Original Article
Curcumin promotes apoptosis of breast cancer cells by down-regulating DJ-1-PTEN/PI3K/AKT signaling pathways

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Received April 2, 2019; Accepted June 11, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: Enhanced activity of the PI3K/AKT signal pathway is correlated with tumorigenesis. As a negative regulator of PTEN, DJ-1 over-expression is associated with the occurrence of multiple tumors. Curcumin (Cur) is a phenol group compound with anti-tumor effects. Previous studies have shown its role in mediating the PTEN-PI3K/AKT pathway. However, it is still unclear whether Cur regulates DJ-1-PTEN/PI3K/AKT activity and proliferation or apoptosis of breast cancer cells. Cultured normal human breast epithelial cells MCF-10A, breast cancer cells MDA-MB-231 and MCF-7 were compared for DJ-1 and PTEN mRNA expression using qRT-PCR, and protein expression by Western blot. MCF-7 cells were treated with 0, 5, 10 and 20 μM Cur, and CCK-8 assay measured cell proliferation, followed by Western blot for DJ-1 and PTEN protein expression. MCF-7 cells were divided into control, 20 μM Cur treatment and Cur + PTEN inhibitor SF1670 groups, for comparing DJ-1, PTEN and p-AKT expression. Cell apoptosis and proliferation were tested by flow cytometry and EdU staining. Compared to MCF-10A cells, breast cancer cells showed an up-regulation of DJ-1, plus suppressed PTEN expression. MDA-MB-231 cells had higher DJ-1 expression and lower PTEN levels than MCF-7 cells. Cur treatment remarkably inhibited MCF-7 cell proliferation in a dosage-dependent manner. Gradient Cur suppressed DJ-1 expression and increased PTEN. Combined treatment with SF1670 enhanced phosphorylation activity of AKT, weakened Cur induced inhibition on AKT phosphorylation, and alleviated pro-apoptosis or anti-inflammatory functions of Cur. Cur can inhibit breast cancer cell proliferation and facilitate cell apoptosis via down-regulating DJ-1 expression and modulating PTEN/PI3K/AKT pathway activity.

Keywords: Curcumin, DJ-1, PTEN/PI3K/AKT, cell proliferation, apoptosis, breast cancer

Introduction
Breast cancer (BC) is a malignant tumor originating from breast gland epithelium. BC is the most commonly occurring cancer in females and has the highest incidence among all female cancers [1, 2]. BC is responsible for about 20%-30% of all female systemic tumors, having a 2%-3% annually increased rate of incidence, making it the most threatening malignant tumor for women [1, 2]. It is estimated that about 1.5 million people are newly diagnosed with BC, and one third (about 0.5 million) of these patients die from BC [3]. BC characteristics include difficulty in early diagnosis, rapid progression at terminal stages and high mortality, thus severely affecting patients life health and quality [4, 5]. Phosphatase and tensin homologus deletion on chromosome ten (PTEN) can negatively regulate the activity of phosphatidylinositol-3 kinase (PI3K)/AKT (protein kinase B, PKB) signal pathway [6-9]. As a negative regulator for the PTEN gene, DJ-1/Parkinson gene 7 (PARK7) can decrease the inhibitory function of PTEN on the PI3K/AKT signaling pathway via suppressing PTEN expression or function, thus indirectly activating the PI3K/AKT signal pathway, and thus playing an important role in decreasing cell apoptosis, facilitating cell proliferation, cell survival and tumor pathogenesis [10]. Curcumin (Cur) is a group of polyphenol substances extracted from the roots and stems of Curcuma longa L. plants. Increasing evidence has demonstrated that Cur has pluripotent anti-tumor effects including suppressing cell proliferation...
Curcumin in breast cancer and facilitating cell apoptosis [11, 12]. Existing evidence has demonstrated that Cur plays a role in the regulation of the PTEN-PI3K/AKT signaling pathway [13, 14]. Considering the role of PTEN in the occurrence or development of tumors, whether or not Cur plays a role in treatment of breast cancer via the PTEN/PI3K/AKT pathway remains poorly understood. This study investigated if Cur modulates DJ-1-PTEN/PI3K/AKT pathway activity, and affects BC cell proliferation or apoptosis.

Materials and methods

Major reagent and materials

Normal breast epithelial cells MCF-10A, BC cell lines MDA-MB-231 and MCF-7 were purchased from Beina Bio (China). RPMI 1640 and fetal bovine serum (FBS) were purchased from Hyclone (US). Transfection reagent Lip2000 and Trizol were purchased from Invitrogen (US). PrimeScript™ RT reagent Kit was purchased from Takara (China). Cur was purchased from Sigma (US). Edu flow cytometry test reagent was purchased from RioBio (China). Rabbit anti-human DJ-1, AKT, p-AKT and β-actin polyclonal antibodies were purchased from Abcam (US). Rabbit anti-human PTEN polyclonal antibody was purchased from CST (US). HRP conjugated secondary antibody was purchased from Sangon Bio (China). BCA protein quantification kit, CCK-8 assay kit and Annexin V/PI cell apoptosis kit were purchased from Beyotime Bio (China). PTEN inhibitor SF1670 was purchased from MedChemExpress (US). FORMA model 3131 incubator was purchased from Thermo (US).

Cell incubation

MCF-10A, MDA-MB-231 and MCF-7 cells were kept in RPMI 1640 medium containing 10% FBS, in a 37°C chamber with 5% CO₂. After cell growth on the plate, cells were passed at a 1:3~1:4 ratio. Cells in log-growth phase were used for experiments.

Cell treatment and grouping

In vitro cultured MCF-10A, MCF-7 and MDA-MB-231 cells were inoculated into 96-well plates (10,000 cells per well, in 6 parallel wells). After 24 h attached growth, cells were treated with 0, 5, 10 and 20 μM Cur. Seventy-two hours later, 10 μL CCK-8 solution was added into each well. After a 4 h reaction, absorbance values (A) were measured at 450 nm wavelength. Relative proliferation activity (%) = (A450 of drug treatment group - A450 of empty well)/(A450 of control group - A450 of empty well) × 100%.

Cur and SF1670 treatment of MCF-7 cells

MCF-7 cells were treated with 10 μM EdU for 120 min, and were assigned into three groups: control group, Cur (20 μM) treatment group, and Cur + SF1670 (200 nM) treatment group. After 48 h of incubation, cells were digested by trypsin. After collection, cells were fixed in 4% paraformaldehyde for 15~30 min and were neutralized in 2 mg/mL glycine for 5 min. After PBS rinsing, 500 μL Apollo staining buffer was added for resuspension of cells, which were then incubated in the dark at room temperature for 10 min. Cells were then centrifuged at 300 g for 5 min, and the staining buffer was discarded. Followed by rinsing in 0.5% Triton X-100 permeabilizing buffer at room temperature for 1~3 rinses, cells were then resuspended in PBST and were measured with FC500 MCL flow cytometry (Beckman, US).

Cell apoptosis assay

Cells from all treatment groups were digested and collected, followed by PBS rinsing and centrifugation. Cells were resuspended in 100 μL Annexin V Binding Buffer and were sequentially mixed with 5 μL Annexin V-FITC and 5 μL PI dyes. After 15 min room temperature incubation, 400 μL Annexin V Binding Buffer was added for measuring cell apoptosis by flow cytometry.

qRT-PCR for measuring gene expression

RNA was extracted by the Trizol method. cDNA was synthesized from RNA using reverse transcription by PrimeScript™ RT reagent Kit. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase. In a 10 μL system, we added 5.0 μL 2X SYBR, 0.5 μL forward/reverse primer (5 μm/L), 1 μL cDNA and ddH₂O up to 10.0 μL. Reverse transcription conditions were: 50°C for 15 min, and 85°C for 5 min. PCR conditions were: 95°C to denature for 5 min, followed by 40 cycles each consisting of 95°C for 15 sec and 60°C for 1 min. PCR was performed on a Bio-Rad CFX96 real time fluorescent quantitative PCR cycler. Data were stored for further use.
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Figure 1. Up-regulation of DJ-1 and down-regulation of PTEN in BC cells. A: qRT-PCR assay for mRNA expression. B: Western blot for cellular protein expression. *P<0.05 compared to MCF-10A cells; #P<0.05 compared to MCF-7 cells.

Table 1. Effects of Cur on the proliferation activity of MCF-10A, MCF-7 and MDA-MB-231 cells. Cells were treated with different concentrations of Cur followed by analysis of cell proliferation by flow cytometry

<table>
<thead>
<tr>
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<th>0 μM</th>
<th>5 μM</th>
<th>10 μM</th>
<th>20 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10A</td>
<td>100%</td>
<td>96.3±5.1%</td>
<td>105.2±5.4%</td>
<td>93.5±4.9%</td>
</tr>
<tr>
<td>MCF-7</td>
<td>100%</td>
<td>72.6±3.6% †</td>
<td>51.3±3.1% †</td>
<td>38.9±2.6% †</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>100%</td>
<td>87.3±4.1% †, #</td>
<td>68.4±3.9% †, #</td>
<td>57.2±3.3% †, #</td>
</tr>
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Compared with corresponding 0 μM, *P<0.01; Compared with corresponding value for MCF-7 cells, #P<0.05.

Western blot

Cell precipitations were mixed with 100 μL RIPA lysis buffer for 15 min to lysis on ice. After 10,000 g centrifugation for 5 min, proteins in the supernatant were transferred to new Eppendorf tubes. After BCA quantification for quality and quantity for proteins, 40 μg of sample was loaded and separated in 10% SDS-PAGE, and were transferred to a PVDF membrane (300 mA, 100 min). The protein membrane was blocked with 5% nonfat milk powder. Primary antibodies (DJ-1 at 1:2,000, PTEN at 1:1,000, AKT at 1:1,000, p-AKT at 1:500, and β-actin at 1:10,000) were added for 4°C overnight incubation. On the next day, the membrane was rinsed in PBST three times, and HRP conjugated secondary antibody (1:10,000 dilution) was incubated for 60 min at room temperature. The membrane was rinsed in PBST three times and was washed with chromogenic reagent. After 2-3 min of dark incubation, the membrane was exposed, developed and scanned for data storage.

Statistical analysis

SPSS 18.0 was used for data statistics. Measurement data were presented as mean ± standard deviation (SD). Measurement data between the two groups were compared by t-test. The comparison of means across multiple groups was first performed by analysis of variance (ANOVA), followed by Bonferroni comparison between the two groups. A statistical significance was defined when P<0.05.

Results

DJ-1 upregulation and PTEN down-regulation in BC cells

qRT-PCR results showed that compared to normal breast gland epithelial cells MCF-10A, the BC cell lines MCF-7 and MDA-MB-231 showed significantly elevated DJ-1 mRNA expression, whilst PTEN mRNA expression was remarkably decreased. Moreover, MDA-MB2-31 cells with higher malignancy presented higher DJ-1 expression compared to MCF-7 cells which had relatively lower malignancy, and PTEN expression was lower than in the MCF-7 cells (Figure 1A). Western blot results showed that BC cell lines MCF-7 and MDA-MB-231 had significantly higher DJ-1 protein expression than normal breast gland epithelial cells MCF-10A, and that PTEN protein expression was remarkably lower in BC cells (Figure 1B).

Cur treatment remarkably inhibited proliferation activity of BC cells

CCK-8 assay showed that different concentrations of Cur had no major effect on proliferation activity of normal breast gland epithelial cells MCF-10A (Table 1). The proliferation activity of BC cells, however, was significantly inhibited by 5, 10 or 20 μM Cur. Higher Cur dosage further suppressed proliferation activity. Compared to MDA-MB-231 cells with higher malignancy, MCF-7 cells with relatively lower malignancy presented higher drug sensitivity towards Cur under the same dosage, as presented by lower proliferation activity (Table 1).

Cur suppressed DJ-1 expression and increased PTEN expression in the BC cell line MCF-7

qRT-PCR results showed that Cur treatment remarkably suppressed DJ-1 mRNA expression
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in MCF-7 cells, with lower RNA expression at higher dosage (Figure 2A). Compared to 0 μM treatment group the 5, 10 and 20 μM Cur treated MCF-7 cells had significantly elevated PTEN mRNA expression, with more potent increase of PTEN mRNA expression in the high dosage groups (Figure 2B). Western blot results showed that compared to the untreated group, Cur treated MCF-7 cells had remarkably decreased DJ-1 protein expression, whilst PTEN protein expression was remarkably increased (Figure 2C).

Curcumin significantly inhibited BC cell proliferation and induced cell apoptosis whilst SF1670 antagonized Cur effects

qRT-PCR results showed that Cur treatment remarkably decreased DJ-1 mRNA expression in MCF-7 cells and up-regulated PTEN mRNA expression. Combined treatment using SF1670 did not affect DJ-1 or PTEN mRNA expression (Figure 3A and 3B). Western blot results showed that SF1670 combined Cur treatment did not affect the modulatory effect of Cur on DJ-1 or PTEN protein expression, but remarkably up-regulated the p-AKT protein expression (Figure 3C). Flow cytometry results showed that Cur remarkably inhibited the proliferation of MCF-7 cells (Figure 3D) and induced cell apoptosis (Figure 3E). Combined treatment of Cur and SF1670 antagonized the effect of Cur on the inhibition of proliferation of MCF-7 cells and induction of cell apoptosis.

Discussion

PI3K/AKT is a signal pathway that is widely distributed in multiple tissues and cells. When PI3K/AKT is activated, PI3K can catalyze the production of phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 4,5-trisphosphate (PIP2), the former of which can directly phosphorylate and activate AKT to further activate downstream signaling molecules to modulate various important biological processes including cell growth, survival and apoptosis via regulating target gene transcription and expression [15]. Previous studies showed that over-activation of the PI3K/AKT signaling pathway was correlated with the occurrence, progression, metastasis and drug resistance of multiple tumors such as endometrial carcinoma [16], gastric cancer [17] and pulmonary carcinoma [18]. PTEN is the only tumor suppressor gene that has been discovered with dual activities including protein lipase and phosphatase. By dephosphorylation of its substrate PIP3, PTEN can inhibit AKT phosphorylated activation by PI3K via PIP3, thus blocking the PI3K/AKT pathway transduction, and negatively regulating its activity [19]. DJ-1/Parkinson gene 7 (PARK7) located on human chromosome 1p36.2-36.3, being around 24 kb length, and encoding a 21 kDa protein consisting of 189 amino acids [10]. As a negative regulator of PTEN, DJ-1 can weaken the inhibitory role on the PI3K/AKT signaling pathway by PTEN, via suppressing its expression and function, thus indirectly activating the PI3K/AKT signaling pathway to play a role in alleviating cell apoptosis, facilitating cell proliferation, survival and tumorigenesis, making it one oncogenic factor [10].

Cur is a polyphenol compound with the characteristics of high efficiency, wide spectrum of anti-tumor functions, gastrointestinal actions and minimal toxic effects, plus pharmacological effects including anti-inflammation, antioxidation, and clearance of free radicals. Increasing evidence show that Cur has anti-
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Figure 3. Curcumin significantly inhibited BC cell proliferation and induced cell apoptosis, whilst SF1670 antagonized Cur effects. A: qRT-PCR for DJ-1 mRNA expression in cells. B: qRT-PCR for PTEN mRNA expression. C: Western blot for cellular protein expression. D: Flow cytometry for measuring cell proliferation. E: Flow cytometry for cell apoptosis. *, $P<0.05$ compared to control group. #, $P<0.05$ compared to Cur group.

tumor effects including suppression of tumor cell proliferation, facilitation of cell apoptosis, anti-angiogenesis, anti-invasion and anti-migration [11, 12]. The anti-tumor effects of Cur are drawing increasing attention from people, and has become a hot topic in tumor prevention and treatment. Previous studies showed that Cur played a role in modulating the PTEN/PI3K/AKT pathway [13, 14]. Whether Cur can modulate the PTEN/PI3K/AKT pathway via DJ-1 as a negative regulator upstream of PTEN, is still unclear. This study thus investigated whether Cur modulates the DJ-1-PTEN/PI3K/AKT pathway activity, and proliferation and/or apoptosis of BC cells.

Our results showed that compared to normal breast gland epithelial cell line MCF-10A, BC cells showed significantly elevated DJ-1 expression plus lower PTEN expression. MDA-
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MB-231 cells had higher DJ-1 expression than MCF-7 cells, and lower PTEN expression. Results showed that DJ-1 up-regulation played an important role in suppressing PTEN expression and BC pathogenesis, and was related to malignancy of BCs. Klawitter et al, found that lovastatin can enhance PTEN functional activity via suppressing DJ-1 expression, and inhibit AKT phosphorylation to suppress BC cell proliferation [19]. Ismail et al, found that compared to non-invasive BC cell lines MCF-7 and T47D cells, the invasive BC cells MDA-MB-231 and MDA-MB-435 had significantly elevated DJ-1 expression [20]. Kawate et al, found that compared to healthy control patients, BC patients had abnormally elevated DJ-1 protein in blood samples, and its expression level was related with clinical stage of tumors, and Ki-67 cell proliferation index in tumor tissues [21]. Zhang et al, also found that compared to tumor adjacent tissues, BC tissues showed remarkably elevated DJ-1 expression [22]. All these results reveal the oncogenic role of DJ-1 up-regulation in BC, as supported by observations from this study.

Treatment of different concentrations of Cur remarkably inhibited the proliferation activity of cells, and suppressed DJ-1 expression in BC cell line MCF-7, plus up-regulated PTEN expression. Results indicated that Cur might enhance the expression of tumor suppressor gene PTEN via inhibiting oncogene DJ-1 expression, thus exerting pro-apoptotic role of BC cells. Further studies were performed using the PTEN inhibitor SF1670 in combined with Cur. Results showed that such combined treatment remarkably enhanced the phosphorylation activity of AKT, weakened the inhibitory role of Cur on downstream AKT phosphorylation, and suppressed the pro-apoptotic and anti-proliferation function of Cur. These results indicate the role of Cur in inhibiting the PI3K/AKT pathway activation, facilitation of BC cell apoptosis and inhibition of cell proliferation, via DJ-1 down-regulation and PTEN up-regulation. In a study of the relationship between DJ-1 and its biological effects on BC, Ismail et al, found that over-expression of DJ-1 or siRNA interference of DJ-1 remarkably enhanced or weakened, respectively, the cell invasion potency and Snail expression of BC cell lines MCF-7 and MDA-MB-231, along with the facilitatory effects on BC cell invasion by DJ-1 being mediated via KLF17 down-regulation [20]. Zhang et al, found that siRNA interference on DJ-1 expression in BC cell line MCF-7 remarkably facilitated cell apoptosis, and enhanced the Adriamycin sensitivity of cells via modulating PI3K/AKT signal pathway [22]. However, these studies were performed via genetic approaches that interfere with DJ-1 expression, in sharp contrast to our studies. In a study on the effect of DJ-1 expression, Ismail et al, found that Paclitaxel can up-regulate the expression of a negative regulator of cell invasion KLF17 via suppressing DJ-1 expression, which weakens BC cell invasion potency [23]. Yang et al, also showed that bis-demethoxycurcumin can remarkably suppress DJ-1 expression in pancreatic carcinoma cells and facilitate PANC-1 cell apoptosis [24]. These studies, however, were not simultaneously targeting Cur and BCs. Our study revealed that Cur can affect PTEN/PI3K/AKT pathway activity via suppressing DJ-1 expression, thus exerting anti-proliferation and pro-apoptotic roles of BC cells for anti-tumor effects: all of which have not been previously reported and are thus a novelty of this study. However, the treatment effects of Cur on BC and its in vivo modulatory effects are largely unclear and require further animal studies to fulfill the weakness of the current studies.

Conclusion

Cur can exert anti-proliferation and pro-apoptotic roles on BC cells in an anti-tumor manner via suppression of DJ-1 expression to modulate the PTEN/PI3K/AKT pathway activity.

Disclosure of conflict of interest

None.

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