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Ultrasound reverses adriamycin-resistance in non-small cell lung cancer via positively regulating BRAF activated non-coding RNA

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Abstract

Objectives: Multi-drug resistance (MDR) remains one of the major obstacles to successful chemotherapy in patients with cancers. BRAF activated non-coding RNA (BANCR) was reported to be regulated in non-small cell lung cancer (NSCLC). In present study, we investigated the effects of ultrasound on BANCR expression and its possible mechanism were investigated in vitro and in vivo.

Methods: The Adriamycin-resistance A549/ADM cells and its transplantation tumor model of A549/ADM in nude mice were established. Real-time PCR was used to quantify the BANCR expression in cells and in mice administrated with or without ultrasound. P-gp and MRP proteins expression were measured by western blot. The cell viability was detected by MTT assay. Results: BANCR expression was significantly elevated by ultrasound in A549/ADM cells and tumor tissue from xenograft mice. In vitro experiments, P-gp and MRP levels were markedly reduced by ultrasound. When the cancer cells up-regulated BANCR by transfection of pCDNA-BANCR, P-gp and MRP levels were inhibited. And the down-regulation of BANCR by si-BANCR transfection elevated their expression. Conclusion: Ultrasound reversed adriamycin-resistance A549/ADM cells via regulating BANCR expression, and P-gp and MRP regulation were involved in this process.

Keywords: Ultrasound; multi-drug resistance; lung cancer; lncRNAs

1 Introduction

Non-small cell lung cancers (NSCLCs), accounts for 85% of lung cancer patients [1], is the highest malignancy of all lung cancer types and is the leading cause of cancer deaths worldwide [2]. Multi-drug resistance (MDR) remains one of the major obstacles to successful chemotherapy, with more than 90% of tumor patients died of MDR [3]. Consequently, it is still an urgent need for exploring viable strategies to overcome MDR. Ultrasound, a form of energy, have been applied to the tumor ablation and reversal of tumor cells [4]. In addition, ultrasound is reported to be more effective and safer to reversal of MDR in tumor cells [5, 6]. However, the molecular mechanism of ultrasound treatment on cancers is still not fully understood.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides and take part in the regulation of gene transcription and expression [7]. The emerging evidences have been shown that lncRNAs are involved in the complex network of tumors and play important roles in tumorigenesis and progression [8, 9]. BRAF-activated non-coding RNA (BANCR), an 693-bp lncRNA on chromosome 9 was reported to regulate tumor cell proliferation and migration [10, 11]. The recent study found that BANCR was dysregulated in tumor tissues of lung cancer and non-small cell lung cancers [12, 13]. Specifically, the BANCR expression was closely related to the stages of tumor development.

P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1), is an important protein of the cell membrane that pumps many foreign substances out of cells thus involved in the MDR [14]. Multidrug resistance-associated protein (MRP) is another membrane glycoprotein to be implicated in MDR [15]. We hypothesize that P-gp and MRP expression is probably regulated by BANCR, which related to the reversal of MDR in NSCLC. In present study, the hypothesis was proved in vitro and in vivo.

2 Materials and Methods

2.1 Establishment of A549/ADM cells

The NSCLC adenocarcinoma cell lines A549 was obtained from . The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA) at 37°C with 5% CO2. The logarithmic phase cells were harvested and single-cell suspension (1×107/ml) was prepared. To establish the drug resistance cells, different concentrations (0.001μg/ml-1.0μg/ml) of Adriamycin (ADM) were supplemented in RPMI-1640 medium to incubate with cells at 37°C for 120 days. The medium was replaced every three days. Kept the cells grown well in medium at a concentration of 1.0μg/ml ADM and considered to be A549/ADM cells.

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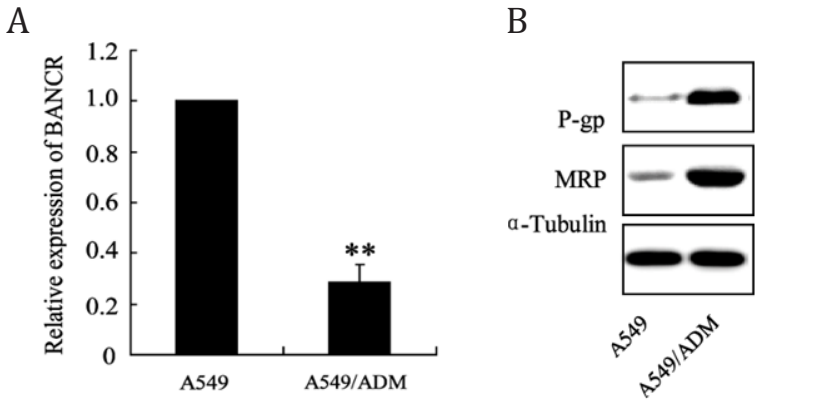

Figure 1 . The BANCR expression in A549/ADM and A549 cells. (A) The relative expression of BANCR was detected by real-time PCR; (B) the protein expression of P-gp and MRP were measured by western blot; ** versus with the level of A549 cells, P<0.01.

2.2 Establishment of transplantation tumor model of A549/ADM in nude mice

Harvested the logarithmic phase A549/ADM cells and prepared the cell suspension with normal saline at a concentration of 1×107 cells/ml. 32 male BALB/C-nu/nu nude mice aged 4 to 6 week-old were purchased form Chinese Academy of Medical Sciences Laboratory Animal (Beijing, china). All the mice were anesthetized with 430mg/kg chloral hydrate first. 150 μl A549/ADM cell suspension were injected subcutaneously into the right forelimb armpit of each mouse. The injection lasted for 8 days, and all mice were housed under SPF conditions. After the model establishment, the tumor volume was measured by vernier caliper and calculated according the formula: V= length×width2/2.

2.3 MTT assay

The cell viability was detected by MTT assay using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China) according to its introduction. The RPMI-1640 medium without cells considered as control.

2.4 Plasmid generation and cell transfection

The BANCR was synthesized and cloned into the pCDNA3.1 (Invitrogen, Shanghai, China) vector. Ectopic expression of BANCR was achieved through pCDNA-BANCR transfection, with an empty pCDNA3.1 vector used as a control. Plasmid vectors with or without BANCR were prepared using PureLink® HiPure Plasmid (Life technologies, USA), and transfected into A549/ADM cells. siRNA-BANCR (si-BANCR) and siRNA-negative control (si-control) were prepared by Hanbio Co., Ltd (Shanghai, China). The si-BANCR and si-control were transfected into A549/ADM cells using Lipofectamine 2000 (Invitrogen, USA). 48 h after the transfection, the cells were harvested for further detection.

2.5 RNA extraction and Real-time PCR

Total RNA of cells or tumor tissues was isolated with TRIzol reagent (Invitrogen, USA) according to the instructions

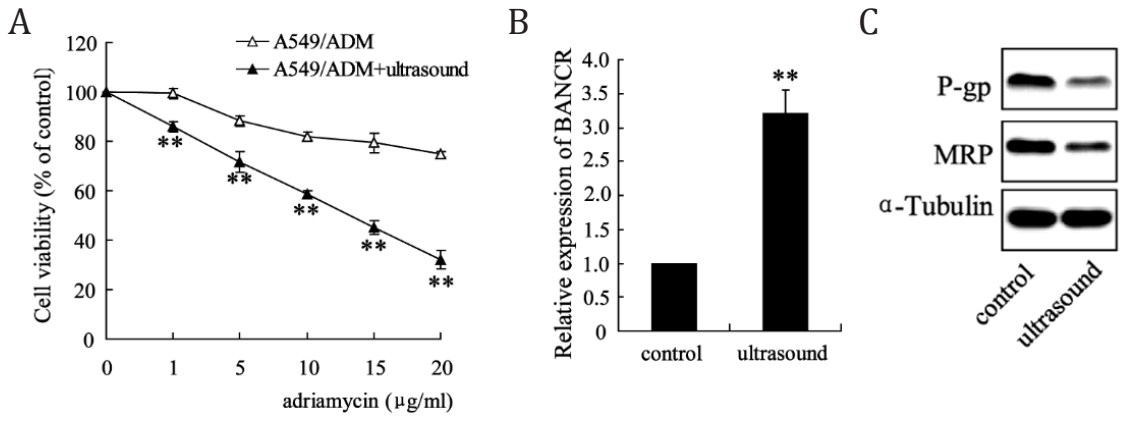

Figure 2 The effect of ultrasound on cell viability and BANCR expression of A549/ADM. The A549/ADM cells were performed with ultrasound of 0.8MHz, pulse ratio 60% and 0.4302W/cm2 sound intensity for 1 min; (A) the cell viability was detected by MTT assay; (B) the relative expression of BANCR was quantified by real-time PCR; (C) the proteins expression of P-gp and MRP were detected by western blot with α-Tubulin as control; ** versus with A549/ADM cells without ultrasound treatment, P<0.01.

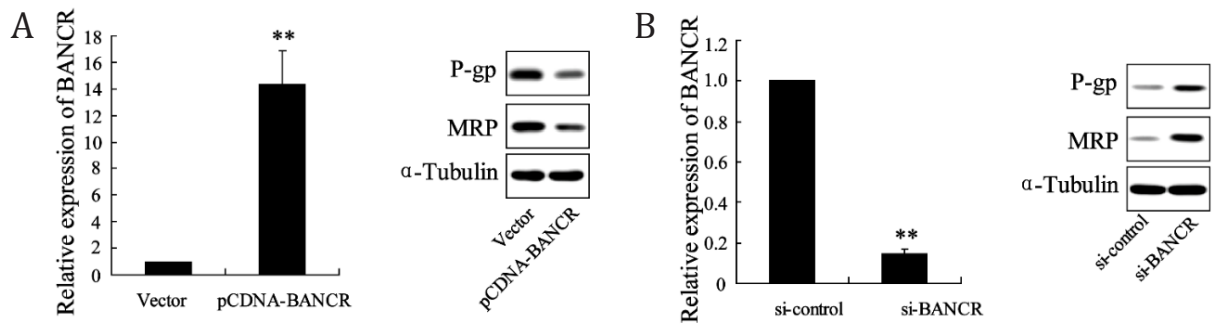

Figure 3 BANCR level regulates the P-gp and MRP expression in A549/ADM cells. (A) The BANCR level was up-regulated by pCDNA-BANCR, and the P-gp and MRP expression were measured by western blot; (B) the BANCR level was dwon-regulated by si-BANCR, and the P-gp and MRP expression were measured by western blot; ** versus with A549/ADM cells without ultrasound treatment, P<0.01.

of manufacturer. RNA was reverse transcribed to cDNA using ImProm-II™ Reverse Transcription System (Promega, USA). The BANCR expression was quantified by Applied Biosystems 7900HT Fast Real-Time PCR System with Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). GAPDH was used to normalized the BANCR expression. All specific primers were synthesized by Shanghai Generay Biotech Co., Ltd (Shanghai, China).

2.6 Western blot

The A549 cells or A549/ADM cells were washed once in ice-cold PBS and lysed with RIPA Lysis Buffer (Beyotime, China). The total protein of cells were abstracted by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) and protein concentration was quantified by BCA Protein Assay Kit (Beyotime, China). 40μg total protein were separated by 10% SDS gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes (Millipore, USA) and incubated with primary antibodies (Cell Signaling Technology, USA). The horseradish peroxidase (HRP)-conjugated secondary antibody were incubated with membranes at room temperature for 1h. The bands were visualized using the enhanced chemiluminescent (ECL) substrate. Protein loading was normalized by α-Tubulin.

2.7 Statistical analysis

All statistical analysis was performed using SPSS 18.0 software. The measurement data were presented as mean ± standard deviation. Independent T-test was used to compare the difference between two groups. One-way ANOVA followed by Student's t-test was conducted to compare the difference between any two means. Two-tailed P values of <0.05 was regarded as statistically significant.

3 Results

3.1 The BANCR expression in A549/ADM and A549 cells

We first detected the relative expression of BANCR of A549/ADM and A549 cells. As shown in Figure 1A, it was significantly decreased in A549/ADM cells compared with A549 cells. In addition, the protein expression of P-gp and MRP were marked up-regulated in A549/ADM cells (Figure 1B).

3.2 The effect of ultrasound on cell viability and BANCR expression of A549/ADM

Administrated A549/ADM cells with ultrasound for 1 min, the cell viabilities were significantly decreased, and the drops were enhanced with the increase of Adriamycin concentrations (Figure 2A). The relative expression of BANCR in cells was elevated after ultrasound treatment (Figure 2B). In addition, ultrasound down-regulated the P-gp and MRP proteins expression (Figure 2C).

3.3 BANCR level regulates the P-gp and MRP expression in A549/ADM cells

To overexpress the BANCR level of A549/ADM cells, the proteins of P-gp and MRP were significantly decreased (Figure 3A). In addition, to inhibit the expression of BANCR, the proteins of P-gp and MRP were markedly increased (Figure 3B).

3.4 The effect of BANCR down-regulation on A549/ADM cell viability

Si-BANCR was used down-regulate BANCR expression of A549/ADM cells. As shown in Figure 4, the cell viability was significantly elevated by down-regulation of BANCR.

3.5 The effect of ultrasound on tumor growth and BANCR expression in vivo

The model of transplantation tumor of A549/ADM in nude mice was established. Administrated mice with different treatments, the results showed that 5mg/kg Adriamycin alone had no effect on tumor volume decrease, while it was

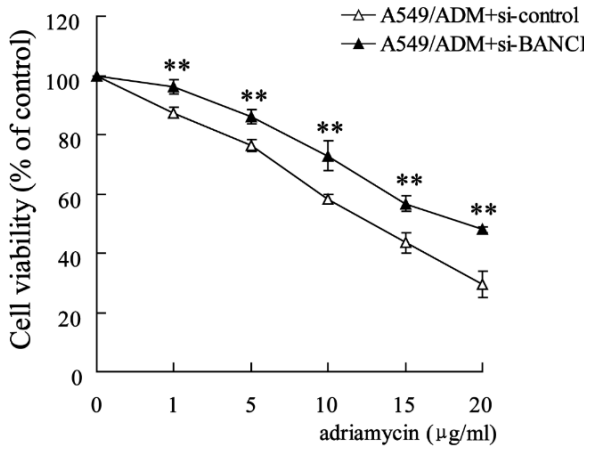

Figure 4 The effect of BANCR down-regulation on A549/ADM cell viability. The cell viability was detected by MTT assay. ** versus with A549/ADM + si-control group, P<0.01

significantly reduced by ultrasound treatment. In addition, the tumor volume was marked lowered in the combination therapy of Adriamycin and ultrasound compared with ultrasound treatment alone (Figure 5A). The real-time PCR data presented that ultrasound significantly elevated BANCR expression of tumor tissue and its level was even higher in the tumor treated with combination therapy (Figure 5B).

4 Discussion

LncRNAs, a newly discovered class of noncoding genes, have been recognized to be involved in the epigenetics, transcription and post-transcriptional regulation in past few years [16]. The role in gene regulatory processes were concerned in various human diseases, especially in cancer development [17]. Although a lots of studies have demonstrated the functions of lncRNAs, the involvement of lncRNAs in MDR of non-small cell lung cancer is still not fully understood. In present study, we found A549/ADM cells had lower level of BANCR expression compared with A549 cells, which implied that BANCR might take part in the regulation of tumor development with MDR.

Ultrasound have been reported to be an effective approach to the reversal of MDR in tumor cells. Low-intensity ultrasound can result in a series of biological reactions of histiocytes, including the alteration of material motion, volume and endochylema flow. The previous studies demonstrated that ultrasound reduced tumor growth, and the combination of ultrasound with chemotherapeutics could enhance the antitumor effects [18, 19]. In present study, we found ultrasound significantly decreased the viabilities of Adriamycin-resistant A549/ADM cells in vitro, and lowered the tumor volume of A549/ADM induced xenograft mice in vivo.

We also investigated the ultrasound effect on BANCR expression. The real-time PCR data presented that BANCR level was markedly elevated by ultrasound treatment in both in A549/ADM cells and in mice, suggesting BANCR regulation was involved the ultrasound treatment of NSCLC tumor. In previous study, Sun et al [13] demonstrated BANCR expression was reduced and associated with larger tumor size, advanced pathological stage, metastasis distance, and shorter overall survival of NSCLC patients. In addition, they also found knockdown of BANCR expression promoted cell migration and invasion in intro. Jiang et al [12] showed that BANCR levels were down-regulated in lung cancer cells, and the overexpression of BANCR could suppress the tumor growth.

P-gp and MRP, belong to ATP-binding cassette transporters, is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. The overexpression of P-gp and MRP are considered to be one of the mechanisms in MDR development [14, 20]. In present study, we also found an elevation of P-gp and MRP protein

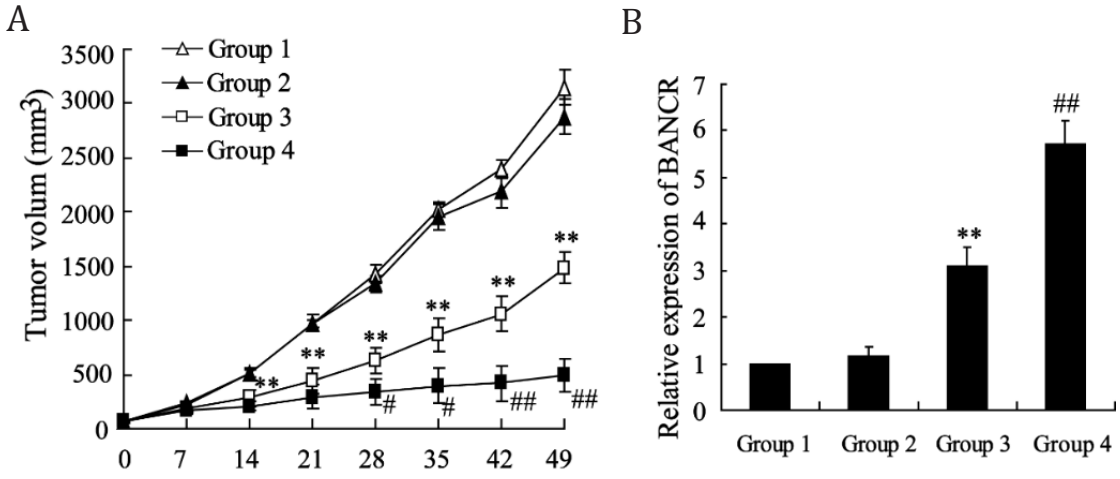

Figure 5 The effect of ultrasound on tumor growth and BANCR expression in vivo. Group 1: tail vein injection with phosphate buffer; Group 2: tail vein injection with 5mg/kg Adriamycin; Group 3: the tumor was performed with ultrasound therapy; Group 4: the mice performed with tail vein injection with 5mg/kg Adriamycin and ultrasound therapy; (A) The tumor volume was recorded at the beginning of treatment; (B) the BANCR expression was quantified by real-time PCR; ** versus with the Group 1, P<0.01; # versus with the Group 3, P<0.05; ## versus with the Group 3, P<0.01.

expression in A549/ADM cells compared with A549 cells. In addition, a reduction levels of them were observed in cells treated with ultrasound. In vitro experiments, the overexpression of BANCR could decrease the levels of P-gp and MRP, while the down-regulaiton of BANCR increase their expression. Therefore, it was believed that BANCR took part in A549/ADM cell viability probably via regulation of P-gp and MRP expression.

In conclusion, the expression of BANCR was markedly down-regulated in Adriamycin-resistance NSCLC adenocarcinoma cell lines A549 cells. Ultrasound treatment could elevated BANCR expression in A549/ADM cells and in its xenograft mice, with inhibition of P-gp and MRP expression was one of the potential mechanisms. Our study promotes the understanding the pathogenesis of NSCLC development and the mechanism of ultrasound treatment for cancer. These findings are also significant for therapeutics against cancers via BACNR regulation. However, the further molecular mechanisms underlying the effects of lncRNAs on cancers are needed to be further investigated.

Conflict of interest

All authors declare that they have no competing interests.

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