell culture

BC cell lines (MCF7 and MDA-MB-231) were obtained from the Cell Bank of the Chinese Academy of Science. MCF-7 cells were cultivated in DMEM containing 10% fetal bovine serum (FBS). MDA-MB-231 cells were incubated in a Leibovitz medium with the addition of 10% FBS. The human embryonic kidney 293T (HEK293T) cell line offered by National Institutes for Food and Drug Control was grown in RPMI-1640 medium added with 10% FBS and 2 mM glutamine. The culture medium was also added with 100 units/ml of penicillin and 100 mg/ml of streptomycin. The culture dishes involved were placed in the incubator having an atmosphere of 37°C and 5% CO₂.

Establishment of radio-resistant BC cells

Radio-resistant BC cells (labeled as MCF7-R and MDA-MB-231-R) were established based on previous descriptions (4, 16). In brief, parental MCF-7 cells were treated with 6 MV X-rays irradiation at a rate of 3 Gy/min. As to one cycle, MCF-7 cells were subjected to irradiation at 5 Gy twice a week, along with a 3-6-week recovery period. Following seven-cycle irradiation (accumulated dose 70 Gy), radio-resistant MCF-7 cells were established. Parental MDA-MB-231 cells were incubated till reaching 70-80% cell confluence. Subsequently, irradiations were conducted at a dose rate of 1.25 Gy/min. Following a 25-cycle of 2Gy irradiation for five weeks, the remaining cells were involved in the following investigation.

Plasmid transfection

The short hairpin RNA (sh-RNA) specifically targeting circ-ABCC1 (sh-circ-ABCC1), along with the negative control (sh-NC), was prepared ahead. The pcDNA3.1 vectors inserted with circ-ABCC1 or ABCC1 sequence were generated as well. The empty vector pcDNA3.1 was involved as an NC vector. Additionally, miR-627-5p mimics/inhibitor was made for miR-627-5p augmentation/inhibition, respectively. After the 24-hour cell culture, the cell transfection was completed utilizing Lipofectamine 3000. The supplier’s guidelines were strictly observed. Functional assays were performed following 48-hour transfection.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA extracted from cell samples with the application of Trizol reagent was first subjected to cDNA synthesis with the application of a reverse transcription kit. The user’s manual was carefully referred. Subsequently, the RNA expression level was monitored utilizing a qRT-PCR kit. The 2^(-ΔΔCt) method was involved in calculating the relative gene expression. As for the internal reference to miRNAs, U6 was involved in the research, while to circRNAs and mRNAs, GAPDH was applied.

Cell counting kit-8 (CCK-8) assay

Cell viability was examined via this assay. Cell samples were plated into 96-well plates, following X-ray irradiation at 0Gy or 3Gy. Seventy-two hours later, CCK-8 reagent was added into the culture plate, followed by a 2-hour incubation. Ultimately, the optical density (OD) at 450 nm was monitored utilizing a microplate reader.

Colony formation assay

Cell samples stably transfectsed with indicated plasmids were plated into 6-well plates, followed by two-week incubation. Following PBS washing and paraformaldehyde (PFA) fixation, the cell colonies were stained after using 0.5% crystal violet. Ultimately, the formed cell colonies were counted manually.

Caspase-3 activity assay

Caspase-3 Activity Assay Kit was applied in this assay as per the supplier’s guidelines. First of all, the total protein was extracted from the cell samples with the use of a lysis buffer. The obtained protein extracts were incubated with the reaction buffer containing caspase3 substrate. In the end, the caspase-3 activity was monitored at 405 nm wavelength employing a microplate reader.

Luciferase reporter assay

For the investigation into the regulation of circ-ABCC1 on ABCC1 expression at the transcriptional level, pGL3 plasmids containing the sequence of ABCC1 promoter were prepared ahead, followed by the co-transfections with pcDNA3.1 or pcDNA3.1/circ-ABCC1 into HEK293T cells. For the investigation into the post-transcriptional regulation of circ-ABCC1 on ABCC1 expression, the pmirGLO vectors containing ABCC1 3’UTR sequence were constructed and subsequently co-transfected with sh-NC or sh-circ-ABCC1#1 into HEK293T cells.

In addition, this assay probed the RNA interactions as well as the effectiveness of binding sites predicted from the online database. The sequence of circ-ABCC1 covering the wild-type (WT)/mutated (MUT) binding sites on miR-627-5p was synthesized and labeled as pmirGLO-circ-ABCC1-WT/MUT. In the same way, pmirGLO-ABCC1-WT/MUT vectors containing WT/MUT binding sequence of ABCC1 on miR-627-5p were constructed as well. The aforementioned plasmids, along with mimics NC/miR-627-5p mimics, were co-transfected into HEK293T cells. Following 48-hour transfection, a Luciferase Reporter Gene Assay kit was employed for examining the luciferase activities.

Subcellular fractionation assay

The obtained cell lysates were centrifuged, followed by the separation of cytoplasmic and nuclear parts of BC cells. Afterward, RNA levels of circ-ABCC1, GAPDH (cytoplasmic reference) and U6 (nuclear reference) in different subcellular parts were examined through RT-qPCR.

RNA binding protein immunoprecipitation (RIP) assay

Cell samples were lysed following the treatment of RIP...
lisis buffer. Subsequently, the obtained cell lysates were incubated with the indicated primary antibodies (Anti-IgG and Anti-Ago2), together with magnetic beads. Subsequent to immunoprecipitation, RNAs enriched in the complexes were eluted and purified for quantification via RT-qPCR.

**RNA pull-down assay**

Above all, biotinylated(Bio)-circ-ABCC1, Bio-ABCC1, or control probe Bio-NC was synthesized. The abovementioned biotin-labeled probes were transfected into the cell samples. Subsequent to 48-hour incubation, the cells were collected and washed with PBS. After a 10-minute treatment of lysis buffer, the cell lysates were obtained, followed by further incubation with M-280 streptavidin beads at 4°C overnight. Eventually, RNA-RNA complexes were gathered and purified. The enrichment of pull-down miRNAs was analyzed via RT-qPCR.

**Statistical analysis**

Each independent experiment was carried out thrice. All gathered data were processed and analyzed with the application of SPSS 21.0 software. The quantitative data were presented in the manner of mean ± standard deviation. With regard to statistical differences between the two groups, the student’s t-test was employed. In terms of differences among three or more groups containing one or two variables, one-way or two-way analysis of variance was employed. *P < 0.05* implied that data difference was statistically significant.

**Results**

**Circ-ABCC1 knockdown suppresses BC cell radioresistance**

This research concentrated on circ-ABCC1, attempting to probe its function in the radioresistance of BC cells, together with its latent molecular mechanism. At the very beginning, we detected the expression profile of circ-ABCC1 (Figure 1A), noticing a significant uplift of circ-ABCC1 expression in the radio-resistant BC cells, compared with the corresponding parental BC cells. Accordingly, we silenced circ-ABCC1 in the radio-resistant BC cells ahead (Figure 1B), making preparation for the subsequent loss-of-function assays. As presented in Figure 1C-D, we observed that viability and proliferation of the radio-resistant BC cells were suppressed upon circ-ABCC1 silencing. Noteworthily, the such inhibitory impact was much more significant to be seen in the radio-resistant BC cells treated with 3Gy irradiation. On the contrary, the increase of caspase-3 activity represented that circ-ABCC1 deficiency conduced to the facilitated apoptosis of radio-resistant BC cells. After the irradiation at 3Gy, the influence of circ-ABCC1 depletion on the cell apoptosis was much more stimulated, relative to the cells upon 0Gy treatment (Figure 1E). To conclude, circ-ABCC1 demonstrates a high expression in radio-resistant BC cells, and its down-regulation contributes to restraining the radioresistance of BC cells.

**Circ-ABCC1 up-regulates ABCC1 to strengthen the radioresistance of BC cells**

Increasing evidence has suggested that circRNA is allowed to regulate tumor progression via up-regulating its host gene (17-19). Accordingly, circ-ABCC1 might be able to modulate ABCC1 expression, functionally affecting the radioresistance of BC cells. Our speculation was initially supported by the following RT-qPCR outcomes that circ-ABCC1 silence led to a decline of ABCC1 expression in radio-resistant BC cells (Figure 2A). Furthermore, we excluded the transcriptional regulation of circ-ABCC1 on ABCC1 expression, since the luciferase activity of the ABCC1 promoter was hardly impacted in responding to circ-ABCC1 down-regulation (Figure 2B). Not of note, the luciferase activity of ABCC1 3’UTR was significantly diminished in responding to circ-ABCC1 down-regulation, which meant that circ-ABCC1 could modulate ABCC1 expression at the post-transcriptional level (Figure 2C). Moreover, we noticed that ABCC1 increment antagonized the suppressive impact of circ-ABCC1 silence on the viability and proliferation of the radio-resistant BC cells (Figure 2D-E). Caspase-3 activity detection illustrated the promoting impact of circ-ABCC1 depletion on radio-resistant BC cell apoptosis was counterbalanced responding to ABCC1 up-regulation (Figure 2F). In brief, circ-ABCC1 aggravates the radioresistance of BC cells through increasing ABCC1 expression.

**Circ-ABCC1 sequesters miR-627-5p to up-regulate ABCC1**

Currently, cytoplasmic circRNA has been identified to function as miRNA sponges, further affecting downstream target expression (20, 21). Ahead of the specific mecha-

![Figure 1. Circ-ABCC1 silencing enhances radio-sensitivity of radio-resistant BC cells. (A) RT-qPCR detected circ-ABCC1 expression in parental and radio-resistant BC cells. (B) RT-qPCR tested the knockdown efficacy of sh-circ-ABCC1 in radio-resistant BC cells. (C-D) CCK-8 and colony formation assays monitored viability and proliferation of radio-resistant BC cells upon circ-ABCC1 deficiency. (E) Caspase-3 activity detection assessed the apoptosis of radio-resistant BC cells responding to circ-ABCC1 depletion. *P < 0.05, **P < 0.01.](image-url)
nism analysis, we determined the cytoplasmic distribution of circ-ABCC1 at first (Figure 3A), which made the ceRNA regulation mode tenable in BC cells. Furthermore, the RIP assay uncovered that circ-ABCC1, along with ABCC1, was detected in Anti-AGO2, the kernel of miRNA-induced RNA-induced silencing complex (RISC) (Figure 3B). Given that, we projected common miRNAs potentially binding with circ-ABCC1 and ABCC1 from the starBase website (https://starbase.sysu.edu.cn/). Upon the indicated criteria (CLIP Data: strict stringency; Degradoome Data: high stringency), hsa-miR-122-5p and hsa-miR-627-5p stood out (Figure 3C). Ultimately, miR-627-5p was decided to be the right common miRNA involved in the following investigation, considering that miR-627-5p could be pulled down by Bio-circ-ABCC1 as well as Bio-ABCC1 (Figure 3D). From starBase, the putative binding sites between circ-ABCC1 and miR-627-5p were acquired (Figure 3E). Moreover, the combination relationship and the effectiveness of the binding sites were further supported, since the luciferase activity of circ-ABCC1-WT, instead of circ-ABCC1-MUT, was reduced in response to miR-627-5p augmentation (Figure 3F). In the same way, we performed a luciferase activity assay and verified their binding affinity and sequences as well (Figure 3G-H). Additionally, RT-qPCR analysis indicated that the reduction of ABCC1 expression caused by circ-ABCC1 knockdown was allowed to be restored by miR-627-5p inhibition (Figure 3I). To sum up, circ-ABCC1 acts as the miR-627-5p sponge to elevate ABCC1 expression in BC cells.

Circ-ABCC1 intensifies BC cell radioresistance via competitively binding with miR-627-5p

To further probe the co-effect of circ-ABCC1 and miR-627-5p on the radioresistance of BC cells, we conducted the functional assays in a rescue manner. Based on the data collected, the repressed viability and proliferation resulting from circ-ABCC1 depletion were recovered because of miR-627-5p inhibition (Figure 4A-B). Moreover, we concluded that the inhibiting influence of circ-ABCC1 down-regulation on the cell apoptosis could be abrogated by responding to miR-627-5p repression, as the lowered caspase-3 activity caused by circ-ABCC1 deficiency was seen as a significant upturn following miR-627-5p inhibitor transfection (Figure 4C). In short, circ-ABCC1 reinforces the radioresistance of BC cells via sponging miR-627-5p.

Discussion

Of all female cancer-related death, the mortality resul-
Circ-ABCC1 is a vital modulator implicated in the radio-sensitivity of lung cancer cells are facilitated (25). CircPITX1 works as a miR-329-3p sponge to prompt NEK2 expression (26). Up to now, limited research work has been published to unveil the molecular mechanism underlying radioresistance in BC. As verified by Zhao et al., circ-ABCC1#1 is allowed to enhance radioresistance in BC via serving as the miR-223-3p sponge and positively regulating downstream PIFN2 expression (27). Herein, we revealed a novel circRNA-miRNA-mRNA ceRNA axis engaging in the regulation of BC cell radioresistance. Specifically, circ-ABCC1 could bind with miR-627-5p to elevate ABCC1 expression. The inhibitory impact of circ-ABCC1 depletion on the radioresistance of BC cells could be abrogated after miR-627-5p inhibition or ABCC1 overexpression.

In conclusion, this study represents circ-ABCC1 manifested a high expression level in radioresistant BC cells and its down-regulation lowers BC cell radioresistance. The novelty of this work is that we first determine the positive and indirect regulation of circ-ABCC1 on its host gene ABCC1. Briefly, Circ-ABCC1 aggravates the radioresistance of BC cells through decoying miR-627-5p and inducing ABCC1 up-regulation. Clinical samples and in vivo studies are not involved in this study. Therefore, our follow-up investigation will be discussing more clinical implications concerning the novel pathway we currently discovered.

Figure 4. Circ-ABCC1 aggravates the radioresistance of BC cells by sequestering miR-627-5p. Radio-resistant BC cells were transfected with indicated plasmids, including sh-NC, sh-circ-ABCC1#1, and sh-circ-ABCC1#1+miR-627-5p inhibitor. (A-B) CCK-8 and colony formation assays detected the viability and proliferation of radio-resistant BC cells with different transfections. (C) Caspase-3 activity assay monitored the alterations of radio-resistant BC cell apoptosis upon indicated conditions. *P < 0.05, **P < 0.01.

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References

12. Ou R, et al., circAMOTL1 Motivates AMOTL1 Expression to


