

Original article:

LOW-INTENSITY ULTRASOUND ENHANCED THE ANTITUMOR EFFECTS OF CISPLATIN IN NON-SMALL CELL LUNG CANCER CELLS VIA INHIBITING MIR-124/STAT3 SIGNALING

Liqing Peng¹, Zhijian Luo¹, Dan Liu¹, Gang Feng², Mingxing Li^{1,*}

¹ The Department of Ultrasound, The Affiliated Hospital of Southwest Medical University, Sichuan Luzhou, 646000, People's Republic of China

² Institute of Ultrasound Imaging, Second Affiliated Hospital of Chongqing Medical University, Chongqing, 400010, People's Republic of China

* Corresponding author: Mingxing Li, The Department of Ultrasound, The Affiliated Hospital of Southwest Medical University, No. 25 Taiping street, Luzhou, 646000, People's Republic of China. Tel: +86- 0830-3165200; E-mail: mingxingli66@163.com

<http://dx.doi.org/10.17179/excli2017-910>

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>).

ABSTRACT

Cisplatin (CDDP) is one of the most widely employed first-line chemotherapeutic agent against human non-small cell lung cancer (NSCLC). However, most NSCLC patients are eventually resistant to chemotherapy. Recently, the combined effects of low-intensity ultrasound (US) and several anti-cancer drugs have often been reported, but the underlying mechanism remains largely unknown. This study aimed to test whether low-intensity ultrasound could enhance the anti-tumor effects of cisplatin in NSCLC cells and reveal its underlying molecular mechanism. First, the effects of low-intensity US on cisplatin mediated cell viability, induction of apoptosis and reactive oxygen species (ROS) production were determined by CCK-8 assay, flow cytometry analysis and fluorescent probe (DCFH-DA), respectively. To profile the expression of miRNAs that are involved in the combined effects of cisplatin and low-intensity US, miRNA microarray was applied and the result was verified by quantitative reverse transcription PCR (qRT-PCR). Loss-of-function study was performed to determine the role of miR-124 in the combined effect of cisplatin and low-intensity US exposure. A luciferase reporter assay was performed to validate the interaction between miR-124 and signal transducer and activator of transcription 3 (STAT3) followed by qRT-PCR and Western blot confirmation. Afterward, we further explored whether the effects of miR-124 on the anti-tumor activity of US combined with cisplatin are mediated by STAT3 signaling pathway. Our study showed that low-intensity US enhanced the anti-tumor effects of cisplatin in A549 cells, as evidence by the reduction of cell viability and the induction of apoptosis and ROS levels compared with cisplatin alone treatment. Subsequently, blockade of ROS generation by NAC significantly reversed low-intensity US induced the expression of miR-124, suggesting that cisplatin combined with low-intensity US up-regulated the expression of miR-124 through generation of ROS. Knockdown of miR-124 inhibited the synergistic effects of cisplatin combined with low-intensity US in A549 cells. STAT3, an oncogene, was identified as a direct target of miR-124 and its expression was negatively regulated by miR-124 at mRNA and protein levels. Furthermore, cisplatin combined with low-intensity US decreased the expressions of STAT3 and its downstream genes, phosphorylated STAT3 (p-STAT3), B-cell lymphoma 2 (Bcl-2) and Bcl-xL, whereas knockdown miR-214 reversed the inhibitory effects of cisplatin combined with low-intensity US in A549 cells. In conclusion, our study unraveled low-intensity US enhanced the anti-tumor effects of cisplatin on NSCLC cells and elucidated the participation of miR-124/STAT3 signaling pathway in the synergistic effects of cisplatin combined with low-intensity US. Our findings suggest that low-intensity US can be considered to be a potential strategy for overcoming cisplatin resistance in NSCLC.

Keywords: low-intensity ultrasound, cisplatin, non-small cell lung cancer, reactive oxygen species, microRNA-124, STAT3

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and the leading causes of all cancer-related deaths. Although significant improvement in surveillance and targeted therapy, the 5-year overall survival rate of NSCLC cases is less than 5 % when accompanied by metastasis (Yu et al., 2013). At present, chemotherapy is one of the most effective treatments for NSCLCs, and cisplatin (CDDP) is the standard first-line chemotherapy drug for NSCLCs; however, most NSCLC patients are eventually resistant to chemotherapy (Li et al., 2016). Therefore, new therapeutic methods should be developed to improve the survival rate.

Therapeutic ultrasound (US), especially low-intensity US in combination with several anti-cancer drugs, has been successfully used for cancer therapy (McHale et al., 2016). Low-intensity ultrasound can suppress cell proliferation and clone formation, improve the effects of anticancer chemicals and deactivate cells via indirect mechanisms (Lejbkiewicz et al., 1993; Lejbkiewicz and Salzberg, 1997). For example, Yoshida et al. found that low-intensity ultrasound combined with doxorubicin caused a synergistic enhancement in cell killing and an additive enhancement in apoptosis induction in human lymphoma U937 cells (Yoshida et al., 2008). A recent study from low-intensity ultrasound combined with 5-fluorouracil (5-FU) produced much enhanced synergistic anti-tumor effects via enhanced intracellular reactive oxygen species (ROS) production in treating hepatocellular carcinoma (HCC) (Hu et al., 2016). Although applications of low-intensity ultrasound are still in the process of investigation, low-intensity ultrasound has distinct potential as a technique for cancer treatment, especially in cisplatin resistance. However, the underlying mechanisms about the synergistic effects of cisplatin combined low-intensity ultrasound are yet to be elucidated.

MicroRNAs (miRNAs) are a class of endogenous small (19–24 nucleotide) noncoding RNAs that repress translation of target messenger RNAs (mRNAs) or induce degradation of target mRNAs by binding to the 3'-UTR of target mRNAs (Bartel, 2004). Emerging data have suggested that miRNAs are involved in a wide range of biological processes, such as cellular proliferation, apoptosis and drug resistant (Croce, 2009). Recent data revealed that microRNA expression alters in response to ROS exposure (He and Jiang, 2016). Furthermore, a marked increase of ROS production accounted for the significantly enhanced anticancer effects of low-intensity ultrasound combined with 5-FU, suggesting the potential role of ROS in the synergistic effects of chemotherapeutic drug combined with low-intensity ultrasound (Hu et al., 2016). Therefore, we speculated that low-intensity ultrasound would enhance the anti-tumor effects of cisplatin through regulating miRNAs expression in NSCLC cells.

In this study, we evaluated the promoting effect of low-intensity ultrasound on the anti-tumor activity of cisplatin and further explored the involvement of possible molecular mechanisms, particularly the participation of miR-124/STAT3 signaling pathway in the anti-cancer activity of cisplatin combined with low-intensity US. Our findings suggest that cisplatin combined with low-intensity US could be a valid treatment option for NSCLC.

MATERIALS AND METHODS

Chemicals and antibodies

Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and the stock solution was prepared at a concentration of 2 mM in 0.95 % NaCl solution, pH 7.4 which was stored at -20 °C. N-acetylcysteine (NAC; purity by TLC: ≥ 99 %) were also purchased from Sigma-Aldrich. Antibodies against STAT3, p-STAT3, B-cell lymphoma 2 (Bcl-2), Bcl-xL and β -actin were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Cell culture

HEK293T and NSCLC cells, A549, were obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM (HEK293T) (Invitrogen, Carlsbad, CA, USA) or RPMI 1640 (A549 cell lines) (Thermo Fisher Scientific, Waltham, MA, USA) culture medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 2 mM GlutaMAX-1, 100 mg/mL streptomycin and 100 U/mL penicillin (Sigma, St. Louis, MO, USA) in an incubator with a humidified atmosphere and 5% CO₂ at 37 °C. Cells were treated with the indicated concentrations of cisplatin. In some experiments, the ROS scavenger NAC (10 mM), were added 1 hour prior to the administration of cisplatin, immediately followed by ultrasound exposure.

Ultrasound device and intensity measurement

The system SonoPore KTAC-4000 (NepaGene, Chiba, Japan) with a fixed duty factor of 25 % and with 100 HZ pulse repetition frequency (PRF) was used. The sonication was conducted at an intensity of 0.5 W/cm² and exposure time of 60 sec as previously described (Masui et al., 2013). The intensity and time were used in all of the sonication experiments. To keep the transducer-facing directory upward for the sonication procedure, the transducer with a diameter of 5.0 cm, was fixed with a clamp attached to a metal stand. A 3.5 cm culture dish was placed on the center of the transducer intermediate with gel. The setup was previously described elsewhere by Masui et al. (2013).

Cell viability assays

Cell viability was monitored using a Cell Counting Kit (CCK-8; Dojindo, Kumamoto, Japan). Briefly, A549 cells were seeded in 96-well plates at a density of 5000 cells/well. After 48 h of treatment, 10 µl of CCK-8 solution was added to each well, and the plates were incubated for 3 h at 37 °C. The optical density (OD) levels were measured at 450

nm using SpectraMax M3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell apoptosis detection by flow cytometry

An Annexin-V FITC-PI Apoptosis Kit (Invitrogen) was applied to determine the apoptotic rate by flow cytometry. A549 cells were collected after treatment, and washed twice with PBS at 4 °C. Then, the cells were re-suspended in 490 µl binding buffer containing 5 µl Annexin V (Bio-Science, Co. Ltd, Shanghai, China) and 5 µl PI. After incubation at 4 °C in the dark for 30 min, samples were analyzed by a FACS Aria flow cytometry (BD Biosciences, San Jose, CA, USA).

Reactive oxygen species (ROS) analysis

The intracellular ROS level was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). Treated or untreated cells were trypsinized and washed with PBS, and 1×10⁶ cells were subsequently re-suspended in Hanks' balanced salt solution containing 10 µM DCFH-DA and incubated for 45 min at 37 °C then the cells were collected and analyzed by a flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

microRNA expression profiling

Total RNA was extracted from A549 cells treated with cisplatin in the presence or absence of low-intensity US using the miR-Neasy mini kit (Qiagen, West Sussex, UK). Samples were labeled with a label kit (miR-CURY™ Hy3™/Hy5™ Power labeling kit and hybridized to the miRCURY LNA™ Array (v.16.0) (Agilent Technologies). The chips were scanned with the Axon GenePix 4000B Microarray Scanner. The procedure and images process method as described previously (Xu et al., 2017). The miRNA expressions of all differentially expressed samples were clearly displayed by a hierarchical clustering heat map.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from A549 cells using the miRNeasy mini kit (Qiagen, West Sussex, UK) and TRizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR was carried out using a standard SYBR Green PCR kit (TaKaRa, Dalian, China) protocol on Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems). Results were normalized to the expression of U6 and GAPDH. The specific primer was designed with Primer 5.0 software and synthesized by the Shanghai Sangon Biotech Company: miR-124 forward 5'-CTAGTCTAGAGTC-GCTGTTATCTCATTGTCTG-3', and reverse 5'-CGCGGATCCTCTGCTTCTGTC-ACAGAATC-3'; U6 forward, 5'-AAAGAC-CTGTACGCCAACAC-3' and reverse, 5'-G-TCATACTCCTGCTTGCTGAT-3'; STAT3 forward, 5'-GAAGGACATCAGCGGTAA-GA-3' and reverse, 5'-AGATAGACCAGT-GGAGACAC-3'; GAPDH forward, 5'-GAAGATGGTGATGGGATTTC-3' and reverse, 5'-GAAGGTGAAGGTCGGAGT-3'.

Cell transfection

MiR-124 mimic, miR-124 inhibitor and the corresponding negative control (mimics NC and inhibitor NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The miRNA mimics, miRNA inhibitor, and the negative control miRNA oligonucleotides were transfected into the HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Luciferase reporter assay

The 3'-UTR of STAT3 with wild-type or mutant (Mut) binding sites for miR-124, was amplified and cloned into the pGL3 vector (Promega, Madison, WI, USA) to generate the plasmid pGL3-WT-STAT3-3'-UTR or pGL3-Mut-STAT3-3'-UTR, respectively. For the luciferase reporter assay, HEK293T cells were co-transfected with the luciferase reporter vectors and miR-124 mimics, miR-

124 inhibitor or corresponding negative control (GenePharma, Shanghai, China). The pRL-TK plasmid (Promega) was used as a normalizing control. After 48 h of incubation, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega).

Western blotting analysis

Cells were lysed using RIPA protein extraction reagent (Beyotime, Beijing, China). The concentration of proteins was determined using a BCA assay kit (Pierce, Rockford, IL, USA). Protein extracts (50 µg) were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes (Sigma) and then incubated at room temperature with primary antibodies against STAT3 (1:1,000) and β-actin (1:3,000) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). β-actin was used as control. Membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. ECL chromogenic substrate was used to visualize the bands and the intensity of the bands was quantified by densitometry (Quantity One software; Bio-Rad).

Statistical analysis

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ^2 test and the other data were evaluated by Student's t-test and expressed as the mean \pm SD from three independent experiments. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Low-intensity ultrasound enhances the anticancer activity of cisplatin in vitro

Previous studies showed that low-intensity ultrasound could enhance the anticancer activity of several anticancer drugs, such as cetuximab, cisplatin (CDDP) and doxorubicin (DOX) (Watanabe et al., 2008,

Yoshida et al., 2008; Masui et al., 2013). Thus, we sought to test whether low-intensity US enhances the anticancer activity of cisplatin in NSCLC cells. First, we measured the IC₅₀ of cisplatin in A549 cells and found that cisplatin inhibited cell viability in our assay with an IC₅₀ of 15 μM (data not shown), which is consistent with literature value (IC₅₀: 15 μM) (Wu et al., 2015). It must be noted that in our experiments we used cisplatin in a low final concentration of 10 μM under sonication for highlighting the synergistic effects of cisplatin combined with low-intensity US. According to the results of cell viability, the growth of A549 cells was significantly inhibited in US, CDDP and US + CDDP groups compared with Blank group (Figure 1A). Notably, the growth of A549 cells was markedly reduced in US + CDDP when compared with that in the group treated with CDDP only (Figure 1A).

Recent studies have reported that reactive oxygen species (ROS) plays a key role in CDDP-mediated anti-tumor effects (Ma et al., 2015; He et al., 2016). Here we detected intracellular ROS levels and found that ROS production in A549 cells was significantly promoted in US, CDDP and US + CDDP groups compared with Blank group (Figure 1B). Meanwhile, ROS production in US + CDDP was markedly increased when compared with CDDP group (Figure 1B). Furthermore, the induction of apoptosis was assessed by flow cytometry. Results of apoptosis studies revealed that CDDP stimulated did not result in considerable elevation of apoptosis, but A549 cells exposed to low-intensity US in the presence of CDDP showed the highest level of apoptosis (Figure 1C, D). All these results indicated that low-intensity US enhanced the anticancer activity of cisplatin in NSCLC cells, evidenced by the reduction of cell viability and the promotion of ROS levels and apoptosis.

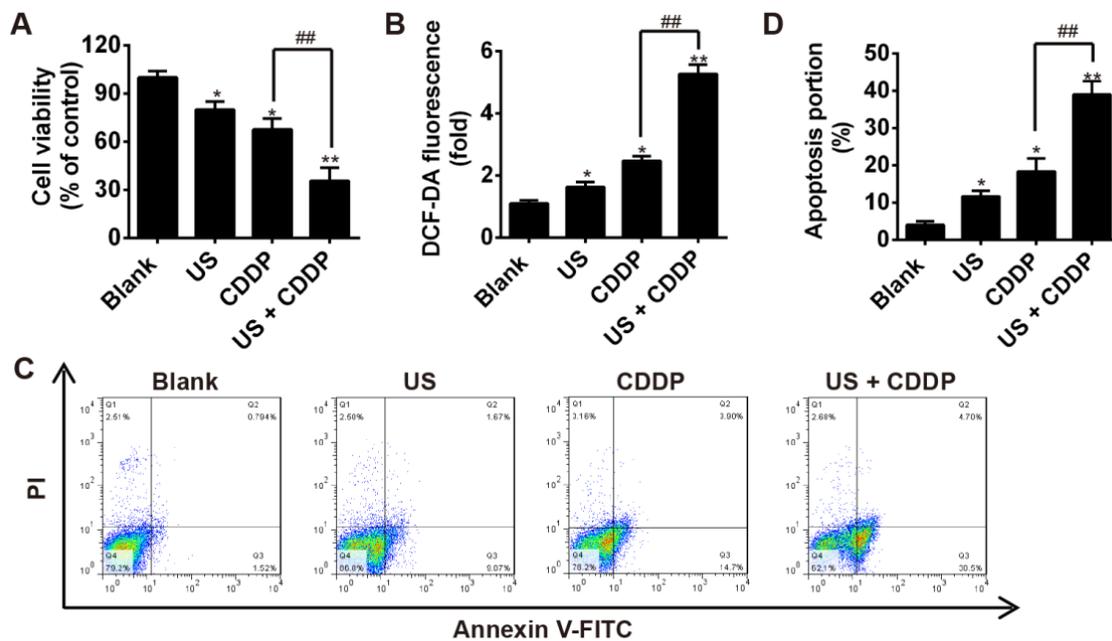


Figure 1: Low-intensity US enhanced the anti-tumor activity of cisplatin in A549 cells. A549 cells (1×10^6) in 1.5 ml medium were seeded in a 3.5 cm dish and incubated at 37 °C for 12 h. Experiments were conducted in 4 groups: (1) non-treated (Blank), (2) CDDP treated (CDDP), (3) US treated (US), and (4) combined (CDDP + US). In CDDP + US, cells were exposed to 10 μM CDDP for 30 min and sonicated by 1 MHz pulsed US (PRF 100 Hz, DF 25 %) at intensities of 0.5 W/cm² for 60 s. The cells were washed and incubated for 6 h. **A.** Cell viability was measured by CCK-8 assay. **B.** The intracellular ROS level was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). **C** and **D.** Cell apoptosis was detected by flow cytometry. Data represent the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. Blank group; ### $p < 0.01$ vs. CDDP group

Low-intensity ultrasound combined with CDDP increased the expression of miR-124 via ROS production

It is reported that US could effectively enhance ROS generation inside the cells (Hu et al., 2016), and that ROS can alter the expression of several microRNAs (miRNAs) (Jajoo et al., 2013; Melnik, 2015; He and Jiang, 2016). Thus, we sought to determine whether low-intensity US combined with CDDP exerts the anticancer activity via regulating the expression of miRNAs. First, we studied the differentially expressed miRNAs in A549 cells by microarray under the influence of separate and combined cisplatin and low-intensity US far field exposure. The

results of microarray analysis revealed a significant downregulation of 36 miRNAs and up-regulation of 19 miRNAs in US + CDDP group when compared with CDDP group (Figure 2A). From the list of differentially expressed miRNAs, we focused on miR-124, as it was the most up-regulated miRNA in A549 cells treated with low-intensity US and CDDP when compared with CDDP treatment alone. Moreover, miR-124 has been reported to play important roles as a tumor suppressor in diverse cancers including NSCLC (Hao et al., 2017; Hu et al., 2017; Zhao et al., 2017). Therefore, we chose miR-124 for further study.

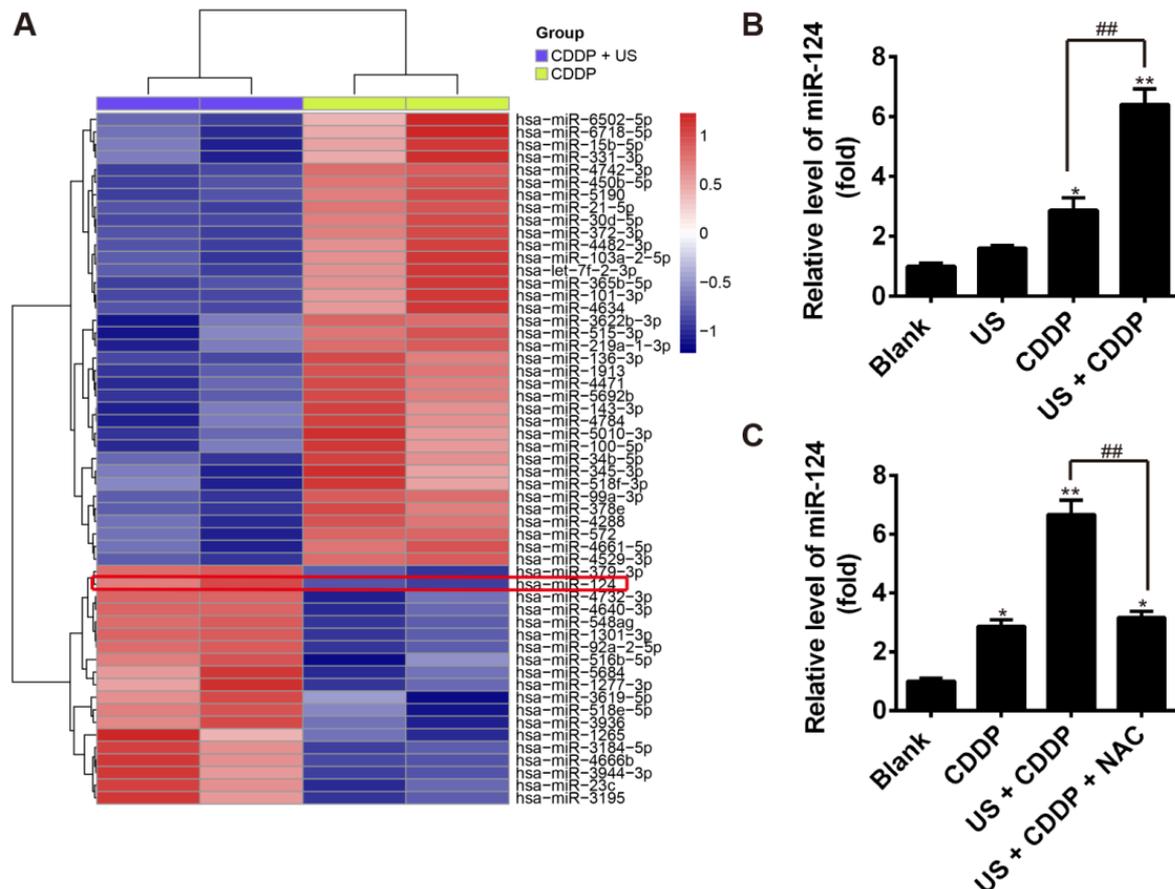


Figure 2: miR-124 is up-regulated in A549 cells after cisplatin combined with low-intensity US treatment. **A.** Heatmap of normalized expression levels of miRNAs in A549 cells after cisplatin and/or low-intensity US treatment. **B.** qRT-PCR was performed to determine the expression levels of miR-124 in A549 cells after cisplatin and/or low-intensity US treatment. Data represent the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. US or Blank group; ### $p < 0.01$ vs. CDDP group. **C.** qRT-PCR was performed to determine the expression levels of miR-124 in A549 cells after cisplatin and/or low-intensity US treatment in the presence or absence of the ROS scavenger NAC. Data represent the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. Blank group; ### $p < 0.01$ vs. CDDP +US group

To validate the microarray data, quantitative real-time PCR (qRT-PCR) was performed. The results of qRT-PCR showed that the expression of miR-124 in A549 cells under cisplatin combined with low-intensity US treatment was significantly increased compared with the expression of miR-124 in CDDP treatment alone (Figure 2B). Previous studies showed that some microRNAs were regulated by oxidative stress to mediate the expression levels of their direct targets in response to ROS (Simone et al., 2009; He and Jiang, 2016). Thus, we tested the impact of ROS generation on miR-124b level in A549 cells under cisplatin combined with low-intensity US treatment. Using the antioxidant NAC, the increased miR-124 expression induced by cisplatin combined with low-intensity US was becoming low, which is close to the levels in the CDDP alone group (Figure 2C), suggesting that cisplatin combined with low-intensity US increased the expression of miR-124 via ROS production.

Knockdown of miR-124 inhibited the synergistic anti-tumor effects of cisplatin combined with low-intensity US

To examine whether low-intensity US enhances the anti-tumor effects of cisplatin through up-regulation of miR-124 in A549 cells, the miR-124 inhibitor and mimics were transfected into A549 cells, and the expression level of miR-124 was significantly decreased (Figure 3A) or enhanced after transfection (Figure 3B). Then, we examined the alteration of cell viability and apoptosis after miR-124 knockdown. As shown in Figure 3C, inhibition of miR-124 restored the reduction of cell viability induced by cisplatin combined with low-intensity US. Moreover, knockdown of miR-124 reversed the promoting effect of cisplatin combined with low-intensity US on the cell apoptosis (Figure 3D). These data indicate that miR-124 is involved in the synergistic anti-tumor effects of cisplatin combined with low-intensity US.

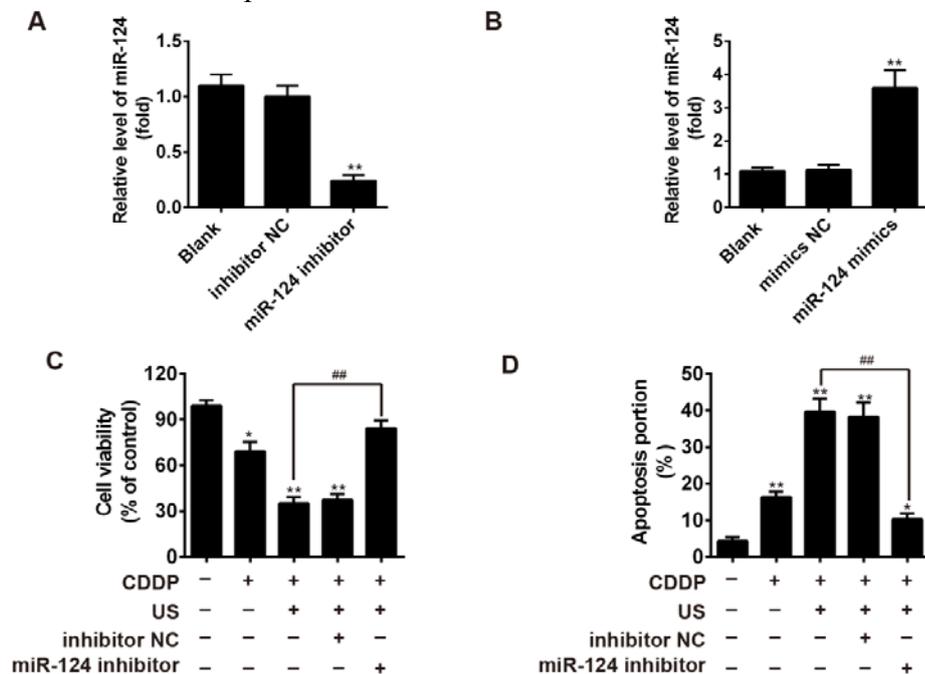


Figure 3: Knockdown of miR-124 inhibited the synergistic anti-tumor effects of cisplatin combined with low-intensity US. A549 cells were transfected with miR-124 inhibitor or inhibitor negative control (NC), followed by cisplatin and/or low-intensity US treatment. After 48 h transfection, cells were harvested for cell viability and cell apoptosis detection. **A, B.** The expression level of miR-124 was measured by qRT-PCR after transfection of miR-124 inhibitor or miR-124 mimics in A549 cells. Data represent the mean \pm SD of three independent experiments. ** $p < 0.01$ vs. inhibitor NC or mimics NC. **C.** Cell viability was measured by CCK-8 assay. **D.** Cell apoptosis was detected by flow cytometry. Data represent the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. Blank group; ## $p < 0.01$ vs. CDDP +US group

MiR-124 suppresses STAT3 expression by directly targeting its 3'-UTR

To explore the molecular mechanism by which miR-124 functions in the anti-tumor effects of cisplatin combined with low-intensity US, bioinformatics tools were used to predicate the putative targets of miR-124. According to the results of these analyses, we found that STAT3, a well-known oncogene, might be a potential target of miR-124 and the target site located in the 3'-UTR of STAT3 mRNA (Figure 4A). Previous studies have been reported that miR-124 functions as a tumor suppressor in diverse types of cancers through targeting STAT3 signaling

pathway (Lu et al., 2013; Cheng et al., 2015; Liu et al., 2016; Xu et al., 2016). To test whether STAT3 is negatively regulated by miR-124, STAT3 expression was analyzed by Western Blot analysis and qRT-PCR. We found that transfection with miR-124 mimics significantly downregulated the protein and mRNA levels of STAT3 in A549 cells, whereas an increase in STAT3 protein and mRNA levels was shown in A549 cells treated with miR-124 inhibitor (Figure 4B, C, D, E). To further confirm that whether miR-124 suppresses STAT3 expression by directly targeting its 3'-UTR, a luciferase reporter assay was conducted in HEK293T

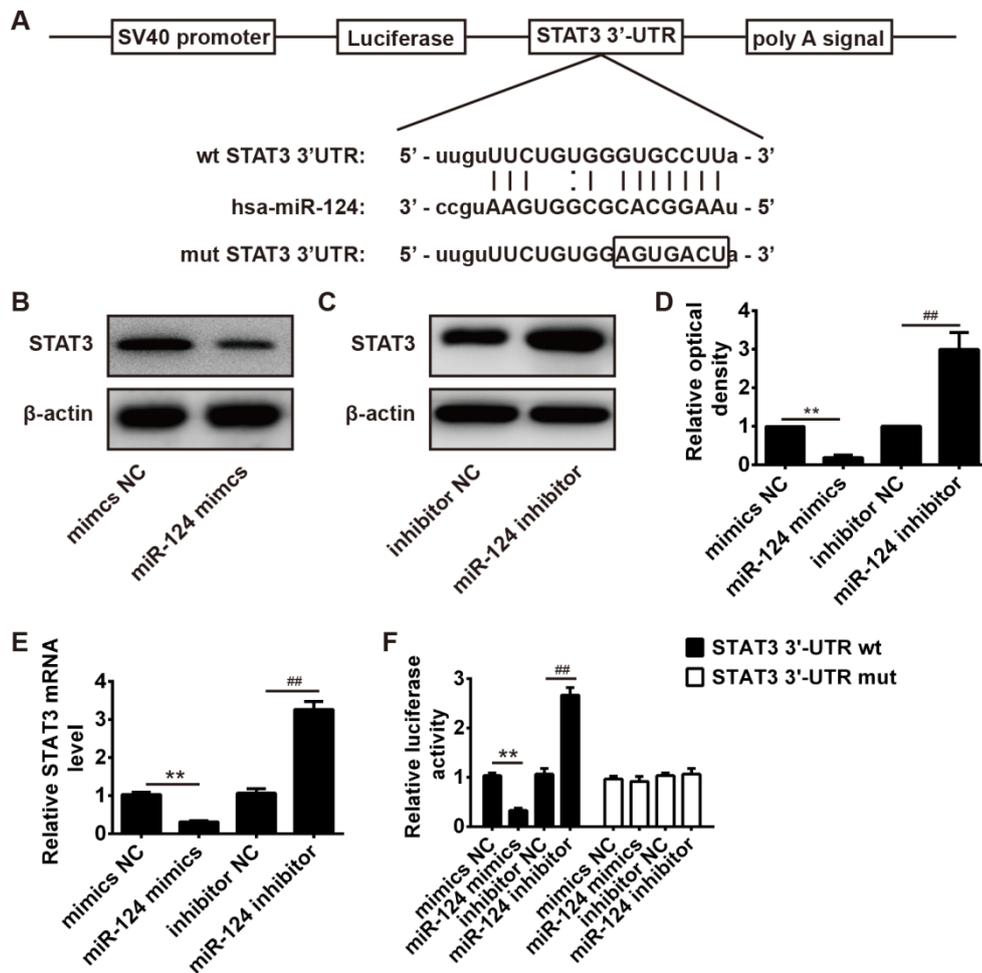


Figure 4: STAT3 was a direct target of miR-124 in A549 cells. **A.** The predicted miR-124 binding sites on STAT3. **B-E.** miR-124 mimics, miR-124 inhibitor and controls were transfected into A549 cells, then after 48 h transfection, the protein and mRNA levels of STAT3 were detected by Western Blot and qRT-PCR assays. ** $p < 0.01$ vs. mimic NC; ## $p < 0.01$ vs. inhibitor NC group. **F.** Luciferase activity in HEK293T cells was co-transfected with miR-124 mimics, miR-124 inhibitor and luciferase reporters containing STAT3 wild type or mutant type (MUT) 3'-UTR. Histogram indicates the values of luciferase measured 48 h after transfection. Data represent the mean \pm SD of three independent experiments. ** $p < 0.01$ vs. mimic NC; ## $p < 0.01$ vs. inhibitor NC group

cells. The results showed that over-expression of miR-124 significantly decreased the luciferase activity of wt-STAT3-3'UTR, whereas knockdown of miR-124 increased luciferase activity. Likewise, cells co-transfected with miR-124 mimics, miR-124 inhibitor, and STAT3-mut-3'UTR, showed no obvious change in their luciferase activity (Figure 4F), indicating that STAT3 could be regulated post-transcriptionally by miR-124 in A549 cells.

Cisplatin combined with low-intensity US inhibited the STAT3 pathway through up-regulation of miR-124

Previous studies have been demonstrated that STAT3, an important target of miR-124, plays important roles in many aspects of tumorigenesis, including proliferation, apoptosis, angiogenesis, and metastasis, especially apoptosis (Jove, 2000; Kasprzycka et al., 2006). These reports prompted us to study whether STAT3 is involved in miR-124 mediated the anti-tumor effect of cisplatin combined with low-intensity US. To elucidate the molecular mechanisms underlying the miR-124/STAT3 axis-mediated the combined effect of cisplatin and low-intensity US treatment, A549 cells were transfected with miR-124 inhibitor and treated with cis-

platin and low-intensity US. Cisplatin alone significantly inhibited STAT3 protein expression compared with Blank group, and US enhanced the inhibitory effects of cisplatin. While after inhibition of miR-124 expression, STAT3 level were notably increased, closely to the level of CDDP alone. In addition, since STAT3 has been reported to participate in facilitating cell proliferation and cycle progression, and also in antagonizing cell apoptosis by enhancing down-stream genes expression, such as Bcl-xL (Ting et al., 2012) and Bcl-2 (Bhattacharya et al., 2005), we also explored the effects on the expression of these genes upon indicated treatment. As shown in Figure 5A, B, cisplatin alone inhibited p-STAT3 expression and its downstream genes, Bcl-xL and Bcl-2 protein expression compared with Blank group, and US enhanced the inhibitory effects of cisplatin on these downstream genes. While after inhibition of miR-124 expression, p-STAT3, Bcl-xL and Bcl-2 levels were notably increased, closely to the level of CDDP alone. These results indicates that cisplatin combined with low-intensity US induces miR-124 up-regulation and then inhibited the activation of STAT3 signaling, which in turn facilitates the anti-tumor effect of cisplatin combined with low-intensity US.

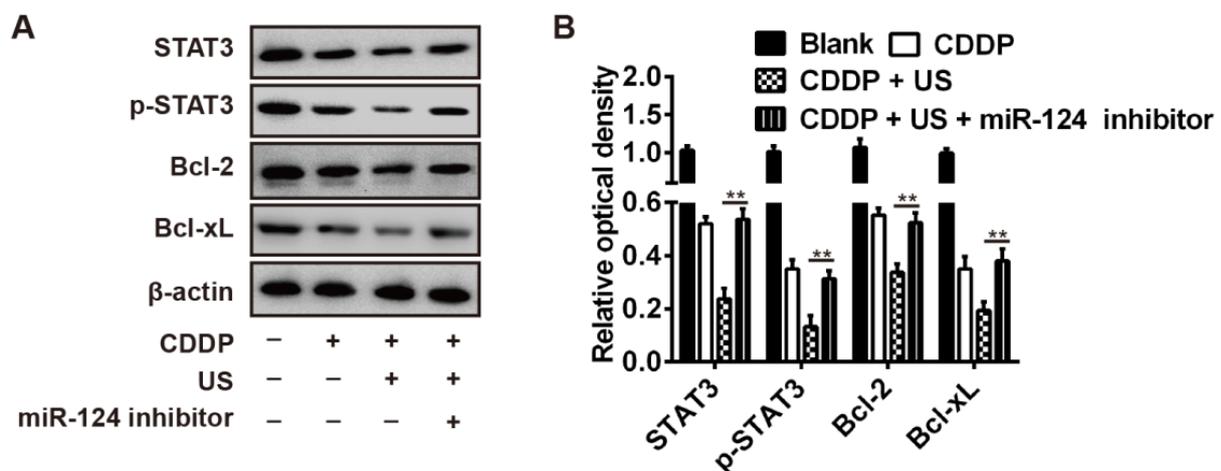


Figure 5: Cisplatin combined with low-intensity US inhibited the STAT3 pathway through up-regulation of miR-124. A549 cells were transfected with miR-124 inhibitor or inhibitor negative control (NC), followed by cisplatin and/or low-intensity US treatment. **A.** The expressions of STAT3 expression and its downstream genes, Bcl-xL and Bcl-2 protein were analyzed by Western Blot. **B.** The bands were semi-quantitatively analyzed by using Image J software, normalized to β-actin density. Data represent the mean ± SD of three independent experiments. ***p* < 0.01 vs. CDDP + US group

DISCUSSION

In the present study, we demonstrated that low-intensity US could enhance the anti-tumor activity of cisplatin in NSCLC cells. We also found that cisplatin combined with low-intensity US treatment promoted the expression of miR-124 and knockdown of miR-124 could inhibit the synergistic anti-tumor effects of cisplatin combined with low-intensity US. Furthermore, we demonstrated that cisplatin combined with low-intensity US exerts the synergistic anti-tumor activity through miR-124/STAT3 signaling pathway.

In recent years, a great number of studies have reported the mechanism of the synergistic anti-tumor activity of low-intensity US, including non-thermal effect, cavitation effect and direct mechanical force (Dalecki, 2004). For example, Li et al. had demonstrated that low-intensity US combined with scutellarin allowed drugs to be delivered to cancer cells by increasing the frequency and contact area between the drug and cells (Li et al., 2013). Sundaram et al. reported that the key mechanism of ultrasound-enhanced chemotherapy might be due to cavitation-generated ROS production (Sundaram et al., 2003). A recent study from Hu et al. showed that ROS generation play an important role in synergistic antitumor effects of low-intensity ultrasound combined with 5-FU in hepatocellular carcinoma (HCC) cells (Hu et al., 2016). Herein, we found low-intensity US enhanced the anti-tumor activity of cisplatin by inhibiting cell viability, promoting ROS production and cell apoptosis in NSCLC cells. The present findings suggest that increased ROS production is the main mechanism responsible for the combined effects of low-intensity US and cisplatin. However, the possible molecular mechanism needs further research to be understood deeply.

Recent studies verified that ROS could alter the expression of several microRNAs. For example, H₂O₂ exposure led to the up-regulation of miR-21 while decreased the expressions of miR-29b, miR-328 and so on

(He and Jiang, 2016). A recent study from Jajoo et al. found that high ROS contributed to increased metastatic potential of the prostate cancer cells through its regulation of miR-21 (Jajoo et al., 2013). Based on the above-mentioned findings, we assume that there exists novel miRNAs that could play an important role in the synergistic antitumor effects of cisplatin and low-intensity US. In the present study, we demonstrated a comprehensive miRNA expression profile in A549 cells after cisplatin combined with low-intensity US treatment and found that miR-124 was one of the most being up-regulated miRNAs. Moreover, the up-regulation of miR-124 can be abrogated when ROS are depressed, indicating that cisplatin combined with low-intensity US increased the expression of miR-124 via ROS production. More recently, several studies have reported that miR-124 functions as a tumor suppressor in a variety of human cancers, such as prostate cancer (Wu et al., 2017), nasopharyngeal carcinoma (NPC) (Hu et al., 2017), breast cancer (Feng et al., 2016) and colorectal cancer (Zhou et al., 2016). Subsequently, we investigated the function of miR-124 and found that knockdown of miR-124 inhibited the synergistic antitumor effects of cisplatin combined with low-intensity US by promoting cell viability and suppressing apoptosis. These results suggested that cisplatin combined with low-intensity US exerts the synergistic antitumor effects through ROS mediated up-regulation of miR-124.

The signal transducer and activator of transcription (STAT) pathway has been implicated in various human cancers including NSCLC (Wu et al., 2013; Khan et al., 2015; Lee et al., 2015), suggesting that STAT3 may contribute to the tumor progression of NSCLC. Moreover, the STAT3 pathway is an important anti-apoptotic pathway that is frequently activated in different cancer cells (Du et al., 2012; Jorvig and Chakraborty, 2015; Maryam et al., 2017). Wang et al. found that the activation of STAT3 signaling was involved in the suppression of CXCL12

on cisplatin-induced apoptosis in human non-small-cell lung cancer cells (Wang et al., 2017). Recent research has demonstrated that miR-124 over-expression suppressed proliferation and induced apoptosis in HepG-2 cells by targeting STAT3 (Lu et al., 2013). In the present study, we demonstrated that knockdown of miR-124 reactivated the STAT3 signaling pathway that was blocked by cisplatin combined with low-intensity US treatment, therefore STAT3, as a target for miR-124, may be involved in the synergistic anti-tumor effect of cisplatin combined with low-intensity US.

In conclusion, the present findings demonstrated that low-intensity US can enhance the anti-tumor activity of cisplatin in A549 cells and miR-124/STAT3 axis plays important roles in the much enhanced synergistic anti-tumor activity of cisplatin combined with low-intensity US. These results generated a rationale for further investigation of low-intensity US combined with cisplatin as a potential therapeutic strategy for patients with NSCLC.

Acknowledgements

None.

Conflict of interest

We all declare that we have no conflict of interest.

REFERENCES

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281-97.

Bhattacharya S, Ray RM, Johnson LR. STAT3-mediated transcription of Bcl-2, Mcl-1 and c-IAP2 prevents apoptosis in polyamine-depleted cells. *Biochem J*. 2005;392:335-44.

Cheng Y, Li Y, Nian Y, Liu D, Dai F, Zhang J. STAT3 is involved in miR-124-mediated suppressive effects on esophageal cancer cells. *BMC Cancer*. 2015;15:306.

Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*. 2009;10:704-14.

Dalecki D. Mechanical bioeffects of ultrasound. *Annu Rev Biomed Eng*. 2004;6:229-48.

Du W, Hong J, Wang YC, Zhang YJ, Wang P, Su WY, et al. Inhibition of JAK2/STAT3 signalling induces colorectal cancer cell apoptosis via mitochondrial pathway. *J Cell Mol Med*. 2012;16:1878-88.

Feng T, Shao F, Wu Q, Zhang X, Xu D, Qian K, et al. miR-124 downregulation leads to breast cancer progression via LncRNA-MALAT1 regulation and CDK4/E2F1 signal activation. *Oncotarget*. 2016;7:16205-16.

Hao C, Xu X, Ma J, Xia J, Dai B, Liu L, et al. MicroRNA-124 regulates the radiosensitivity of non-small cell lung cancer cells by targeting TXNRD1. *Oncol Lett*. 2017;13:2071-8.

He J, Jiang BH. Interplay between Reactive oxygen Species and MicroRNAs in Cancer. *Curr Pharmacol Rep*. 2016;2:82-90.

He G, He G, Zhou R, Pi Z, Zhu T, Jiang L, et al. Enhancement of cisplatin-induced colon cancer cells apoptosis by shikonin, a natural inducer of ROS in vitro and in vivo. *Biochem Biophys Res Commun*. 2016;469:1075-82.

Hu Z, Lv G, Li Y, Li E, Li H, Zhou Q, et al. Enhancement of anti-tumor effects of 5-fluorouracil on hepatocellular carcinoma by low-intensity ultrasound. *J Exp Clin Cancer Res*. 2016;35:71.

Hu H, Wang G, Li C. miR-124 suppresses proliferation and invasion of nasopharyngeal carcinoma cells through the Wnt/beta-catenin signaling pathway by targeting Capn4. *Onco Targets Ther*. 2017;10:2711-20.

Jajoo S, Mukherjea D, Kaur T, Sheehan KE, Sheth S, Borse V, et al. Essential role of NADPH oxidase-dependent reactive oxygen species generation in regulating microRNA-21 expression and function in prostate cancer. *Antioxid Redox Signal*. 2013;19:1863-76.

Jorvig JE, Chakraborty A. Zerumbone inhibits growth of hormone refractory prostate cancer cells by inhibiting JAK2/STAT3 pathway and increases paclitaxel sensitivity. *Anticancer Drugs*. 2015;26:160-6.

Jove R. Preface: STAT signaling. *Oncogene*. 2000;19:2466-7.

Kasprzycka M, Marzec M, Liu X, Zhang Q, Wasik MA. Nucleophosmin/anaplastic lymphoma kinase (NPM/ALK) oncoprotein induces the T regulatory cell phenotype by activating STAT3. *Proc Natl Acad Sci U S A*. 2006;103:9964-9.

- Khan M, Maryam A, Qazi JI, Ma T. Targeting apoptosis and multiple signaling pathways with icaraside II in cancer cells. *Int J Biol Sci.* 2015;11:1100-12.
- Lee JH, Kim C, Sethi G, Ahn KS. Brassinin inhibits STAT3 signaling pathway through modulation of PI-AS-3 and SOCS-3 expression and sensitizes human lung cancer xenograft in nude mice to paclitaxel. *Oncotarget.* 2015;6:6386-405.
- Lejbkowitz F, Salzberg S. Distinct sensitivity of normal and malignant cells to ultrasound in vitro. *Environ Health Perspect.* 1997;105(Suppl 6):1575-8.
- Lejbkowitz F, Zwiran M, Salzberg S. The response of normal and malignant cells to ultrasound in vitro. *Ultrasound Med Biol.* 1993;19:75-82.
- Li H, Fan H, Wang Z, Zheng J, Cao W. Potentiation of scutellarin on human tongue carcinoma xenograft by low-intensity ultrasound. *PLoS One.* 2013;8:e59473.
- Li Y, Qi K, Zu L, Wang M, Wang Y, Zhou Q. Anti-apoptotic brain and reproductive organ-expressed proteins enhance cisplatin resistance in lung cancer cells via the protein kinase B signaling pathway. *Thorac Cancer.* 2016;7:190-8.
- Liu S, Hu C, Wang Y, Shi G, Li Y, Wu H. miR-124 inhibits proliferation and invasion of human retinoblastoma cells by targeting STAT3. *Oncol Rep.* 2016;36: 2398-404.
- Lu Y, Yue X, Cui Y, Zhang J, Wang K. MicroRNA-124 suppresses growth of human hepatocellular carcinoma by targeting STAT3. *Biochem Biophys Res Commun.* 2013;441:873-9.
- Ma X, Dang C, Kang H, Dai Z, Lin S, Guan H, et al. Saikosaponin-D reduces cisplatin-induced nephrotoxicity by repressing ROS-mediated activation of MAPK and NF-kappaB signalling pathways. *Int Immunopharmacol.* 2015;28:399-408.
- Maryam A, Mehmood T, Zhang H, Li Y, Khan M, Ma T. Alantolactone induces apoptosis, promotes STAT3 glutathionylation and enhances chemosensitivity of A549 lung adenocarcinoma cells to doxorubicin via oxidative stress. *Sci Rep.* 2017;7:6242.
- Masui T, Ota I, Kanno M, Yane K, Hosoi H. Low-intensity ultrasound enhances the anticancer activity of cetuximab in human head and neck cancer cells. *Exp Ther Med.* 2013;5:11-6.
- McHale AP, Callan JF, Nomikou N, Fowley C, Callan B. Sonodynamic therapy: concept, mechanism and application to cancer treatment. *Adv Exp Med Biol.* 2016;880:429-50.
- Melnik BC. MiR-21: an environmental driver of malignant melanoma? *J Transl Med.* 2015;13:202.
- Simone NL, Soule BP, Ly D, Saleh AD, Savage JE, Degraff W, et al. Ionizing radiation-induced oxidative stress alters miRNA expression. *PLoS One.* 2009;4:e6377.
- Sundaram J, Mellein BR, Mitragotri S. An experimental and theoretical analysis of ultrasound-induced permeabilization of cell membranes. *Biophys J.* 2003;84:3087-101.
- Ting CM, Wong CK, Wong RN, Lo KW, Lee AW, Tsao GS, et al. Role of STAT3/5 and Bcl-2/xL in 2-methoxyestradiol-induced endoreduplication of nasopharyngeal carcinoma cells. *Mol Carcinog.* 2012;51: 963-72.
- Wang M, Lin T, Wang Y, Gao S, Yang Z, Hong X, et al. CXCL12 suppresses cisplatin-induced apoptosis through activation of JAK2/STAT3 signaling in human non-small-cell lung cancer cells. *Onco Targets Ther.* 2017;10:3215-24.
- Watanabe Y, Aoi A, Horie S, Tomita N, Mori S, Morikawa H, et al. Low-intensity ultrasound and microbubbles enhance the antitumor effect of cisplatin. *Cancer Sci.* 2008;99:2525-31.
- Wu K, Chang Q, Lu Y, Qiu P, Chen B, Thakur C, et al. Gefitinib resistance resulted from STAT3-mediated Akt activation in lung cancer cells. *Oncotarget.* 2013;4: 2430-8.
- Wu DW, Lee MC, Hsu NY, Wu TC, Wu JY, Wang YC, et al. FHIT loss confers cisplatin resistance in lung cancer via the AKT/NF-kappaB/Slug-mediated PUMA reduction. *Oncogene.* 2015;34:3882-3.
- Wu Z, Huang W, Chen B, Bai PD, Wang XG, Xing JC. Up-regulation of miR-124 inhibits invasion and proliferation of prostate cancer cells through mediating JAK-STAT3 signaling pathway. *Eur Rev Med Pharmacol Sci.* 2017;21:2338-45.
- Xu S, Zhao N, Hui L, Song M, Miao ZW, Jiang XJ. MicroRNA-124-3p inhibits the growth and metastasis of nasopharyngeal carcinoma cells by targeting STAT3. *Oncol Rep.* 2016;35:1385-94.
- Xu H, Wu Y, Li L, Yuan W, Zhang D, Yan Q, et al. MiR-344b-1-3p targets TLR2 and negatively regulates TLR2 signaling pathway. *Int J Chron Obstruct Pulmon Dis.* 2017;12:627-38.

Yoshida T, Kondo T, Ogawa R, Feril LB Jr, Zhao QL, Watanabe A, et al. Combination of doxorubicin and low-intensity ultrasound causes a synergistic enhancement in cell killing and an additive enhancement in apoptosis induction in human lymphoma U937 cells. *Cancer Chemother Pharmacol.* 2008;61:559-67.

Yu HG, Wei W, Xia LH, Han WL, Zhao P, Wu SJ, et al. FBW7 upregulation enhances cisplatin cytotoxicity in non- small cell lung cancer cells. *Asian Pac J Cancer Prev.* 2013;14:6321-6.

Zhao X, Lu C, Chu W, Zhang B, Zhen Q, Wang R, et al. MicroRNA-124 suppresses proliferation and glycolysis in non-small cell lung cancer cells by targeting AKT-GLUT1/HKII. *Tumour Biol.* 2017;39:1010428317706215.

Zhou L, Xu Z, Ren X, Chen K, Xin S. MicroRNA-124 (MiR-124) inhibits cell proliferation, metastasis and invasion in colorectal cancer by downregulating Rho-Associated Protein Kinase 1 (ROCK1). *Cell Physiol Biochem.* 2016;38:1785-95.