Original Article
High intensity focused ultrasound inhibits breast cancer cell proliferation and promotes cell apoptosis via miR-222-3p/p27Kip1 axis

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Abstract: Background: High intensity focused ultrasound (HIFU) is a novel therapeutic way to treat solid tumors without damaging the surrounding tissues. However, the mechanism that underlies the effect of HIFU on breast cancer progression is largely unclear. Methods: Cell proliferation was measured after treatment of HIFU by MTT, colony formation, flow cytometry or western blot, respectively. Cell apoptosis was analyzed after treatment of HIFU by flow cytometry, Caspase 3 activity analysis and western blot assay of apoptosis-related protein. The level of miR-222-3p was examined in breast cancer cells. The association between p27Kip1 and miR-222-3p was explored via luciferase activity. Murine xenograft experiment was performed to investigate the function of HIFU on breast cancer growth. Results: The treatment of HIFU inhibited proliferation and triggered apoptosis of breast cancer cells. After treatment of HIFU, miR-222-3p was declined and p27Kip1 protein was up-regulated. P27Kip1 was validated to be targeted via miR-222-3p. Up-regulation of miR-222-3p restored proliferation and restrained apoptosis in HIFU-treated breast cancer cells, which was reversed by introduction of p27Kip1. In addition, treatment of HIFU suppressed tumor growth by inhibiting miR-222-3p and increasing p27Kip1. Conclusion: Treatment of HIFU inhibited proliferation and contributed to apoptosis by regulating miR-222-3p and p27Kip1 in breast cancer, indicating novel theoretical foundation for application of HIFU in the treatment of breast cancer.

Keywords: Breast cancer, HIFU, miR-222-3p, p27Kip1, proliferation, apoptosis

Introduction
Breast cancer is a common disorder for women with high incidence [1]. In the past few decades, great advance has been achieved in understanding the development of breast cancer, including risk factors, signaling pathways and molecular pathogenesis [2]. Patients with breast cancer exhibit better survival possibly due to its unnecessariness for human survival, while the breast surgery may endanger their health because of the mental and emotional disturbances. Hence, new strategies for therapeutic intervention of breast cancer are wanted.

High intensity focused ultrasound (HIFU) is a novel way inducing coagulative necrosis at precise focal point without damaging the adjacent structures, which has been increasingly applied to treat solid tumors [3]. Multiple evidences have reported the application of HIFU as new ablative therapeutics of many advanced cancers, such as glioma, liver cancer and pancreatic adenocarcinoma [4-6]. Importantly, apart from the standard breast conserving surgery, HIFU has been indicated as a novel ablative technique to improve treatment of breast cancer [7]. Nevertheless, the mechanism underlying HIFU influence on breast cancer progression remains largely unknown.

The networks of microRNAs (miRNAs) and their targeted complementary mRNAs have been suggested as main molecular pathways in numerous types of cancer, including breast cancer [8]. Moreover, miRNAs (~22 nucleotides) exhibit pivotal roles in therapy of breast cancer [9]. A previous study reports that miR-222 could promote drug-resistance of breast cancer cells via modulation of protein kinase B/forkhead box O1 (FOXO1) signaling pathway.
Moreover, Zong et al. have reported miR-222 as an oncogene to facilitate tumor growth and repress apoptosis in breast cancer [11]. Wang et al. have revealed that miR-222 enhances the Adriamycin resistance of breast cancer cells by inhibiting p27Kip1 [12]. P27Kip1 is a negative mediator of cell cycle, which is correlated with varying women’s diseases [13]. In addition, He et al. revealed that imbalance of p27Kip1 is involved in breast carcinogenesis [14]. However, whether miR-222-3p (a main mature form of miR-222) and p27Kip1 is implicated in the anti-cancer role of HIFU in breast cancer remains undetermined. In this work, we analyzed the function of HIFU on proliferation and apoptosis of breast cancer cells and explored the association between HIFU and miR-222-3p/p27Kip1 axis.

Materials and methods

Cell culture and HIFU treatment

MCF-7 and MDA-MB-468 cells were provided by BeNa Culture Collection (Beijing, China). The cells grew in DMEM (Solarbio, Shanghai, China) with 10% fetal bovine serum (Thermo Fisher, Wilmington, DE, USA), and 1% penicillin/streptomycin (Thermo Fisher) in 5% CO₂ at 37°C. For HIFU exposure, cells were added into polyethylene centrifuge tube and then sonicated for 0, 2, 4, 6, 8 or 10 s at 142.7 W/cm² under the HIFU therapeutic apparatus (Haifu medical technology, Chongqing, China).

Cell transfection

P27Kip1 overexpression vector (p27Kip1), pcDNA empty vector (vector), mimic negative control (miR-NC) and miR-222-3p mimic were generated by Genomeditech (Shanghai, China). These oligonucleotides or vectors were transfected into MDA-MB-468 and MCF-7 cells using Lipofectamine 2000 (Thermo Fisher) for 24 h.

Cell viability

Following the exposure of HIFU, 1 x 10⁴ cells were plated into 96-well plates. At ending point, cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution (Beyotime, Shanghai, China) for 4 h. Next, the formazan was dissolved by 100 μl DMSO (Solarbio). The absorbance was examined through a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. The relative viability was normalized to control group.

Colony formation

After the treatment of HIFU, cells (600 cells/well) were cultured in 6-well plates for 10 d. Clones were fixed and stained with 0.05% crystal violet (Solarbio). A microscope (Olympus, Tokyo, Japan) was applied to observe the colony formation.

Cell cycle analysis

After the treatment of HIFU for 24 h, cells were fixed with ethanol and then incubated with propidium iodide (PI; Solarbio) for 25 min. Cells at different phases were detected with a flow cytometer (Agilent, Hangzhou, China). The entire experiment was repeated 3 times.

Western blot

Total proteins were isolated with RIPA buffer (Beyotime) and the concentration was detected via BCA protein assay kit (Solarbio). 20 μg proteins were heated at 100°C for 5 min and then separated via SDS-PAGE, followed by membrane transfer with polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). 5% skim milk was exploited to block the membranes. And then the membranes were interacted with primary antibodies overnight at 4°C and secondary antibody conjugated with horseradish peroxidase. The antibodies against CDK2 (#2546, 1:1000 dilution), Cyclin E (#20808, 1:2000 dilution), Cleaved Caspase 3 (#9664, 1:500 dilution), Bcl-2 (#4223, 1:1000 dilution), Bax (#5023, 1:1000 dilution), p27Kip1 (#2552, 1:1000 dilution), β-actin (#4970, 1:2000 dilution) and secondary antibody (#5127, 1:5000 dilution) were provided by Cell Signaling Technology (Danvers, MA, USA). The protein blot was developed by ECL chromogenic substrate (Beyotime) and the levels of proteins were shown as the ratio of targeted protein and β-actin.

Cell apoptosis

The flow cytometry was applied to determine cell apoptosis with Annexin V-FITC/PI kit (Solarbio). After treatment of HIFU for 24 h, MCF-7 and MDA-MB-468 cells were harvested, resus-
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pended in binding buffer, and incubated with 5 μl Annexin V-FITC and 5 μl PI for 10 min. The stained cells were examined via flow cytometer. The sample of each group was prepared in triplicate.

Caspase 3 activity analysis

The two cell lines of each group were harvested and incubated in lysis buffer. After the centrifugation at 600 g for 3 min, the supernatant was collected for caspase 3 activity analysis using a caspase 3 assay kit (Beyotime) following the instructions of manufacturer.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted via TRIzol reagent (Solarbio) and applied to cDNA synthesis using the miRNA first-strand cDNA synthesis kit (Fulengen, Guangzhou, China). The qRT-PCR was carried out with the miRNA qRT-PCR detection kit (Fulengen) with the procedure: 95°C for 5 min, and 35 cycles of 95°C for 20 s and 60°C for 30 s. The special primers of miR-222-3p (F: 5'-GGGGAGCTACATCTGGCT-3', R: 5'-TGCGTGTCGTGGAGTC-3') and U6 (F: 5'-CACCACGUUUAUCACGCAGUG-3', R: 5'-CGCTTCACGAATTTCGCTGTCA-3') were generated by Sangon (Shanghai, China). The relative abundance of miR-222-3p was detected with U6 as a control using 2^ΔΔCt method [15].

Luciferase activity assay

MDA-MB-468 and MCF-7 cells were co-transfected with pmirGLO constructs and miR-222-3p or miR-NC. The pmirGLO constructs carrying the 3’-UTR sequences of p27Kip1 with the wild-type (Wt) or mutant (Mut) miR-222-3p seed sites were obtained via pmirGLO vectors (Promega, Madison, WI, USA). After 48 h of post-transfection, luciferase activity was examined using a dual-luciferase assay kit (Promega).

Murine xenograft model

BALB/c nude mice (female, 4-week-old) were subcutaneously injected with MCF-7 cells (5 × 10⁶/mouse). Tumor volume was examined every three days using a formula (volume (mm³) = width (mm)² × length (mm)/2). When the length reached 8-10 mm (at 12 d), the mice were randomly divided into HIFU or sham-HIFU (Ctrl) group (n=6 per group). The treatment of HIFU was administered to tumor nodule for 100 s with a safety distance of 1 mm from tumor margin to avoid injury in adjacent tissues. The Ctrl group was treated with a sham HIFU procedure. The experiments have been approved by the Animal Research Committee of Xingtai People’s Hospital. After 30 d following the inoculation, the mice were euthanized and tumor tissue samples were weighed. Then the samples were collected for detection of miR-222-3p and p27Kip1 protein levels.

Statistical analysis

The experiments were conducted 3 times. Data were shown as the mean ± standard deviation. The difference was analyzed by Student’s t-test or one-way ANOVA. It was statistically significant when P value < 0.05.

Results

HIFU exposure inhibits proliferation of breast cancer cells

To assess the function of HIFU in breast cancer, the proliferative ability of MCF-7 and MDA-MB-468 cells was measured after treatment of HIFU. As shown in Figure 1A and 1B, cell viability was obviously reduced after exposure of HIFU in a time dependent manner. The IC₅₀ of HIFU at 142.7 W/cm² was 5.266 s or 5.643 s in MCF-7 and MDA-MB-468 cells, respectively. Hence, cells with a 6 s-exposure of HIFU were used for further experiments. After the treatment of HIFU for 6 s, cell viability was obviously inhibited in the two cell lines (Figure 1C and 1D). Furthermore, exposure of HIFU greatly suppressed colony formation in the two cell lines (Figure 1E and 1F). In addition, flow cytometry analysis revealed that the proportion of cells at G0/G1 stage was notably enhanced in the two cell lines after exposure of HIFU (Figure 1G). Besides, data of cell cycle-related protein expressions demonstrated that treatment of HIFU markedly decreased the protein levels of CDK2 and Cyclin E (Figure 1H and 1I).

HIFU treatment promotes cell apoptosis in breast cancer cells

Moreover, cell apoptosis was examined in the two breast cancer cell lines after exposure of HIFU. Analysis of flow cytometry revealed ele-
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vated apoptotic rate in HIFU-treated cells in comparison to that in Ctrl group (Figure 2A and 2B). Furthermore, treatment of HIFU obviously enhanced the activity of Caspase 3 (Figure 2C). Additionally, the great increase of cleaved Caspase 3 and Bax protein levels and loss of Bcl-2 expression were displayed in the two cell lines after exposure of HIFU (Figure 2D and 2E).

HIFU regulates miR-222-3p and p27Kip1 expressions

To elucidate the mechanism underlying HIFU’s effect on breast cancer, the promising targeted miRNA and mRNA were probed. After treatment of HIFU, MCF-7 and MDA-MB-468 cells showed low expression of miR-222-3p in comparison to those in Ctrl group (Figure 3A). Moreover, bioinformatics analysis predicted two regions of p27Kip1 (CDKN1B) 3'-UTR with the seed sites of miR-222-3p by TargetScan (Figure 3B). To confirm this prediction, the Wt or Mut luciferase reporter vector was constructed. The luciferase reporter assay uncovered that addition of miR-222-3p remarkably decreased the luciferase activity of p27Kip1-Wt reporter vector, while its efficacy was weakened via the mutant of putative binding sites 1 or 2 and even lost in combined mutant of station 1 and 2 group (Figure 3C and 3D). Additionally, the function of miR-222-3p on p27Kip1 protein level was assessed in breast cancer cells. Results displayed that addition of miR-222-3p induced an obvious loss of p27Kip1 protein abundance (Figure 3E). Besides, the level of p27Kip1 was remarkably increased by treatment of HIFU in the two cell lines, which was abated by introduction of miR-222-3p (Figure 3F).
miR-222-3p restores cell proliferation by targeting p27Kip1 in HIFU-treated breast cancer cells

To further elucidate the potential mechanism, cells were transfected with miR-NC, miR-222-3p, miR-222-3p and vector or p27Kip1 and then treated with HIFU. After the transfection, overexpression of miR-222-3p enhanced the viability of MCF-7 and MDA-MB-468 cells after treatment of HIFU, which was abated by introduction of p27Kip1 (Figure 4A and 4B). Moreover, colony formation was promoted by addition of miR-222-3p in HIFU-treated cells, while it was attenuated by restoration of p27Kip1 (Figure 4C). The analysis of cell cycle distribution revealed that miR-222-3p delayed HIFU-induced arrest of cell cycle at G0/G1 stage, whereas introduction of p27Kip1 weakened this effect (Figure 4D and 4E). Western blot assay revealed that abundant accumulation of miR-222-3p resulted in down-regulation of p27Kip1 as well as up-regulation of CDK2 and Cyclin E in HIFU-treated cells, which was alleviated by introduction of p27Kip1 (Figure 4F-I).

miR-222-3p attenuates apoptosis via regulating p27Kip1 in HIFU-treated breast cancer cells

Additionally, we evaluated whether miR-222-3p was involved in HIFU-induced apoptosis. Addi-
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The exposure of HIFU regulates the expressions of miR-222-3p and p27Kip1 in breast cancer cells. A. The expression of miR-222-3p was measured in MCF-7 and MDA-MB-468 cells after treatment of HIFU. B. The potential binding sites of miR-222-3p and p27Kip1 (CDKN1B) were predicted by TargetScan. C and D. Luciferase activity was analyzed in MCF-7 and MDA-MB-468 cells co-transfected with miR-222-3p or miR-NC and p27Kip1-Wt, p27Kip1-Mut-1, p27Kip1-Mut-2 or p27Kip1-Mut-1/2. E. The protein level of p27Kip1 was detected in MCF-7 and MDA-MB-468 cells transfected with miR-222-3p or miR-NC. F. The effect of HIFU treatment on p27Kip1 protein abundance was investigated in MCF-7 and MDA-MB-468 cells. *P < 0.05.

Figure 3. The exposure of HIFU regulates the expressions of miR-222-3p and p27Kip1 in breast cancer cells. A. The expression of miR-222-3p was measured in MCF-7 and MDA-MB-468 cells after treatment of HIFU. B. The potential binding sites of miR-222-3p and p27Kip1 (CDKN1B) were predicted by TargetScan. C and D. Luciferase activity was analyzed in MCF-7 and MDA-MB-468 cells co-transfected with miR-222-3p or miR-NC and p27Kip1-Wt, p27Kip1-Mut-1, p27Kip1-Mut-2 or p27Kip1-Mut-1/2. E. The protein level of p27Kip1 was detected in MCF-7 and MDA-MB-468 cells transfected with miR-222-3p or miR-NC. F. The effect of HIFU treatment on p27Kip1 protein abundance was investigated in MCF-7 and MDA-MB-468 cells. *P < 0.05.

Treatment of HIFU suppresses tumor growth via regulating miR-222-3p and p27Kip1 expression

To better understand the effect of HIFU in breast cancer, we established MCF-7 xenograft model with HIFU treatment. After 30 days following the inoculation, the tumor volume was...
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![Figure 4](image)

**Discussion**

Recent advance of HIFU has raised its popularity in clinical application for therapy of different cancers, including pancreas, prostate, liver, kidney, and breast cancer [16]. What’s more, Peek et al. have revealed that HIFU exposure could induce coagulative necrosis in breast cancer [17]. Nevertheless, the function of HIFU and its potential mechanism remain elusive. In the present research, we analyzed the effect of HIFU treatment on breast cancer cell proliferation and apoptosis and explored the underlying mechanism.

Cyclin E and CDK2 are responsible for cell cycle process through G1 phase into S phase, lack of which prevents cells from entry into S phase [18]. In this research, treatment of HIFU decreased the expressions of CDK2 and Cyclin E protein, indicating that HIFU prevents breast cancer cells from entry into S stage, which is in agreement with the data of flow cytometry analysis.
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Figure 5. miR-222-3p attenuates apoptosis by regulating p27Kip1 in HIFU-treated breast cancer cells. Cell apoptosis (A), Caspase 3 activity (B) and related protein levels (C-F) were detected in MCF-7 and MDA-MB-468 cells transfected with miR-NC, miR-222-3p, miR-222-3p and vector or p27Kip1 after exposure of HIFU. *P < 0.05.

analysis that disclosed increase of cells at G0/G1 stage and loss of proportion at S stage. Moreover, HIFU treatment led to reduction of cell viability and colony formation in the two breast cancer cell lines. These findings reflected that HIFU treatment inhibited breast cancer cells proliferation. Besides, flow cytometry, Caspase 3 activity and pro-apoptotic or anti-apoptotic protein expression assays uncovered that HIFU treatment resulted in increase of apoptosis of breast cancer cells. However, little is known about how HIFU suppresses breast cancer progression. The former work showed that treatment of HIFU could induce dysregulation of miRNAs. For instance, Li et al. suggested that HIFU repressed cell migration and metastasis by regulating miR-21/PTEN/AKT pathway in melanoma [19]. Yuan et al. reported that HIFU treatment might increase anti-tumor immunity by regulating miR-134 and CD86 in melanoma [20]. Moreover, they showed low level of miR-222-3p in HIFU-treated melanoma tissues. In the current research, HIFU treatment reduced the abundance of miR-222-3p, suggesting that HIFU might mediate breast cancer progression by regulating miR-222-3p.

miR-222-3p has been reported to contribute to proliferation, migration and invasion in breast cancer [21]. Moreover, accruing literatures displayed that miR-222-3p level was elevated in breast cancer [22, 23], which uncovered that miR-222-3p might also serve as a carcinogenic miRNA in breast cancer. Here we found that miR-222-3p contributed to proliferation but repressed apoptosis in HIFU-treated breast cancer cells. These data uncovered the importance of miR-222-3p in HIFU-mediated breast cancer progression. The function of miRNAs is to regulate the targeted gene expressions via binding with their 3’-UTR. Thus, to further figure out the regulatory mechanism, the promising target of miR-222-3p was explored. Numerous works have shown the interaction of miR-222-3p and p27Kip1 in different conditions, such as lung, ovarian, glioma and breast cancer [24-27]. Therefore, we hypothesized that p27Kip1 might be responsible for HIFU-mediated breast cancer progression by miR-222-3p targeting. Analysis of luciferase reporter validated that p27Kip1 was targeted via miR-222-3p in breast cancer cells. Subsequent expression assay revealed that HIFU treatment enhanced the protein expression of p27Kip1 and introduction of miR-222-3p attenuated the abundance.

Lu et al. reported that miR-24-3p contributed to proliferation but repressed apoptosis by target-
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Figure 6. Treatment of HIFU suppresses tumor growth by regulating miR-222-3p and p27Kip1 expression in vivo. A. Tumor volume was measured every three days. B. Tumor weight was measured in each group at ending point. C and D. The expressions of miR-222-3p and p27Kip1 protein were measured in tumor tissues of each group. *P < 0.05.

In conclusion, HIFU treatment repressed cell proliferation and increased apoptosis in breast cancer cells. Moreover, HIFU resulted in reduction of miR-222-3p and increase of p27Kip1 protein level. P27Kip1 was targeted via miR-222-3p. Up-regulation of miR-222-3p restored proliferation and restrained apoptosis in HIFU-treated breast cancer cells, which was alleviated by introduction of p27Kip1. Besides, HIFU treatment attenuated tumor growth by regulating miR-222-3p and p27Kip1. Considering all these results, HIFU treatment impeded proliferation and facilitated apoptosis by regulating miR-222-3p and p27Kip1 in breast cancer, providing new theoretical foundation for application of HIFU in the treatment of breast cancer.

Disclosure of conflict of interest

None.

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