

## Original Article

# Role of DJ-1 siRNA in reverse sensitivity of breast cancer cells to chemotherapy and its possible mechanism

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Received April 3, 2015; Accepted May 19, 2015; Epub June 1, 2015; Published June 15, 2015

**Abstract:** Breast cancer which has a high incidence rate is the 2<sup>nd</sup> lethal diseases only followed by lung cancer in women. How to improve the recovery rate is the principal problem should be solved in clinical. Previous studies demonstrated the importance of DJ-1 in the existence of breast cancer for the secreted of protein into serum by breast cancer cells both *in vitro* and *in vivo*. So the DJ-1 probably could be selected as the target in breast cancer treatment. Adriamycin resistance breast cancer cells MCF-7 and DJ-1 siRNA plasmid were employed to explore the potential clinical application of DJ-1 in this study. Our results showed that the sensitivity of cancer cells to chemotherapeutics was significantly improved with the transfection of DJ-1 siRNA. Further mechanism studies indicated the role of PI3K/AKT/MTOR pathway in the improvement of apoptosis after treatment with adriamycin in DJ-1 siRNA group.

**Keywords:** DJ-1, breast cancer, chemotherapeutics, PI3K/AKT/mTOR pathway

## Introduction

Breast cancer is the most common cancer among women, accounting for nearly 1 in 4 cancers diagnosed in the US women [1]. In 2009, an estimated 192,370 new cases of invasive breast cancer will be diagnosed among women, as well as an estimated 62,280 additional cases of in situ breast cancer [1]. Except the high incidence rates, the high death rates of breast cancer is a harder problem need to be solved in clinic. It was estimated that approximately 40,170 women died from breast cancer, only lower than the death caused by lung cancer among women in 2009 [1, 2].

Surgery, radiotherapy, chemotherapy, hormone therapy and/or biologic therapy are available therapeutic methods in clinic at the present [3]. But all these treatments have deficiencies. Take the surgery for instance, both lumpectomy and mastectomy, can only remove the cancerous tissue, has no effect to the metastasizing cancer cells. Radioresistance is the challenge that radiotherapy has to conquer. As to the chemotherapy, to find an effective and specific molecular target to improve the efficacy and

reduce the side effects is the hot issue in this research field.

DJ-1 is a 20-kDa protein whose sequence is conserved among prokaryotic and eukaryotic cells [4] and ubiquitously expressed in many mammalian tissues [5]. Functionally, DJ-1 has been implicated in fertilization [6, 7], the regulation of androgen receptor signaling [8, 9] and oxidative stress [3, 10]. Notably, previous studies also demonstrated that DJ-1 was associated closely with tumor progression and carcinogenesis. For instance, elevated levels of circulating DJ-1 and anti-DJ-1 autoantibodies have been found in breast cancer patients [11]. Other proteomic studies have found the increased level of DJ-1 in primary non-small cell lung carcinoma and prostate cancer [12, 13].

An important function of DJ-1 in carcinogenesis is protection against apoptosis resulting from various stimuli, which works through the directly regulation of some apoptosis pathways such as Nrf 2 and Bax signaling pathways [14, 15]. Moreover, DJ-1 is also a negative regulator of phosphatase and tensin homologue deletion on chromosome 10 (PTEN), which exerts tumor

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suppressor activity through inhibition of protein kinase B (PKB-Akt)-mediated cell survival [16].

Considering the important role of DJ-1 in the survival of breast cancer cells, we set it as a new therapeutic target to solve the problem of multidrug resistance (MDR) caused by chemotherapy in cancer treatment. In present work, DJ-1 siRNA plasmid was transfected into MCF-7 cells to decrease the expression of protein and the mRNA and protein expression level of DJ-1 was detected by RT-PCR and western blot, respectively. The cellular sensitivity to chemotherapeutics was measured by CCK-8. The results revealed that DJ-1 siRNA effectively reverses the MDR of human breast cancer Adriamycin resistant cell line MCF-7, which provides a novel approach for reversing breast cancer MDR. Further mechanism also studied in the present work.

### Materials and methods

#### *Cell culture and transfection*

Human breast cancer cells MCF-7 were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified incubator with an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. For plasmid DNA transfection, the cells were plated at a density of 70-80% confluence, grown overnight, and transfected with appropriate expression vectors in the presence of indicated combinations of plasmid DNAs by using the calcium phosphate method or Lipofectamine-plus reagent. The MCF-7 cells were stably transfected with pcDNA3 empty vector or pcDNA3 Flag-DJ-1, using Lipofectamin-Plus reagent. After 48 h of transfection, the cells were replated at a dilution of 1/20 and were maintained in complete medium containing 500 mg/ml of G418 (Invitrogen-GIBCO, USA) to select the neomycin-resistant cells.

#### *Real-time-PCR*

RT-PCR was carried out to analyze the expression of mRNA levels of the DJ-1 gene. A total of 1×10<sup>6</sup> cells were collected, and total RNA isolated using Trizol reagent. RNA quantification was determined using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). cDNA were synthesized with 2 µg total RNA.

The PCR primers of the DJ-1 gene, and internal reference gene (β-actin) were synthesized by Sangon Biotech (Sangon, Shanghai, China). The DJ-1 primers were 5'-CGCACAGAATTTATCTGAGTC-3' (forward) and 5'-GTCTTAAGAACAAGTGGAGC-3' (reverse); its product was 334-bp long. The β-actin primers were 5'-GTGGGGCGCCAGGCACCA-3' (forward) and 5'-CTCCTTAATGTCACGCACGATTTTC-3' (reverse); its product was 540-bp long. After denaturation at 94°C for 4 min, amplification was carried out at 94°C for 30 s, 58°C for 30 s, 72°C for 50 s, for 35 cycles, followed by incubation at 72°C for 10 min. Amplified products were separated on 1% agarose gel and observed under illumination by UV light.

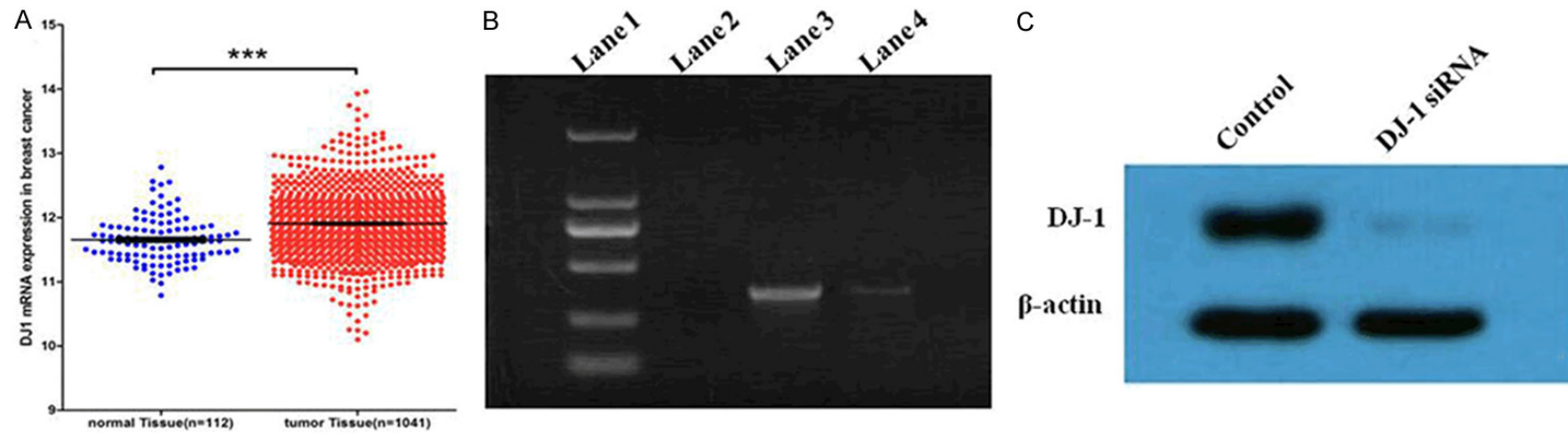
#### *Western blot*

Western blot was carried out to examine the expression of DJ-1, caspase-3, caspase-9, Bcl-xL, Bad and p-AKT. In briefly, MCF-7 cells were harvested and lysed in radioimmunoprecipitation (RIPA) buffer. Protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Perbio Science Deutschland, Bonn, Germany). Western blotting was then done as previously described [17]. The blotted proteins were then probed with anti-human DJ-1 antibody, anti-human caspase-3, caspase-9, Bcl-xL, p-AKT (phospho S473) and Anti-mTOR (phospho S2448) antibodies (Abcam, Cambridge, England), followed by incubation with horseradish peroxidase-labeled secondary antibodies (Santa Cruz, CA, USA). The expression of β-actin (Sigma-Aldrich) was used as a normalization control for protein loading. The blots were developed using enhanced chemiluminescence (ECL).

#### *Cell viability*

A cell count kit-8 (CCK-8 Beyotime, China) was employed in the experiments to quantitatively identify the sensitivity of cancer cells to adriamycin before and after siRNA. 0.2, 0.4, 0.8, 2.4 and 4.8 µmol/L adriamycin were used to treat MCF-7 (Group 1) and DJ-siRNA MCF-7 cells (Group 2). The plasmid vector transfected MCF-7 cells but without any drug treatment group was defined as Group 3. OD value at 450 nm was measured after treated for 72 h. The inhibition ratio was calculated using the following equation to quantify the role of DJ-1 in adriamycin sensitivity.

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**Figure 1.** Expression level of DJ-1 in breast cancer cells. A. DJ-1 was high expression tumor tissues when compared to adjacent tissues by NCBI's Gene Expression Omnibus analysis. B. DJ-1 mRNA expression level was decreased after DJ-1-siRNA. Lane 1: DNA marker, Lane 2: blank; Lane 3: normal MCF-7/AMD cells and Lane 4: siRNA group. C. The DJ-1 protein expression was decreased after DJ-1-siRNA.

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**Table 1.** The inhibition ratio of MCF-7 and DJ-1 siRNA cells after treatment with adriamycin for 72 h

Group	Adriamycin ( $\mu\text{mol/L}$ )	Inhibition ratio after 72 h (%)
MCF-7 cells	0.2	10.62 $\pm$ 1.23
	0.4	21.40 $\pm$ 2.57
	0.8	37.51 $\pm$ 2.16
	2.4	45.67 $\pm$ 2.54
	4.8	62.74 $\pm$ 3.41
MCF-7 DJ-1 siRNA cells	0.2	35.24 $\pm$ 2.77
	0.4	58.07 $\pm$ 2.13
	0.8	79.80 $\pm$ 3.76
	2.4	85.19 $\pm$ 1.59
	4.8	92.79 $\pm$ 5.76

The inhibition ratio = [(OD of Group 3)-(OD of Group 1 or 2)]/(OD of Group 3).

### Cell apoptosis assay

Flow cytometry was employed to determine the sensitivity of MCF-7/AMD cells to adriamycin with or without siRNA. The flipping of phosphatidylserine from the inside to the outside of plasmamembrane of apoptotic cells was determined by using labeled annexin V-fluorescein isothiocyanate (FITC, BD Biosciences, San Jose, USA), a  $\text{Ca}^{2+}$ -dependent phospholipid binding protein with high affinity for phosphatidylserine [18]. The assay was performed as outlined by the manufacturer and the cells were counterstained with propidium iodide (PI) to distinguish apoptosis from necrosis. The bound FITC and necrotic cells (which were stained with both PI and FITC) were gated out so that an accurate determination of the percentage of apoptotic cells could be made.

### Statistical analysis

Statistical analysis was performed on data obtained from at least three independent experiments. All results are presented as means  $\pm$  SD. Significance level is assessed using Student's t-test and differences corresponding to  $P < 0.05$  are considered statistically significant.

## Results

### Expression of DJ-1 in tumor and normal tissue

To study the biological role of DJ-1 in human breast cancer, we first data from NCBI's Gene

Expression Omnibus to analysis the expression levels of DJ-1 in breast cancer patients' tissues. As shown in **Figure 1A**, DJ-1 expression level was higher in tumor tissues compared with adjacent normal tissue control ( $P < 0.001$ ).

### Decrease of DJ-1 mRNA and protein expression after DJ-1-siRNA

The DJ-1 mRNA and protein expression was examined by RT-PCR before and after siRNA, and results are shown in **Figure 1B** and **1C**. The mRNA and protein expression levels were decreased to a relatively low level after transfection. It was considered that siRNA transfection could be used as an effective method to inhibit the expression of DJ-1.

### Increased sensitivity of cancer cells to adriamycin after transfection

CCK-8 assays were used to determine the change of sensitivity to adriamycin after silence of DJ-1 expression in MCF-7 cells. A significant inhibiting effect of adriamycin on cell viability was observed in transfection group (as shown in **Table 1**). The inhibition ratio has shown a dose-dependent manner. An excellent inhibition effect was observed in DJ-1 siRNA group at the dose of 4.8  $\mu\text{mol/L}$ . About 92% of the MCF-7 cells were inhibited in DJ-1 siRNA group while this value is about 62% in MCF-7 group.

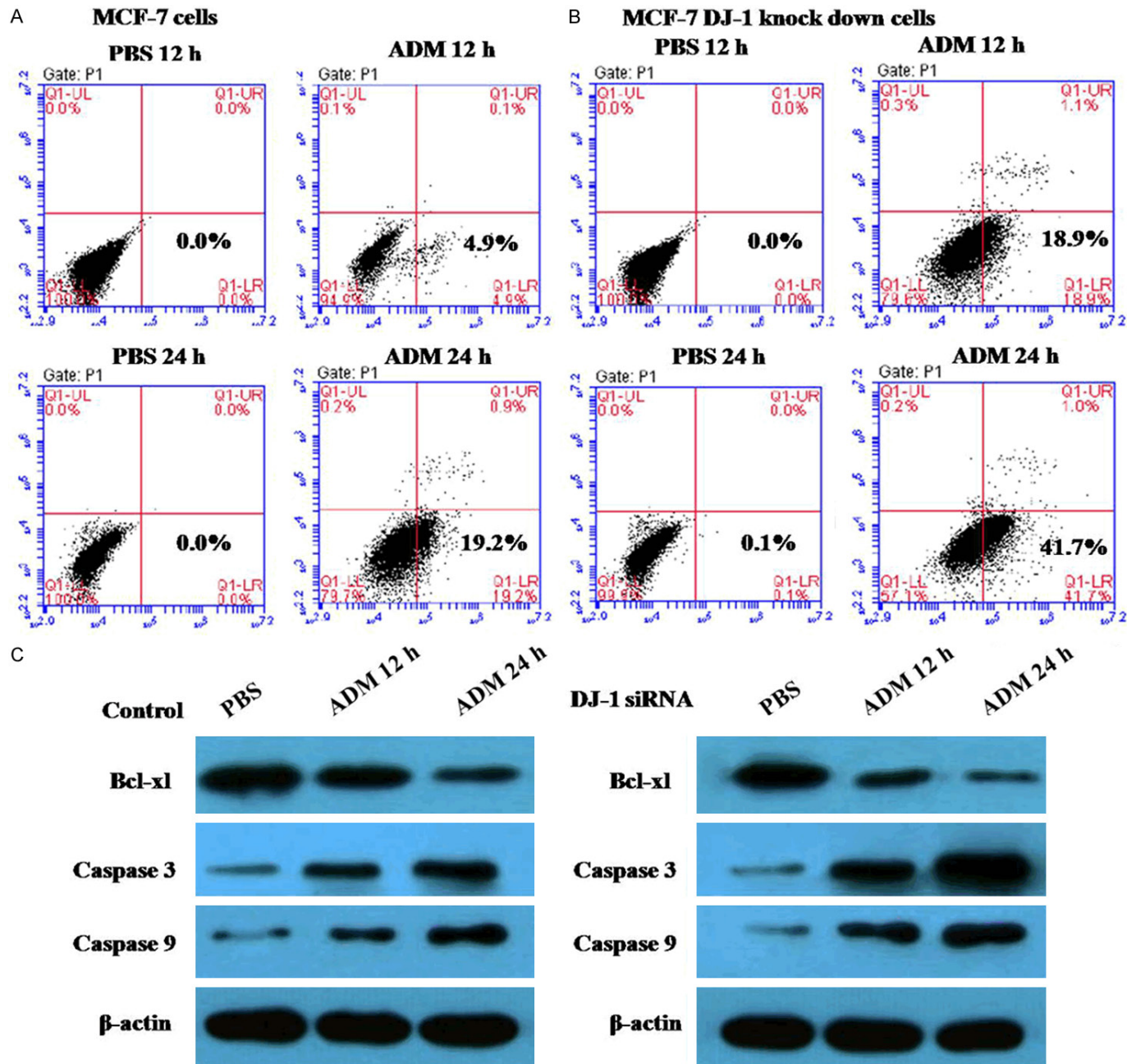
### Increases of apoptosis in DJ-1 silence MCF-7 cells

The apoptosis assay was studied to investigate which way of adriamycin to increase the sensitivity of cancer cells. As expected the number of apoptotic cells, which are annexin V-positive (FL1-H), was increased to a significant level at 12 h and 24 h post adriamycin treatment (**Figure 2A** and **2B**). The altering of apoptosis relative proteins expression proved the results (**Figure 2C**). These results indicated that the improvement of cancer cells' sensitivity to adriamycin might be through the increase of apoptosis.

### Role of PI3K/AKT/mTOR pathway in DJ-1 siRNA induced adriamycin sensitivity

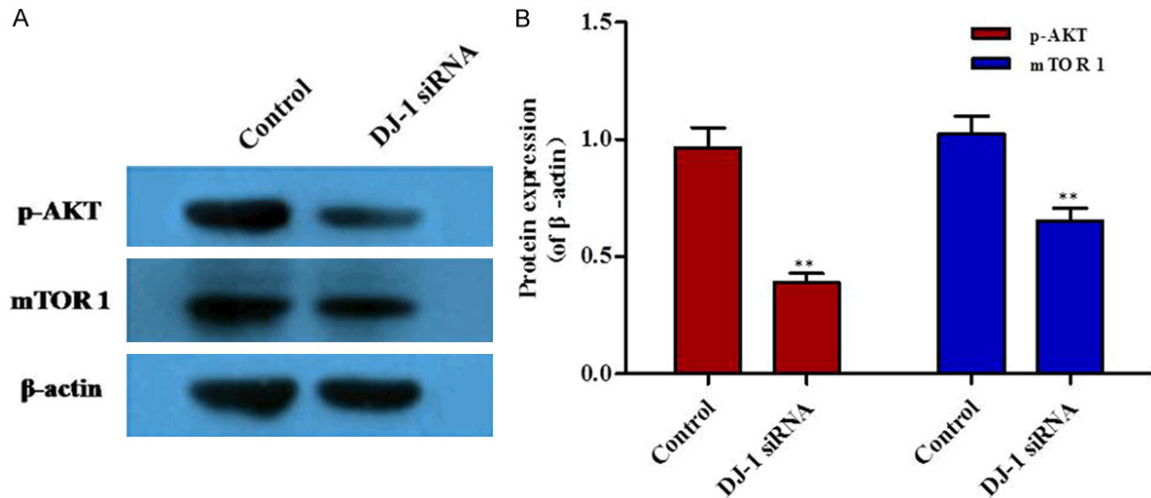
The PI3K/AKT/mTOR pathway related phosphorylated protein expression was studied to reveal the possible mechanism of DJ-1 silencing induced adriamycin sensitivity. As illustrat-

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**Figure 2.** The apoptosis induced by adriamycin in MCF-7 cells increased after silence of DJ-1 protein. (A) Wild type MCF-7 cells and (B) DJ-1 silence group (C) apoptosis relative proteins, including pro-apoptosis protein caspase 3 and caspase 9 expression were increased after DJ-1 silence; while the Bcl-xL protein expression was decreased during this process.



**Figure 3.** Role of PI3K/AKT/mTOR pathway in DJ-1 siRNA induced adriamycin sensitivity. A. Representative results. B. Data analysis p-AKT and mTOR1 protein expression level. \*\* $P < 0.01$ .

ed in **Figure 3**, both of the phosphorylated Akt (p-AKT) and phosphorylated mTOR1 (m-TOR1) expression were decreased in DJ-1 siRNA group.

### Discussion

About 94% of death with malignant tumor related to multi-drug resistance (MDR). MDR is the main reason for failure of tumor chemotherapy, which results in less effective and shortened patients' survival time. Although the mechanism of MDR is very complicated, many methods were carried out to eliminate this negative effect. One possible trial is to silence the expression of DJ-1 protein.

Studies have shown that DJ-1 is highly expressed in breast cancer, non-small cell lung cancer, esophageal squamous cell carcinoma and ovarian cancer. Meanwhile, high level of DJ-1 expression was found related to poor prognosis in patients with esophageal squamous cell carcinoma [12, 19, 20]. DJ-1 is located in human 1p36. 23 sites and involved in cell transformation, anti-oxidation and inhibition of apoptosis molecular chaperone regulation process. DJ-1 works as the oncogene and has a synergistic effect with the H-Ras in mouse NIH3T3 cells [5]. Also it works as RNA-binding

protein complexes to promote the binding between DJ-1 mRNA cAMP [4]. Moreover, DJ-1 could act as a mitochondrial integral protein to isolate the Daxx from nucleus, prevent the activation of apoptosis signal regulating kinase in cytoplasmic and further to inhibit the apoptosis of cells [21]. So DJ-1 was chosen as the target in the present work.

The cellular sensitivity and apoptosis were significantly increased after the silence of DJ-1 gene in present study. Mechanism studies revealed that hyperphosphorylation of AKT and then mitochondrial related apoptosis signaling pathways was activated during the process. AKT is a serine/threonine-specific protein kinase which plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. It is well known that AKT is involved in the PI3K/AKT/mTOR pathway [22]. Once correctly positioned at the membrane via binding of PIP3, AKT can be phosphorylated by its activating kinases phosphorylates effectors and then activated AKT can go on to activate or deactivate its myriad substrates (e.g. mTOR) via its kinase activity to play a role in the signaling transduction [23]. The phosphorylated AKT (p-AKT) works as a negative regulator in apoptosis. That means the decrease of p-AKT is in

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favor of apoptosis. In our studies, a significant decrease of p-AKT was observed after adriamycin treatment in DJ-1 silence group.

mTOR is an evolutionarily conserved serine/threonine kinase composed of two distinct protein complexes, mTORC1 and mTORC2. Inhibition of both complexes may be necessary for optimally controlling cancer growth [24]. In PI3K/AKT/mTOR pathways, the mTOR works as the substrate of p-AKT in the downstream. In other words, PI3K activation activates AKT which activates mTOR. In many cancers, this pathway is overactive, thus reducing apoptosis and allowing proliferation. The expression of phosphorylated mTOR1 (p-mTOR) was proved to decrease in our study.

In conclusion, all the evidence suggested that DJ-1 played a positive role in the survival of breast cancer cells, especially in the MDR of cancer cells to adriamycin. So silence of DJ-1 was used as an effective method to improve the sensitivity of cancer cells to chemotherapy. Further mechanism studies revealed the important role of PI3K/AKT/mTOR in the improvement of apoptosis in breast cancer cells.

In this study, siRNA technology was used to silence the DJ-1 expression to explore the relationship between the DJ-1 and drug resistance in human breast cancer cells. The results showed that after inhibition of DJ-1 expression, the apoptosis and death rate of MCF-7 to adriamycin was increased in vitro. That means silencing of DJ-1 protein can reverse the sensitivity of cells to chemotherapy drugs to a certain extent. Considering the potential of siRNA in clinical applications and its positive role in reverse resistance to chemotherapy, the present work aims to provide a foundation for potential application of DJ-1 silencing in breast cancer treatment.

### Acknowledgements

This work was supported by the Educational Bureau of Heilongjiang Province (No. 115-3G020) and Natural science research fund of Heilongjiang Province (No. H201333).

### Disclosure of conflict of interest

None.

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