miR-520b inhibits IGF-1R to increase doxorubicin sensitivity and promote cell apoptosis in breast cancer

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Abstract

Doxorubicin (DOX) is currently one of the most widely used and effective drugs for the treatment of breast cancer, but drug resistance in breast cancer often leads to poor efficacy. MicroRNAs (miRNAs) are involved in the development and progression of various tumors and increasing number of studies have confirmed that abnormal miR-520b expression is closely associated breast cancer. We analyzed the clinical features, including miR-520b, of 30 patients with breast cancer. Further, we analyzed the interaction between miR-520b and insulin-like growth factor 1 receptor (IGF-1R) in breast cancer cell. miR-520b expression was significantly increased in chemotherapy-sensitive patients and was positively correlated with the chemotherapeutic efficacy in breast cancer. Cell proliferation assay confirmed that miR-520b promotes DOX-induced breast cancer cell apoptosis by regulating the PI3K/AKT signaling pathway. Moreover, bioinformatics method and dual luciferase reporter assay demonstrated that miR-520b negatively regulates IGF-1R, and IGF-1R overexpression and enhanced activity are closely associated with tumor development, progression, metastasis, and chemotherapy resistance. Similarly, cell proliferation assay showed that IGF-1R is negatively correlated with the efficacy of DOX chemotherapy and affects cell apoptosis mediated by the PI3K/AKT signaling pathway. On the contrary, miR-520b can downregulate the expression of IGF-1R. miR-520b increases DOX sensitivity and promotes cell...
apoptosis in breast cancer by inhibiting IGF-1R expression by the PI3K/AKT signaling pathway.

**Keywords:** miR-520b; IGF-1R; doxorubicin; breast cancer; chemotherapy resistance

**Introduction**

According to the 2012 Globocan statistics on breast cancer incidence and mortality by the International Agency for Research on Cancer, there were new cases of breast cancer and 458,000 deaths every year\(^1\). The number of breast cancer-related deaths has increased by 113,000 just within 7 years. The global incidence of breast cancer has been increasing at an average annual rate of 3\(^\%\) (0.2\%-8\%) in the past decade, and the incidence of breast cancer in China has been increasing more rapidly at an average annual rate of 3\%-4\% since the 1990s\(^3\). Although breast cancer is not gender-specific like many reproductive cancers, women account for more than 99\% of all breast cancer patients. Therefore, breast cancer is the most common cancer that seriously endangers women’s health and is currently a global health problem.

Molecular typing of breast cancer is a common clinical method used to provide targeted treatment and to help with the judgment of prognosis in breast cancer patients. There are four molecular subtypes of breast cancer, namely luminal A, luminal B, triple negative and Her-2 positive\(^4\). Chemotherapy is still the primary treatment for breast cancer, and doxorubicin (DOX) is commonly used in clinical practice\(^5\). However, as the course of treatment progresses, tumors often develop drug resistance which can eventually lead to chemotherapy failure\(^6\). Many studies have attempted to elucidate the development of chemotherapy resistance, but the underlying mechanism remains unclear.

DOX is one of the most widely used and effective anthracycline chemotherapeutic drugs for the treatment of breast cancer\(^6\). Chemotherapy drug resistance is the primary factor that limits the desired therapeutic effect of DOX in breast cancer, and reversion of DOX resistance may help improve its efficacy in breast cancer. However, the mechanism of DOX resistance is still elusive\(^7\).
MicroRNAs (miRNAs) are a type of newly discovered non-coding RNA that are 19-25 base pairs in length\(^8\). Several miRNAs, including miR-21\(^9\), miR-22\(^{20}\), miR-328\(^{11}\) and miR-451\(^{12}\), have been shown to play a regulatory role in the development of drug resistance in breast cancer\(^{13}\). Recent studies have found that miR-520b expression is significantly downregulated in various cancers, such as bladder cancer, liver cancer, and breast cancer\(^{14-16}\). Furthermore, miR-520b regulates the immune escape of breast cancer cells through complement binding proteins and specific signal transduction pathways\(^{16}\).

IGF 1 receptor (IGF-1R) is a member of the insulin-like growth factor (IGF) family. Upon the binding of IGF1 and IGF2, IGF-1R is phosphorylated and activates PI3K and MAPK signaling, which in turn can regulate cell proliferation and differentiation as well as inhibit cell apoptosis\(^{17,18}\). Previous studies have shown that IGF-1R and its ligands are abnormally expressed in patients with various malignancies, including breast cancer, endometrial cancer, prostate cancer and non-small cell lung cancer, and play a supporting role in the development and progression of malignant tumors\(^{19,20}\). Overexpression and enhanced activity of IGF-1R are closely related to tumor development, progression, metastasis, and chemotherapy resistance\(^{21,22}\).

In this study, we analyzed the differential expression of miR-520b between chemotherapy-sensitive and drug-resistant breast cancer patients and examined the relationship between miR-520b expression and chemotherapy efficacy. Moreover, we investigated the regulatory interaction between miR-520b on IGF-1R and its effect on drug resistance in breast cancer.

**Methods**

**Clinical samples**

A total of 30 female patients confirmed with primary invasive breast cancer by paraffin pathology in the Galactophore Department in Chongqing Cancer Hospital between January to December 2017 were enrolled in this study. All patients have completed neoadjuvant anthracycline chemotherapy before surgery and had complete clinical and pathological data. Core needle biopsy
specimens of the tumor lesions of the patients were obtained before surgery and immediately sent to the tumor laboratory specimen bank for -80°C storage. Treatment regimen was selected according to the Chinese Anti-Cancer Association Guidelines and Specifications for the Diagnosis and Treatment of Breast Cancer (2017 Edition). Breast MRI was performed, and treatment response of the target lesions was evaluated using the RICIST 1.1 criteria before and after neoadjuvant chemotherapy before surgery. The efficacy of neoadjuvant chemotherapy was pathologically evaluated using the Miller-Payne grading system after surgery\(^{(5,23)}\). This study was approved by the Ethics Committee of Chongqing Cancer Hospital, and all patients have signed the informed consent form. All patients had no history of other malignancies and treatments.

**Cell lines and culture**

Human breast cancer cell lines MCF-7 and MDA-MB-231 and DOX-resistant cell lines MCF-7/ADR and MDA-MB-231/ADR were donated by Professor Weihua Sheng from the Laboratory of Cell and Molecular Biology, Soochow University\(^{[6]}\). Cells were cultured in DMEM culture medium (Corning, Shanghai, cat: 10-03-CVRC) containing 10% FBS (Corning, Shanghai, cat: 04-001-1A), 100 U/mL penicillin (North China.), and 100 mg/mL streptomycin (Shandong Lukang) at 37°C with 5% CO₂.

**RNA extraction and real-time fluorescence quantitative PCR detection**

Total sample RNA was extracted using the Trizol Total RNA Extraction Kit (Tiangen, Tianjin, cat: DP424) according to the instructions. After the integrity of the total RNA was tested, the concentration (A260) and purity (A260/280) of the extracted RNA samples were measured using a micro nucleic acid analyzer. RNA samples were reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Thermofisher, Chongqing, cat: 28015-013), and miR-520b expression was measured by real-time quantitative PCR (qRT-PCR) using a real-time quantitative PCR kit (Takara, Dalian, cat: RR096A) and U6 as the internal reference gene. In addition, melting curves were used to assess nonspecific amplification. The qRT-PCR results were shown as the Ct (threshold cycle)
and transferred to fold changes \((2^{-\Delta\Delta Ct}}\) or \(2^{-\Delta Ct}\).

**Cell transfection**

miR-520b mimics, miR-520b inhibitor and control oligonucleotide miR-NC plasmids were purchased from Guangzhou RiboBio Co., Ltd. All plasmids were transfected with Lipofectamine 2000 according to the instructions.

**Lentivirus packaging**

IGF-1R overexpression vector was constructed by cloning the IGF-1R ORF sequence into the pGCL vector, and the IGF-1R interfering plasmid IGF-1R-siRNA was constructed by Shanghai Genechem Co., Ltd. The interfering sequence for IGF-1R siRNA (5’-3’) was 5’-AUACGGAUCACAAGUUGAG-3’. The plasmids were transformed into E. coli DH5α, extracted and confirmed by sequencing. The packaging plasmid and recombinant lentivirus plasmids were transfected into 293T cells and the culture medium was collected after 72 hours. After removal of cell debris by centrifugation, the virus stock was collected in an ultracentrifuge tube, centrifuged at 20,000 r/min for 120 min at 4°C, aliquoted and stored at -80°C.

**Screening of stably transfected cell lines**

MCF-7/ADR and MDA-MB-231/ADR cells were seeded at 4x10⁵ cells/well into a 6-well plate and cultured at 37°C and 50% CO₂ until 50%-80% confluency. After 24 h of culture, cells were infected with 4x10⁶ TU lentivirus at MOI=10 in opti-MEM antibiotic-free and serum-free culture medium. After 6 h of infection, cells were washed with PBS and cultured in fresh complete medium containing puromycin for another 72 h to obtain stable cell lines, including MCF-7/ADRIGF-1R, MCF-7/ADRvector and MDA-MB-231/ADRIGF-1R, MDA-MB-231/ADRvector, as well as MCF-7/ADRmiR-520b+IGF-1R and MDA-MB-231/ADRmiR-520b+IGF-1R.

**Determination of cell viability**
Cell viability was determined by the Cell Proliferation Reagent Kit I (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide [MTT]). To determine the effect of miR-520b on DOX sensitivity in breast cancer cells, MCF-7/ADR and MDA-MB-231/ADR cells were cultured in a 6-well plate and transfected with miR-520b mimics or miR-NC for 24 h. To examine how miR-520b regulates IGF-1R to affect DOX sensitivity in breast cancer cells, MCF-7 cells were co-transfected with miR-520b inhibitor+NC or miR-520b inhibitor+si-IGF-1R, and MDA-MB-231/ADR cells were co-transfected with miR-520b mimics+NC or miR-520b mimics+IGF-1R overexpression plasmid. After 24 h of transfection and culture, cells were collected, resuspended into a cell suspension and seeded into a 96-well plate at 5x10^3 cells/well. After 72 h of culture, each group of cells was treated with 8 different concentrations of DOX (60 μg/mL, 30 μg/mL, 15 μg/mL, 7.5 μg/mL, 3.75 μg/mL, 1.875 μg/mL, 0.9375 μg/mL and 0.46875 μg/mL) with 5 replicate wells per concentration. On the other hand, MDA-MB-231/ADR-miR-520 and MDA-MB-231/ADR-NC cells were treated with 40 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL, 0.625 μg/mL or 0.3125 μg/mL DOX. After another 48 h of culture, the culture medium was discarded, and 100 μl of MTT and DMEM mixture was added to each well at a ratio of 1:10. Cells were culture for 1.5 h and the absorbance value at 490nm was measured using a microplate reader.

**Flow cytometry**

MCF-7/ADR and MDA-MB-231/ADR cells were treated with DOX at 24 h after transfection and control cells were treated with DMSO. MCF-7/ADR-NC and MCF-7/ADR-miR-520b cells were treated with 5.0 μg/mL of DOX, while MDA-MB-231/ADR-NC and MDA-MB-231/ADR-miR-520b cells were treated with 3.0 μg/mL DOX. After 48 h of culture, cells were digested with EDTA-free trypsin, centrifuged and resuspended in 100 μL of 1× binding buffer into 1×10^6 cells/mL single-cell suspension. Preparation of single stained cells: Three tubes of 100 μL cell suspension were obtained from each of the above groups. Two tubes were placed in a 70°C water bath for 30 s to induce apoptosis, then one tube was stained with 5 μL of Propidium Iodide (Invitrogen, Beijing,
cat: P3566) and the other was stained with Annexin V-FITC (Invitrogen, cat: A13199) to make single-stain controls. The third tube was not stained. Each tube of cells was mixed gently by pipetting and incubated at room temperature in the dark for 15 min. Preparation of double-stained cells: Each tube of cell suspension was gently mixed and stained with 5 μL of Propidium Iodide (Invitrogen, cat: P3566) at room temperature in the dark for 15 min, then mixed and stained with 5 μL of Annexin V-FITC (Invitrogen, cat: A13199) in the dark for 15 min. Cell apoptosis was analyzed by flow cytometry.

**Western Blot**

Cells were lysed with 100:1 RIPA lysis buffer and PMSF (100 mM), and cell lysates were used for WB detection. Protein concentration was measured using the BCA assay and adjusted to the same concentration across samples. Protein samples were reduced, separated by 10% SDS-PAGE, and transferred onto a PVDF membrane. PVDF membrane was blocked with 5% skim milk on a rocker at room temperature for 2 h or at 4°C overnight, then stained with chromogenic substrates. Protein bands on the PVDF membrane were visualized using the Bio-Rad gel imaging system. Band intensity was normalized to the internal reference GAPDH and the gray values were calculated using Image J. The relative expression of the target protein in each group was calculated by the optical density ratio (OD ratio) of the target protein to GAPDH. Primary antibodies: Rabbit monoclonal to anti-IGF-1R (Abcam, Beijing, cat: ab263907); rabbit monoclonal to PI3CK (Abcam, cat: ab40776); rabbit anti p-AKT (Abcam, cat: 38449); rabbit to AKT (Abcam, cat: ab179463); rabbit anti Bcl-2 (Abcam, cat: ab32124); rabbit anti-Bax (Abcam, cat: ab32503); mouse anti-GAPDH (Abcam, cat: ab8245). Secondary antibodies: goat anti rabbit (Abcam, cat: ab6721); rabbit anti mouse (Abcam, cat: ab190475).

**Dual luciferase reporter assay**

We searched and download datasets related to neoadjuvant chemotherapy for breast cancer from the GEO and EMBL-EBI EGA databases, and used target gene prediction software (PITA,
miRmap, mirWalk3.0 and Encori) to predict miR-520b binding sites on IGF-1R. Wild-type (IGF-1R-WT) and mutant (IGF-1R-MUT) IGF-1R sequences were cloned into the reporter vector pmirGLO to construct pmirGLO-IGF-1R-WT and pmirGLO-IGF-1R-MUT plasmids. MCF-7 cells were cultured in a 24-well plate until 60% confluency, then co-transfected with either one of these plasmids and miR-520b mimics or miR-NC using Lipofectamine 2000. Cells were lysed after 24 h of culture, and the luminescence values of firefly luciferase and renilla luciferase were measured by Varioskan Lux to calculate the relative fluorescence value. Details of the assay can be found in the instructions of the Dual-luciferase Reporter Assay (Promega, cat: E1910).

**Statistical analysis**

All data were processed using the SPSS 24.0 statistical software package and plotted using GraphPad Prism 7.0. Data are expressed as “mean ± standard deviation” and compared between groups using the independent sample t-test. Correlation was analyzed using Pearson correlation analysis. All experiments repeat 3 times. P<0.05 is considered statistically significant.

**Ethics statement**

The present study protocol was reviewed and approved by the Institutional Review Board of (approval No. 2018001). Informed consent was submitted by all subjects when they were enrolled.

**Results**

**miR-520b increased DOX sensitivity in breast cancer cells to DOX and inhibited cell proliferation**

Core needle biopsy specimens were collected from the 30 breast cancer patients before
neoadjuvant chemotherapy (see Table 1 for the clinicopathological data of the patients) and miR-520b mRNA expression was measured by qPCR. The correlation between relative miR-520 expression and post-chemotherapy tumor lesion reduction evaluated by breast MRI before surgery was analyzed. We found that miR-520b expression was positively correlated with the efficacy of neoadjuvant chemotherapy in breast cancer patients (P<0.001), where higher miR-520b expression resulted in better response to neoadjuvant chemotherapy (Fig. 1A). Postoperative tissue specimens of the enrolled patients were pathologically evaluated according to the Miller-Payne grading system to determine the efficacy of neoadjuvant chemotherapy. We assigned the Miller-Payne grades 1-2 to the chemotherapy-resistant group and grades 3-5 to the sensitive group. Our results showed that miR-520b expression was significantly higher in the sensitive group than in the resistant group (P<0.001) (Figure 1B), which indicates that miR-520b can increase chemotherapy sensitivity in patients to improve efficacy. We examined the differential expression of miR-520b between breast cancer cell lines MCF-7 and MDA-MB-231 and their corresponding DOX-resistant cell lines MCF-7/ADR and MDA-MB-231/ADR, and found that miR-520 expression was significantly downregulated in MCF-7/ADR and MDA-MB-231/ADR cells than in MCF-7 and MDA-MB-231 cells (P<0.001) (Fig. 1C and D). Next, we examined the effects of miR-520b on breast cancer cell proliferation using the cell proliferation assay and MTT assay. We transfected DOX-treated breast cancer cells with miR-520b mimics and found that compared with the DOX monotherapy group, the miR520b+DOX group had significantly reduced number of breast cancer cells and inhibited cell proliferation (Fig. 1E). The IC50 of DOX was significantly lower in the miR-520b mimics group than in the control group (P<0.001) (Fig. 1F and G). In addition, cell proliferation assay showed that miR-520b can inhibit cell proliferation by increasing DOX sensitivity in breast cancer cells.

**miR-520b promotes DOX-induced breast cancer cell apoptosis**

To determine the impact of miR-520b on the biological progression of breast cancer cells, we analyzed the effects of miR-520b on breast cancer cell apoptosis by flow cytometry. We found that
miR-520b mimics transfection resulted in significantly increased MCF-7/ADR cell apoptosis in the DOX group compared with the control group (P<0.001). However, there was no significant difference in apoptosis between the DOX group and the control group when the cells were not transfected with miR-520b mimics (Fig. 2A, C). Similarly, miR-520b overexpression also promoted apoptosis in DOX-treated MDA-MB-231/ADR cells (Fig. 2B, D). We subsequently examined the effects of miR-520b on the activity of apoptosis-related caspase-3/7 and caspase-9 in breast cancer cell lines MCF-7/ADR and MDA-MB-231/ADR by qRT-PCR and found that miR-520b mimics transfection significantly increased the activity of caspase 3/7 and caspase 9 both DOX-treated and DOX-untreated MCF-7/ADR and MDA-MB-231/ADR cells (P<0.001) (Fig. 2E-F, G, H). Finally, we used RT-PCR to measure the mRNA levels of apoptosis- and survival-related genes in MCF-7/ADR and MDA-MB-231/ADR cells. Our results showed that bax expression was significantly upregulated while survivin expression was significantly downregulated following miR-520b mimics transfection (P<0.001) (Fig. 2 I, J). Apoptosis assay also confirmed that miR-520b can promote DOX-induced breast cancer cell apoptosis.

**miR-520b influences breast cancer cell apoptosis via the PI3K/AKT pathway**

To further elucidate the exact mechanism by which miR-520b promotes MCF-7/ADR and MDA-MB-231/ADR cell apoptosis, we used Western blot to measure the changes in the expression of PI3K/AKT signaling pathway-related proteins. We found that miR-520b mimics transfection significantly downregulated PIK3CA, p-AKT and Bcl-2 protein expression, upregulated bax protein expression, and had no impact on t-AKT protein expression. The Western blot result of bax expression was consistent with that observed by RT-PCR (Fig. 3 A-C). These findings demonstrate that miR-520b promotes drug-resistant cell apoptosis and inhibits drug resistance in breast cancer cells by downregulating PI3K/AKT signaling pathway-related proteins, inhibiting Bcl-2 expression, and promoting bax expression.

**miR-520b negatively regulates IGF-1R**
We next analyzed the downstream target genes of miR-520b using bioinformatics. Based on the results of the microRNA target gene online prediction databases (PITA, miRmap, mirWalk3.0 and Encori), IGF-1R may be a direct target gene of miR-520b, and the binding sites are shown in Fig. 4A. Furthermore, we used the dual luciferase reporter assay to determine the interactions between miR-520b and IGF-1R, and found that upregulation of miR-520b expression in MCF-7 cells significantly inhibited the expression of the luciferase reporter gene containing IGF-1R 3’UTR, which indicated that IGF-1R is directly regulated by and a direct target gene of miR-520b (Fig. 4B). Moreover, correlation analysis of miR-520b and IGF-1R expression in 1,085 cases of breast cancer in the TCGA database using starBase 3.0 database (http://starbase.sysu.edu.cn/) indicated that miR-520b was negatively corelated with IGF-1R expression (r=-0.087, P<0.001) (Fig. 4C). Furthermore, we measured miR-520b and IGF-1R expression in the breast cancer tissues of 30 patients before neoadjuvant chemotherapy by qPCR, and found that miR-520b expression was negatively correlated with IGF-1R expression (Fig. 4D) and IGF-1R expression was negatively corelated with the efficacy of neoadjuvant chemotherapy in breast cancer patients (P<0.05). These findings suggest that lower IGF-1R expression leads to better chemotherapy efficacy and greater post-chemotherapy reduction in lesion size (Fig. 4E). In addition, correlation analysis of IGF-1R expression and the efficacy of chemotherapy showed that IGF-1R was differentially expressed between the chemotherapy-resistant group and the chemotherapy-sensitive group (P<0.05) (Fig. 4F), which indicates that miR-520b affects the efficacy of chemotherapy in breast cancer patients by negatively regulating IGF-1R expression.

miR-520b inhibits IGF-1R expression in breast cancer cell lines

We previously demonstrated that miR-520b can negatively regulate IGF-1R gene expression in tumor tissues and IGF-1R inhibits chemotherapy sensitivity in breast cancer patients. Next, we measured IGF-1R expression in breast cancer cell lines by qRT-PCR and found that IGF-1R expression was significantly upregulated in the drug-resistant breast cancer cell lines MCF-7/ADR.
and MDA-MB-231/ADR (Fig. 5A and B). Furthermore, IGF-1R expression was significantly downregulated in MCF-7/ADR and MDA-MB-231/ADR cells overexpressing miR-520b than in control cells (Fig. 5C and D). Consistent with the changes in IGF-1R mRNA level, Western blot analysis demonstrated that miR-520b overexpression inhibited IGF-1R protein expression in drug-resistant breast cancer cell lines (Fig. 5E, F).

**IGF-1R suppresses DOX sensitivity in breast cancer cells**

Firstly, we detected IGF-1R expression level in MCF-7 and MDA-MB-231 that transfected with IGF-1R-over plasmid by western blot. The results showed IGF-1R expression level was upregulated (Fig. 6 A, B). We used the MTT assay to analyze the effects of IGF-1R on drug resistance in breast cancer cells and found that IGF-1R-transfected MCF-7 (Fig. 6C) and MDA-MB-231 cells (Fig. 6D) had reduced sensitivity to DOX. In contrast, when transfected with si-IGF-1R, the IC50 of DOX in MDA-MB-231/ADR cells is lower than the control group (Fig. 6E), which indicates that IGF-1R suppresses DOX sensitivity. Moreover, because the negative regulation between miR-520b and IGF-1R, we use miR-520b inhibitor to eliminate inhibition effect of miR-520b. The results show that the IC50s of miR-520b inhibitor and si-IGF-1R co-transfected are lower than mir-520b inhibitor group both in MCF and MDA-MB-231/ADR cells (Fig. 6 F, G).

**miR-520b inhibits IGF-1R to promote breast cancer cell apoptosis**

Analysis of the effects of miR-520b-mediated negative regulation of IGF-1R on breast cancer cell apoptosis by flow cytometry showed that IGF-1R overexpression significantly decreased the apoptosis of DOX-treated MCF-7 and MDA-MB-231/ADR cells (Fig. 7A-D), which suggests that IGF-1R can lead to chemotherapy resistance in breast cancer and affects the apoptotic effects of chemotherapy on breast cancer cells. Similarly, qRT-PCR analysis of the changes in the activity of apoptosis-related caspase-3/7 and caspase-9 showed that miR-520b mimics transfection significantly increased the activity of caspase 3/7 and caspase 9 in DOX-treated MCF-7 and MDA-MB-231/ADR cells (Fig. 7E-H)(P<0.001). Further, when co-transfected with miR-520b mimics
and IGF-1R, the activity of apoptosis-related caspase-3/7 and caspase-9 are slightly reduced than miR-520b mimics. The results indicate miR-520b can promote cell apoptosis in breast cancer through inhibit the function of IGF-1R.

Discussion

According to the GLOBOCAN statistics, there are about 14 million new cancer cases and about 8 million cancer deaths every year, which have imposed a substantial burden on the society. The incidence of breast cancer has been increasing worldwide and ranks first among female malignancies. Although chemotherapy is the primary treatment for breast cancer, it often fails due to the development of drug resistance in tumors24). Therefore, further understanding of the mechanism of drug resistance in breast cancer is paramount to the improvement of chemotherapeutic drug sensitivity and survival in breast cancer patients25).

DOX is currently one of the most widely used and effective anthracyclines for the treatment of breast cancer26,27). However, drug resistance in breast cancer is the main factor that limits the desired therapeutic effect of DOX27). Reversion of resistance in breast cancer is a key to improving the efficacy of DOX, but the potential mechanism of DOX resistance is currently unclear. Recent studies have shown that aberrant miRNA expression is involved in the development of drug resistance in tumors28-30). MiRNA can directly target mRNA to regulate certain genes in cell signaling pathways to increase anthracycline sensitivity in tumor cells31-33).

MiRNA-520b is a miRNA that is recently found to be aberrantly expressed in cancer patients, and its relationship with tumors has become a hot topic of research in the scientific community34). Recent studies have shown that miRNA-520b expression is significantly downregulated in various cancers such as gastric cancer, liver cancer, bladder cancer and breast cancer. In addition, miRNA-520b was reported to regulate breast cancer cell immune escape through complement binding protein and specific signal transduction pathways. IGF-1R is a tyrosine kinase receptor that regulates the biological functions and prevents apoptosis of cells under normal physiological conditions. However, under pathological conditions, IGF-1R can induce the malignant
transformation of cells and promote tumor cell division and proliferation. In fact, IGF-1R is highly expressed in various tumor cells and closely associated with radiotherapy- and chemotherapy-resistant tumor phenotypes. Numerous studies have shown that IGF-1R overexpression induces the development of various tumors by promoting the malignant transformation and preventing the apoptosis of cells.

In this study, we found that miR-520b expression was significantly upregulated in the breast cancer tissues of chemotherapy-sensitive patients and was positively correlated with the efficacy of chemotherapy. Moreover, miR-520b expression was significantly higher in MCF-7 and MDA-MB-231 cells than in the drug-resistant MCF-7/ADR and MDA-MB-231/ADR cells, indicating that miR-520b is closely associated with the efficacy of chemotherapy for patients with breast cancer. Our cell proliferation results revealed that miR-520b expression significantly decreased the proliferation of DOX-resistant breast cancer cells. In addition, the cell apoptosis assay demonstrated that miR-520b expression significantly promoted DOX-induced apoptosis, increased caspase3/7/9 activity, upregulated bax mRNA expression, and downregulated survivin mRNA expression. We also showed that miR-520b promoted drug-resistant cell apoptosis and inhibited drug resistance in breast cancer cells by downregulating PI3K/AKT signaling pathway-related proteins, inhibiting Bcl-2 expression, and promoting bax expression. Altogether, these findings demonstrate that miR-520b can inhibit drug resistance and promote breast cancer cell apoptosis by regulating the PI3K/AKT signaling pathway.

We analyzed the downstream target genes of miR-520b and found that IGF-1R is a target of miR-520b, and their negative regulatory interaction was confirmed by the dual luciferase reporter assay. Correlation analysis showed that IGF-1R expression was negatively correlated with the efficacy of chemotherapy in breast cancer patients, which altogether suggests that miR-520b inhibits chemotherapy resistance in breast cancer by downregulating IGF-1R expression. In order to verify these results, we examined the effects of miR-520b on IGF-1R expression at the gene and protein levels and confirmed that miR-520b inhibited IGF-1R mRNA and protein expression in breast cancer cell lines. Furthermore, the cell proliferation and MTT assays demonstrated that IGF-
1R expression enhanced DOX resistance in breast cancer cells whereas miR-520b expression inhibited the effects of IGF-1R and increased DOX sensitivity in breast cancer cells. Similarly, the cell apoptosis assay confirmed that miR-520b inhibited IGF-1R and affected breast cancer cell apoptosis via the PI3K/AKT pathway.

Reversion of DOX resistance may help improve the efficacy of chemotherapy in breast cancer. Our study examined the effects of miR-520b-mediated negative regulation of IGF-1R on drug resistance in breast cancer and provided new insights to the treatment of breast cancer patients. We will continue to elucidate the molecular mechanism of miR-520b in the regulation of IGF-1R in our subsequent studies.

Disclosure: Conflict of Interest

The authors declare no conflict of interest.

Author contributions:
Hui Zhang contributes conceptualization and experiment and writing. Xiaodong Zheng contributes data curation and investigation. Li Li conducts software and validation. Qi Zhou contributes supervision and review.

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Figure 1. miR-520b inhibits the proliferation of breast cancer cells by increasing the sensitivity of doxorubicin.

A Evaluation of the correlation between the expression of microRNA-520b and the efficacy of neoadjuvant chemotherapy in breast cancer patients;
B Differential expression of microRNA-520b between Sensitive group and Resistant group;
C, D qRT-PCR Detection of Differential Expression of MicroRNA-520b in Breast Cancer Cell Lines. MCF-7, MDA-MB-231 and Adriamycin Resistant MCF-7/ADR and MDA-MB-231/ADR;
E Effect of overexpression of MicroRNA-520b on Proliferation of Breast Cancer Cells;
F, G The IC50 of Doxorubicin of MCF-7/ADR and MDA-MB-231/ADR cells with overexpression of MicroRNA-520b. The IC50 (half maximal inhibitory concentration) of Doxorubicin is shown. Means SDs for triplicate wells are shown. Dashed lines, 50% cell viability. ns, not significant (P > 0.05); *, P < 0.001.
Figure 2. miR-520b promotes doxorubicin-induced apoptosis in drug-resistant breast cancer cells. A, B Flow cytometric analysis of the effect of overexpression of miR-520b on doxorubicin-induced MCF-7 / ADR, MDA-MB-231 / ADR cell line apoptosis. C, D MTT method was used to analyze the effect of overexpression of miR-520b on doxorubicin-induced apoptosis of MCF-7 / ADR and MDA-MB-231 / ADR cell lines. E, F and H, J qRT-PCR analysis of the effect of miR-520b overexpression on doxorubicin-induced apoptosis-related caspase-3/7 and caspase-9 activity in breast cancer cell lines. G Western-Blot detects the effect of overexpression of miR-520b on the expression of caspase-3 protein in breast cancer cell lines MCF-7 / ADR and MDA-MB-231 / ADR. H-K qRT-PCR analysis of the effect of overexpression of miR-520b on apoptosis-related genes in breast cancer resistant cells MCF-7 / ADR, MDA-MB-231 / ADR ns, not significant (P > 0.05); * P < 0.001.
Figure 3. miR-520b on apoptosis related proteins Bcl-2 and Bax on MCF-7/ADR and MDA-MB-231/ADR analyzed by Western Blot (A) and Image J (B-C).

ns, not significant (P > 0.05); ***, P < 0.001. All experiments repeat 3 times.
Figure 4. MiR-520b has a negative regulatory relationship with IGF-1R.
A database (PITA, miRmap, mirWalk 3.0, Enco r) predicts the binding sites of miR-520b and IGF-1R.
B Dual-fluorescence experiment analyses the miR-520b and IGF-1R targeting relationship.
C Analysis of the correlation between 1085 breast cancer miR-520b and IGF-1R in TCGA database.
D Analysis of the expression and correlation of miR-520b and IGF-1R in breast cancer patients.
E qRT-PCR analysis of the relation between IGF-1R expression and neoadjuvant chemotherapy in breast cancer patients.
F qRT-PCR analysis of IGF-1R expression between chemotherapy resistant group and chemotherapy sensitive group.
ns, not significant (P > 0.05); **, P < 0.001.
Figure 5. miR-520b inhibits the expression of IGF-1R in breast cancer resistant cell lines

A, B qRT-PCR analysis of IGF-1R mRNA expression in MCF-7, MCF-7/ADR, MDA-MB-231 and MDA-MB-231/ADR
C, D qRT-PCR analysis of mRNA expression of IGF-1R in MCF-7/ADR and MDA-MB-231/ADR after overexpression of miR-520b
E, F western blot analysis of protein expression of IGF-1R in MCF-7/ADR and MDA-MB-231/ADR after overexpression of miR-520b.

Quantitative analysis of protein by Image J
ns, not significant (P > 0.05); ***, P < 0.001. All experiments repeat 3 times.
**Table 1**

| Treatment                        | IC50 (ug/ml) |
|----------------------------------|--------------|
| MCF-7 IGF-1R-over                | 7.675        |
| MCF-7 NC                         | 2.521        |
| MDA-MB-231 IGF-1R-over           | 5.575        |
| MDA-MB-231 NC                    | 2.221        |
| si-IGF-1R                        | 1.175        |
| miR-520b inhibitor + si-IGF1R    | 0.876        |
| miR-520b inhibitor + control     | 2.543        |
| miR-520b inhibitor + IGF1R       | 0.576        |
| miR-520b inhibitor + control     | 3.943        |

**Graph 1**

- A, B IGF-1R protein expression in MCF and MDA-MB-231 transfected with IGF-1R-over analyzed by Western Blot (A) and Image J (B).
- C-E MTT analysis of the inhibitory effect of doxorubicin on MCF-7/IGF-1R + and MDA-MB-231/IGF-1R +, MDA-MB-231 / ADR/IGF-1R ;
- F, G MTT analysis of the inhibitory effect of doxorubicin on co-transfection of miR-520b inhibitor + si-IGF-1R MCF-7 and MDA-MB-231 / ADR The IC50 of Doxorubicin in each group is shown.

*ns, not significant (P > 0.05); ***, P < 0.001. All experiments repeat 3 times
Figure 7. miR-520b inhibits IGF-1R to promote breast cancer cell apoptosis
A, C Flow cytometry analysis of apoptosis of doxorubicin in overexpressing IGF-1R MCF-7 cells
B, D Flow cytometry analysis of apoptosis of doxorubicin on IGF-1R MDA-MB-231 / ADR cells
E, F qRT-PCR detection of caspase-3 / 7, caspase-9 activity related to apoptosis in MCF-7 cells
G, H qRT-PCR detection of MDA-MB-231 / ADR cell apoptosis-related caspase-3 / 7, caspase-9 activity
ns, not significant (P > 0.05); ***, P < 0.001. All experiments repeat 3 times.
Table 1. Clinical and pathological data of breast cancer patients before neoadjuvant chemotherapy

|                  | Number | %   |
|------------------|--------|-----|
| **Age**          |        |     |
| ≤35              | 3      | 10.0|
| 35-55            | 22     | 73.3|
| >55              | 5      | 16.7|
| **TNM**          |        |     |
| IIB              | 7      | 23.3|
| IIIA             | 14     | 46.7|
| IIIB             | 9      | 30.0|
| **ER**           |        |     |
| +                | 18     | 60.0|
| -                | 12     | 40.0|
| **HER-2**        |        |     |
| +                | 4      | 13.3|
| -                | 26     | 86.7|
| **PR**           |        |     |
| +                | 11     | 36.7|
| -                | 19     | 63.3|
| **Chemo sensitivity** | |   |
| Sensitive        | 20     | 66.7|
| Resistive        | 10     | 33.3|