Over-expression of DJ-1 attenuates effects of curcumin on colorectal cancer cell proliferation and apoptosis

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Abstract. OBJECTIVE: The phosphatase and tensin homologue deleted on chromosome ten (PTEN) acts as a tumor suppressor gene by inhibiting the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. DJ-1, a negative regulator of PTEN, is associated with the pathogenesis of a variety of tumors. Curcumin (Cur) is a phenolic compound that is extracted from various plant rhizomes with various anti-tumor pharmacological effects. This study aimed to investigate the effects of Cur on proliferation and apoptosis of colorectal cancer cells.

MATERIALS AND METHODS: Human normal colorectal epithelial cell line (NCM460) and colorectal cancer cell line (SW480 and SW620) were cultured in vitro. Real-time quantitative PCR (RT-PCR) and western blot were used to detect DJ-1 and PTEN mRNA and protein, respectively. Cell apoptosis was determined with flow cytometry. SW480 cells were divided into control, 20 μM Cur treatment group, Cur+pcDNA3.1-Blank group and Cur+pcDNA3.1-DJ-1 group. Cell proliferation activity was evaluated with EdU staining.

RESULTS: Comparing with NCM460 cells, DJ-1 was significantly increased, while PTEN was significantly declined in SW480 and SW620 cells (p<0.05). Cur treatment significantly inhibited SW480 and SW620 cell proliferation and significantly induced apoptosis compared to control group (p<0.05) but showed no significant effects on NCM460 cells. Cur down-regulated DJ-1 level and enhanced PTEN expressions in SW480 cells with dose dependence. The pcDNA3.1-DJ-1 transfection significantly declined PTEN expression, enhanced p-AKT levels, reduced cell apoptosis, and strengthened cell proliferation in SW480 cells treated by Cur (p<0.05).

CONCLUSIONS: Cur can inhibit colorectal cancer cell proliferation and promote apoptosis by down-regulating DJ-1 expression to regulate the activity of PTEN/PI3K/AKT signaling pathway.

Key Words: Curcumin, DJ-1, PTEN/PI3K/AKT, Proliferation, Colorectal cancer.

Introduction

Colorectal cancer (CRC) is a common clinical malignant tumor in the digestive tract. Its incidence accounts for the third among the malignant tumors of the whole body. CRC is characterized by the occult onset, easy metastasis, and poor prognosis. Although early diagnosis and clinical treatment techniques have been greatly improved, the overall efficacy of CRC is still poor.

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a negative regulator of phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway, and therefore it plays a role in regulating cell proliferation, migration, and invasion. DJ-1/Parkinson gene 7 (PARK7) is a negative regulator of PTEN that can activate phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway through inhibiting PTEN, thus to be involved in cell apoptosis and proliferation as an oncogene. Curcumin (Cur) is a polyphenolic substance extracted from the rhizome of Curcuma longa. Researchers found that Cur regulates cell proliferation, apoptosis, migration, and invasion to play the anti-tumor effect. It was observed that Cur plays a regulatory role in CRC proliferation, apoptosis, and metastasis. It was reported that Cur plays a role in regulating the activity of PTEN/PI3K/AKT signaling pathway. Since DJ-1 is a negative regulator for the PTEN, it is unclear whether

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Cur can regulate PTEN/PI3K/AKT signaling pathway through targeting DJ-1 in CRC.

Materials and Methods

Instruments and Reagents

The human colorectal cancer cell line, SW480 and SW620, and the normal colorectal epithelial cell line (NCM460) were purchased from Beijing Beina Biotechnology Co., Ltd. (Beijing, China). Competent cell JM109 was purchased from Shanghai Shengsheng Biotechnology Co., Ltd. (Shanghai, China). Roswell park memorial institute-L640 (RPMI-L640) and penicillin-streptomycin were purchased from HyClone (South-Logan, UT, USA). Optional Minimum Eagle’s Medium (Opti-MEM) medium and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). TRizol and lipofectamine 2000 were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). PcDNA3.1 vector was purchased from Thermo Scientific Pierce (Rockford, IL, USA). PrimeScript™ RT reagent Kit was purchased from TaKaRa Biotechnology Co. (Dalian, China). Cur was purchased from Sigma-Aldrich (St. Louis, MO, USA). EdU Apollo 488 Flow Cytometry Kit was purchased from Ribobio (Cat. No. C10338-2, Guangzhou, China). Rabbit anti-human DJ-1, protein kinase B (AKT), and phosphorylated AKT (p-AKT) polyclonal antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). Rabbit anti-human PTEN and β-actin polyclonal antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Horse-radish peroxidase (HRP)-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Bicinchoninic acid (BCA) protein quantification kit, Annexin V/propidium iodide (PI) apoptosis detection kit and BeyoECL Plus chemiluminescence reagent were purchased from Beyotime Biotechnology (Shanghai, China). PTEN inhibitor SF1670 was purchased from MedchemExpress (Monmouth Junction, NJ, USA). Cell counting-kit 8 (CCK-8) test kit was purchased from Dojindo Molecular Technologies (Rockville, MD, USA).

Cell Culture

NCM460, SW480, and SW620 cells were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin. The cells were maintained at incubator with 37°C and 5% CO₂ (Mode: FORMA 3131, Thermo Electron Corp, Waltham, MA, USA). The cells were passaged at 1:3-1:4 and were used for experiments in the logarithmic phase. This study was approved by the Ethics Committee of Zibo Central Hospital, Zibo, Shandong, China.

Cell Treatment and Grouping

The NCM460, SW480, and SW620 cells were cultured in vitro and inoculated into 96-well plates (Corning, Corning, NY, USA, 10000 cells/well). After adhering for 24 h, they were treated with 0, 10, or 20 μM of Cur and cultured for 72 h. After that, 10 μl of CCK-8 solution was added to each well and the absorbance of each well at 450 nm was measured after 4 h of reaction (A450). Relative proliferation activity (%) = (A450 value of the treatment group – A450 value of the blank well)/(A450 value of the control group – A450 value of the blank well) × 100%.

PTEN Over-Expression

Plasmid Construction

The SW480 cell whole genome DNA was used as a template to amplify the CDS region of DJ-1 gene. The size of the target fragment was determined by gel electrophoresis. After digested with Xho I and BamH I, it was inserted into the pcDNA3.1 vector to transform competent cell JM109. The positive strain was screened for ampicillin resistance, and the plasmid was extracted after amplification. The inserted target fragment of the DJ-1 gene was determined by sequencing and named as pcDNA3.1-DJ-1. The empty vector pcDNA3.1-Blank was used as a control.

SW480 Cell Transfection

SW480 cells were divided into Cur+pcDNA3.1-Blank group and Cur+pcDNA3.1-DJ-1 group. Lip2000, pcDNA3.1-Blank, and pcDNA3.1-DJ-1 were diluted with Opti-MEM medium, and incubated for 5 min at room temperature, respectively. The mixture was added to the cell culture medium for 6 h. Next, after changing the medium, the cells were further cultured for 72 h. At last, the cells were collected by the enzyme.

SW480 Treatment

SW480 cells were divided into four groups, including control, 20 μM Cur treatment group, Cur+pcDNA3.1-Blank group, and Cur+pcDNA3.1-DJ-1 group. The latter three groups were treated by 20 μM Cur. Before Cur treatment, the cells were added with EdU solution
at 10 μM for 120 min. After Cur treatment, the cells were digested by trypsin (Beyotime Biotech. Shanghai, China) and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15-30 min. Next, the cells were incubated in 500 μl Apollo reaction fluid (Apollo Endosurgery Inc., Austin, TX, USA) at room temperature avoiding the light for 10 min. The cells were tested on FC500 MCL flow cytometry (Beckman, Germany).

**Cell Apoptosis Detection**

The cells were digested by the enzyme and collected. After re-suspended in 100 μl binding buffer, the cells were added with 5 μl Annexin V-FITC and 5 μl PI at room temperature avoiding the light for 15 min. Then the cells were tested on flow cytometry (Mode: FC500, Beckman, Germany).

**Real-Time PCR (RT-PCR)**

The total RNAs of NSCs was extracted by utilizing the TRIzol reagents (Beyotime Biotechnology, Shanghai, China). The complementary DNAs (cDNAs) were synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) and was used to reverse transcribe RNA to complementary DNA (cDNA). The targeting gene mRNAs were amplified by using the PCR reaction system (TaKaRa, Dalian, China), which was composed of 2×SYBR Green Mixture 5.0 μl, 5 μm/l forward and reverse primer 0.5 μl, cDNA 1.0 μl, and ddH2O. The primers of the DJ-1, PTEN, and β-actin genes were synthesized and listed in Table I. The reverse transcription was performed at 50°C for 15 min and 85°C for 5 min. The reaction was performed on Bio-Rad CFX96 at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The relative gene expressions were represented by using a gel scanning system (Mode: GDS8000, UVP, Sacramento, CA, USA) and calculated with 2^-∆∆ct method.

**Western Blot**

The total protein was extracted from the cells by RIPA on ice for 15 min. After quantified by BCA method, a total of 40 μg protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotechnology, Shanghai, China) and transferred to polyvinylidene difluoride (PVDF, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) membrane at 300 mA for 100 min. After being blocked by 5% skim milk at room temperature, the membrane was incubated in primary antibody at 4°C overnight (DJ-1, PTEN, AKT, p-AKT, and β-actin 1:2000, 1:2000, 1:2000, 1:1000, and 1:10000). After washed by phosphate buffer solution Tween-20 (PBST, Beyotime Biotechnology, Shanghai, China), the membrane was further incubated in HRP conjugated secondary antibody at room temperature for 60 min (1:10000). At last, the membrane was treated by enhanced chemiluminescence (ECL, Beyotime Biotechnology, Shanghai, China) reagent and developed.

**Statistical Analysis**

SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was applied for data analysis. The measurement data were presented as mean ± standard deviation (SD). The Student’s t-test was used to compare differences between the two groups. Tukey’s post-hoc test was used to validate the analysis of variance (ANOVA) for comparing measurement data among groups. p<0.05 was depicted as a significant difference.

**Results**

**DJ-1 Up-Regulated, while PTEN Decreased in Colorectal Cancer Cells**

Quantitative real-time PCR (qRT-PCR) showed that compared with normal colorectal epithelial NCM460 cells, DJ-1 mRNA was significantly increased (p<0.05), while PTEN mRNA was significantly declined in SW480 and SW620 cells.

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<th>0 μM</th>
<th>10 μM</th>
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<tr>
<td>Relative cell survival (%)</td>
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<td>100±5.23</td>
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**Table I.** The impact of different concentrations of Cur on cell proliferation.
Curcumin affects colorectal cancer by DJ-1

Curcumin affects colorectal cancer by DJ-1 expression in SW480 cells with dose dependence (Figure 3A, B). Western blot results showed that Cur reduced DJ-1 protein level and up-regulated PTEN protein expression in SW480 cells with dose dependence (Figure 3C).

DJ-1 Over-Expression Antagonized the Impact of Cur on SW480 Cell Proliferation and Apoptosis

qRT-PCR showed that Cur treatment significantly down-regulated DJ-1 mRNA and elevated PTEN mRNA in SW480 cells (Figure 4A, B). pcDNA3.1-DJ-1 transfection significantly increased DJ-1 mRNA and reduced PTEN mRNA expressions in SW480 cells ($p<0.05$). Western blot results indicated that pcDNA3.1-DJ-1 transfection remarkably attenuated the influence of Cur on DJ-1 inhibition and PTEN up-regulation, whereas enhanced p-AKT protein expression (Figure 4C, $p<0.05$). Flow cytometry results showed that Cur significantly inhibited SW480 cell proliferation (Figure 4D, $p<0.05$) and increased cell apoptosis (Figure 4E, $p<0.05$). pcDNA3.1-DJ-1 transfection alleviated the inhibition of Cur on SW480 cell proliferation and significantly reduced apoptosis ($p<0.05$).

Table II. The impact of different concentrations of Cur on cell proliferation.

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Figure 1. DJ-1 upregulated, while PTEN decreased in lung cancer cells. A, qRT-PCR detection of mRNA expression. B, Western blot detection of protein expression. $p<0.05$, compared with NCM460 cells.
When the PI3K/AKT pathway is activated, PI3K can be transformed in phosphatidylinositol 3,4,5-triphosphate (PIP3) by catalyzing phosphatidylinositol 4,5-trisphosphate (PIP2). PIP3 recruits AKT from the cytoplasm to the membrane and phosphorylates loci Ser473 and Thr308 of AKT under the action of phosphoinositide-dependent protein kinase (PDK). Phosphorylation-activated AKT further activates downstream signaling molecules to participate in cell growth, survival, and apoptosis by regulating the transcription and expression of various downstream genes. PTEN is the only tumor suppressor gene discovered that has dual activities of protein esterase and phosphatase. PTEN can dephosphorylate PIP3 to antagonize PI3K impact on AKT activation via PIP3 phosphorylation, thus to negatively regulate PI3K/AKT signaling pathway. The DJ-1/PARK7 gene is located at the chromosome 1p36.2-36.3. The gene is about 24 kb in length and encodes a protein with a molecular weight of 21 kDa consisting of 189 amino acids. DJ-1 is a negative regulator of PTEN, which attenuates the inhibitory effect of PTEN on PI3K/AKT signaling pathway by suppressing the expression and function of PTEN, thereby indirectly activating PI3K/AKT signaling pathway, reducing apoptosis, and promoting cell proliferation.

Increasing evidence indicated that Cur has anti-tumor effects, such as inhibiting tumor cell proliferation, promoting tumor cell apoptosis, anti-angiogenesis, and restraining invasion and migration. It was showed that Cur plays a role in regulating the PTEN-PI3K/AKT pathway. Since DJ-1 is a negative regulator of PTEN, it is unclear whether Cur may affect the PTEN/PI3K/AKT pathway by regulating DJ-1. This study investigated the role of Cur in regulating DJ-1/PTEN/PI3K/AKT pathway activity, colorectal cancer cell proliferation, and apoptosis.
Our results observed that, compared with normal colorectal epithelial NCM460 cells, DJ-1 mRNA was significantly increased, while PTEN mRNA was obviously declined in SW480 and SW620 cells, indicating that DJ-1 up-regulation participates in colorectal cancer pathogenesis and reducing PTEN expression. Lin et al.\(^2\) showed that compared with normal colon tissue, DJ-1 level was significantly increased in CRC tissues and related to poor prognosis. Wu et al.\(^3\) reported that PTEN level was obviously up-regulated in HCT-116 and SW480 cells and CRC tissues, which inhibited CRC cells proliferation, promoted apoptosis, attenuated cell invasion, and restrained tumorigenicity in animals. Ke et al.\(^4\) revealed that PTEN expression was markedly declined in CRC tissues of patients and was associated with poor prognosis. Sun et al.\(^5\) found that PTEN expression in CRC tissues was apparently lower than that in normal colorectal tissues. Over-expression of PTEN in CRC LoVo and SW480 cells significantly inhibited cell proliferation, arrested cell cycle in G1 phase, and enhanced the drug sensitivity of cells to 5-FU. They indicated that abnormal expression of DJ-1 or PTEN plays a role in CRC, which was consistent with our results.
In this study, different concentrations of Cur significantly inhibited SW480 cell proliferation, promoted cell apoptosis, down-regulated DJ-1 expression, and elevated PTEN level. It indicated that Cur exerts an anti-tumor effect on CRC by restraining DJ-1 expression to enhance PTEN level. We further over-expressed DJ-1 in SW480 cells on the basis of Cur. It was observed that pcDNA3.1-DJ-1 transfection significantly reduced PTEN expression and enhanced p-AKT protein level in SW480 cells. PcDNA3.1-DJ-1 transfection alleviated the inhibition of Cur on SW480 cell proliferation and significantly reduced apoptosis, revealing that the down-regulation of DJ-1 and the up-regulation of PTEN by Cur play a role in inhibiting the activity of PI3K/AKT pathway and attenuating the malignant biological characteristics of CRC cells. Lin et al\(^\text{20}\) showed that over-expression of DJ-1 in CRC HCT116 and SW480 cells significantly reduced PTEN expression and enhanced the phosphorylation activity of AKT protein, while small interfere RNA (siRNA) DJ-1 obtained the opposite effect. Zhang et al\(^\text{24}\) revealed that Cur treatment significantly up-regulated the expression of PTEN, inhibited tumor cell proliferation, and induced apoptosis, whereas siRNA PTEN treatment markedly attenuated the proliferation inhibition and apoptosis induction impact of Cur. Yang et al\(^\text{25}\) reported that Cur analog inhibited the proliferation of prostate cancer DU145 cells and induced apoptosis by significantly up-regulating the expression of PTEN via targeting miR-21. Gawde et al\(^\text{26}\) observed that Cur analog inhibited cell proliferation and promoted apoptosis by significantly enhancing the expression of PTEN. It was showed that Cur can affect the biological effects of tumor cells by regulating PTEN. However, there is no report about the direct regulation of DJ-1 by Cur. This study exhibited that Cur can down-regulate the expression of DJ-1, affect the activity of PTEN/PI3K/AKT pathway, and exert anti-therapeutic effects by inhibiting CRC cell proliferation and promoting apoptosis. However, the activity of DJ-1-PTEN/PI3K/AKT pathway and the anti-tumor effect of Cur in animal were unclear.

**Conclusions**

We showed that the Cur inhibited colorectal cancer cell proliferation and promoted apoptosis by down-regulating DJ-1 to regulate the activity of PTEN/PI3K/AKT pathway.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**References**


Curcumin affects colorectal cancer by DJ-1


