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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 antitumor pharmacological effects. This study aimed to investigate the effects of Cur on proliferation and apoptosis of colorectal cancer cells.

MATERIALS AND MATHODS: 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 20 μM Cur treatment group, Cur+pcDNA blank group and Cur+pcDNA3.1-DJ-1 group. Cell proliferation activity was evaluated with EdU staining.

RESULTS: Compared 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 ($p<0.05$). Cur showed no significant effect on NCM460 cells. Cur down-regulated DJ-1 level and reduced 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 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[1]. 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[2-5]. 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[6]. Curcumin (Cur) is a polyphenolic substance extracted from the rhizome of Curcuma longa. Researchers[7,8] 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[9-12]. It was reported that Cur plays a role in regulating the activity of PTEN-PI3K/AKT signaling pathway[13,14]. 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-1640 (RPMI-1640) 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, NJ, 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 Ta Ba Biotechnology Co. (Dalian, China). C purchased from Sigma-Aldrich (St. Loui O, USA). EdU Apollo 488 Flow Cytometry was purchased from Ribobio (Cat. No. C103 2, Guangzhou, China). Rabbit anti-human D 1, protein kinase B (AKT), a orylated AKT (p-AKT) polyclonal odies re purchased from Abcam Biot ology (bridge, MA, USA). Rabbit anti PTE tin polyclonal antibod were d from Cell Signaling Technolo c. (Be MA, USA). Horseradish perc se (HRP)-c ted secondary antibod urchased fro ackson ImmunoRese (W Grove, PA, USA). Bicinchoninic acid (BCA) in quantification kit, Ann V/propidium i (PI) apoptosis detecti t and BeyoECL Plus chemiluminescenc agent purchased from Beyotime Bio olog anghai, China). PTEN inhibitor SI purcha rom MedchemExpress (Monmou ction , USA). Cell counting-kit (CK-8) te purchased from Dojindo Mo ar Tech gies (Rockville, MD, USA).

C

CM460, SW480, and SW620 cells were cul- in RPMI 1640 medium containing 10% FBS a penicillin-streptomycin. The cells were mai ed at incubator with 37°C and 5% CO_2 (Mode: FORMA 3131, Thermo Electron Corp, Waltham, MA, USA). The cells were 1:3-1:4 and were used for experime the arithmic phase. This study was oved by the Ethics Committee of Zibo Cent spital, Zibo, Shandong, China.

Cell Treatment and C ping

The NCM460, SW4 nd SW 20 cells cultured *in vitro* a nocula into 96-well plates (Corning, Co NY A, 100 ells/well). After adh g they w reated with 0, 10, or 2 M of Cu ult for 72 h. After that, 1 of CCK-8 so s added to each well sorbance of n well at 450 nm was asure 4 h of reaction (A450). Relative proliferation ity (%) = (A450 value of t ment group value of the blank w A450 value of the control group – A450 value of the blank w) × 100%.

N Over-Ex ssion id Con ction

whole genome DNA was used as a template to amplify the CDS region of DJ- gene. The size of the target fragment was de- by gel electrophoresis. After digested th 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 neutralized by 2 mg/ml glycine and incubated in 5% TritonX-100 (Biyotime Biotech. Shanghai, China) at room temperature for 10 min. Then, 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. At last, the cell was 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 ext[illegible]d by utilizing the TRIzol reagents (B[illegible] Biotechnology, Shanghai, China). The c[illegible]e-mentary DNAs (cDNAs) were synthesized [illegible] the PrimeScript RT reagent Kit (TaKaRa, Dal[illegible] China) and was used to reverse tran[illegible]cribe RNA [illegible] complementary DNA (cDNA). [illegible]ing gene mRNAs were amplified by [illegible]g the [illegible]R reaction system (TaKaRa, Dal[illegible] China). [illegible]ch was composed of 2×SYBR [illegible] Mixt[illegible] μm/l forward and reve[illegible] pr[illegible], cDNA 1.0 μl, and ddH_2O. The [illegible]mers o[illegible]I-1, PTEN, and β-actin genes [illegible]e synthesize[illegible] listed in Table I. The re[illegible]scription was [illegible]formed at 50°C for 15[illegible] an[illegible] for 5 min. The reaction was pe[illegible]formed on B[illegible] CFX96 at 95°C for 5 mi[illegible]llowed by 40 c[illegible]f 95°C for 15 s, 60°C[illegible]1 min. The relative gene expressions were [illegible]esente[illegible] using a gel scanning system (M[illegible]GDS8[illegible], UVP[illegible]cramento, CA, USA) and ca[illegible]with $2^{-\Delta}$[illegible]ethod.

Western Blot

The total protein was extracted fr[illegible] by RIPA on ice for 15 min. After [illegible]tified BCA method, a total of 40 μg p[illegible] was separated by 10% sodium dodecyl s[illegible]-polyacrylamide gel electrophoresis (SDS-P[illegible]Beyotime Biotechnology, Shanghai, C[illegible]na) and [illegible]sferred to polyvinylidene difluo[illegible] (PVDF, A[illegible] Biosciences, Little Ch[illegible]nt, Bu[illegible]ingham[illegible], UK) membrane at 30[illegible]A for 1[illegible]min. After being blocked by 5% s[illegible]lk [illegible]om tem[illegible]ture, the membrane w[illegible]acu[illegible] prima[illegible]tibody at 4°C overni[illegible] (DJ-1, [illegible] A[illegible] p-AKT, and β-actin[illegible]00, 1:2000, [illegible]:1000, and 1:10000). [illegible]ed by phosph[illegible] buffer solution Tw[illegible]20 (1[illegible] Beyotime Biotechnology, Shanghai, China), th[illegible]brane was further incub[illegible] HRP conjug[illegible]econdary antibody a[illegible]n temperature for 60 min (1:10000). At last, [illegible] membrane w[illegible] treated by enhanced chemi-[illegible]inescence ([illegible], Beyotime Biotechnology, [illegible]ghai, China[illegible]gent and developed.

Statistical Analysis

SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was applied for data analysis. The measure[illegible] were presented as mean ± standard de[illegible]ation (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

Tabl[illegible] The impact [illegible] different concentrations of Cur on cell proliferation.

		0 μM	10 μM	20 μM
[illegible]lative cell survival (%)	NCM460	100±5.23	102.19±9.22	93.81±8.26
	SW480	100±6.03	71.88±6.35	48.79±5.31
	SW620	100±7.56	89.56±7.17	70.35±6.22

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

		0 μM	10 μM	
Relative cell survival (%)	NCM460	100±5.23	102.19±9.22	3.81±8.26
	SW480	100±6.03	71.88±6.35	8.79±5.31
	SW620	100±7.56	89.56±7.17	5±6.22

with malignancy dependence (Figure 1A, B). Western blot demonstrated that DJ-1 protein was markedly enhanced, whereas PTEN protein was apparently declined in SW480 and SW620 cells compared with normal lung epithelial NCM460 cells (Figure 1B).

Cur Treatment Significantly Inhibited Colorectal Cancer Cell Proliferation and Promoted Cell Apoptosis

CCK-8 assay revealed that different concentrations of Cur showed a similar impact on the proliferative activity of normal colorectal epithelial NCM460 cells (Table II). Cur treatment markedly inhibited SW480 and SW620 cell proliferation with dose and malignancy dependence (Table II).

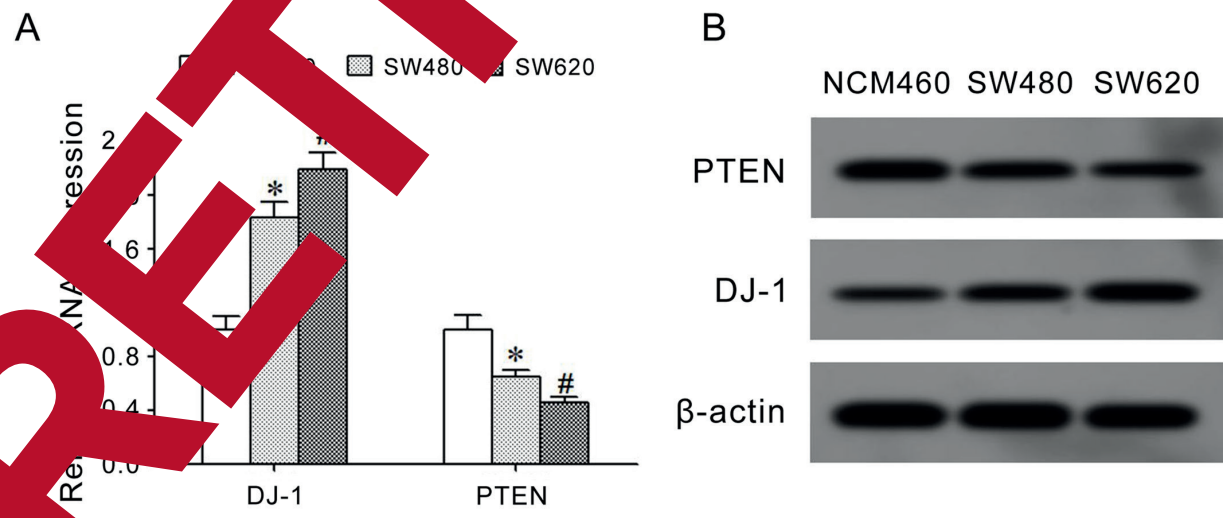
Flow cytometry detection demonstrate[d] [t]hat Cur treatment apparently induced SW[480] SW620 cell apoptosis with dose and mali[gnan]cy dependence (Figure 2A, B).

Cur Treatment Suppressed DJ-1 Expression and Up-Regulated PT[EN] Expression in SW480 Cells

qRT-PCR showed tha[t Cur] dow[n-r]egulated DJ-1 mRNA level and [up-r]ed P[TEN] expression in SW480 [cells] with d[ose] depend[ence] (Figure 3A, B). Wes[tern] blot r[esults] showed that Cur reduced DJ-1 p[rotein] lev[el] and up-[regu]lated PTEN protein [exp]ress[ion] in SW480 [cells] with dose dependen[ce] (Figure 3[B]).

DJ-1 Ov[erexp]ression Antag[oni]zed the Impact [of] Cur [on] SW480 Cell Proliferation and Apoptos[is]

[qRT-PC]R showed tha[t Cur] treatment signifi[cantly] down-regulated DJ-1 mRNA and elevated PTEN mRNA in SW480 cells (Figure 4A, [*p*]cDNA3.1-D[J-1] transfection significantly increased DJ-1 m[RNA] and reduced PTEN mRNA e[xpress]ions in S[W48]0 cells ($p<0.05$). Western blot resu[lts] [sh]ow[ed] that pcDNA3.1-DJ-1 transfection remarkably attenuated the influence of Cur on DJ-1 inhibition and PTEN up-regulation, whereas [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 ($p<0.05$).



Fig[ure 3]. DJ-1 upregulated, while PTEN decreased in lung cancer cells. ***A,*** qRT-PCR detection of mRNA expression. ***B,*** Weste[rn] blot detection of protein expression. $^{*}p<0.05$, compared with NCM460 cells.

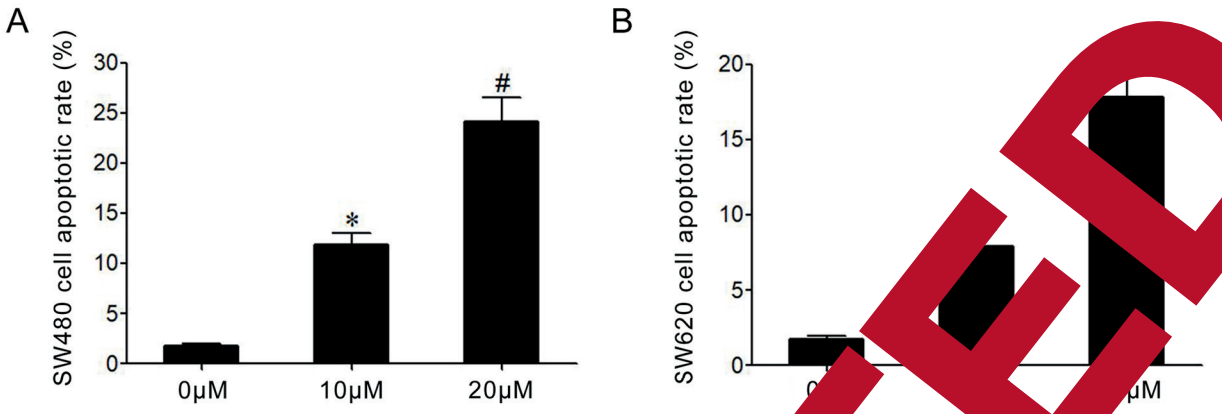
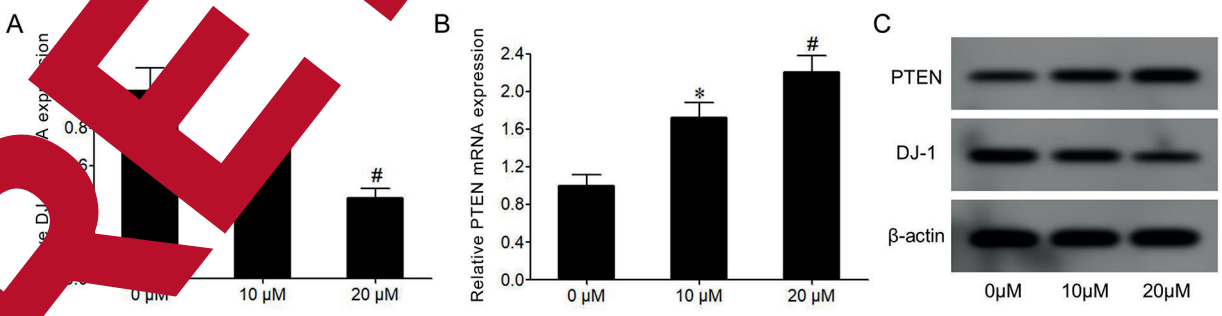


Figure 2. Cur treatment induced CRC cell apoptosis. ***A,*** Flow cytometry detecti… W480 cell apoptos… low cytometry detection of SW620 cell apoptosis. *$p<0.05$, compared with 0 μM.

Discussion

When the PI3K/AKT pathway is activated, P13K 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 membra… d phosphorylates loci Ser473 and Thr308 … under the action of phosphoinositide-dep… nt protein kinase (PDK). Phosphorylation-acti… d AKT further activates downstream signa… molecules to participate in cell g…wth, survi… al, and apoptosis by regulatin… scription… and expression of various d… strea… nes[15-19]. PTEN is the only tumor s… essor g… discovered that has dual activ… f pro… and phosphatase. PT…N … sphorylate PIP3 to antagonize … impa… KT activation via PIP3 pho… rylation, thu… egatively regulate PI3… ignaling pati… y[2]. The DJ-1/PARK7 … is … d at the chromosome 1… -36.3. The gene is about 24 kb in length and …odes a protein …ith a molecular weight of 21 … consisting o… 9 amino acids[6]. DJ-1 is a neg… regulator o… EN, which attenuates the in… effect … TEN on PI3K/AKT signaling path… essing the expression and function of P…EN, thereby indirectly activating PI3K/…KT signaling pathway, reducing apoptosis, and … cell proliferation[6].

…ing 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[7,8]. It was showed that Cur plays a role in regulating the PTEN-PI3K/AKT pathway[13,14]. 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.



…3. Cur treatment suppressed DJ-1 expression and upregulated PTEN expression. ***A,*** qRT-PCR detection of DJ-1 m… pression. ***B,*** qRT-PCR detection of PTEN mRNA expression. ***C,*** Western blot detection of protein expression. *$p<0.…$ompared with 0 μM group.

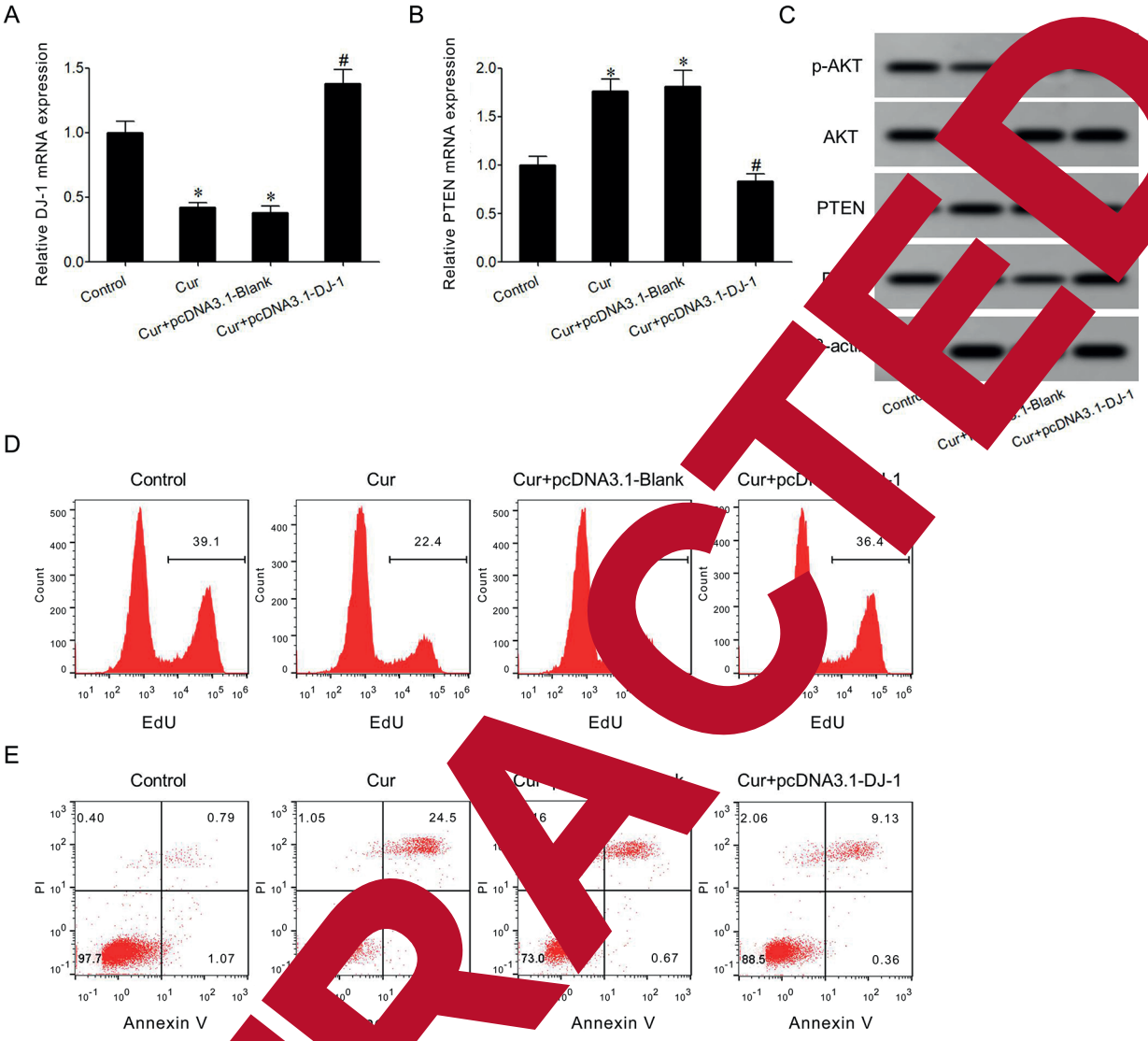


Figure 4. DJ-1 over-e sion ant d the impact of Cur on SW480 cell proliferation and apoptosis. ***A,*** qRT-PCR detection of DJ-1 mR pression. ***B,*** q P detection of PTEN mRNA expression. C, Western blot detection of protein expression. ***D,*** Flow y detection of c feration. ***E,*** Flow cytometry detection of cell apoptosis. $^{*}p<0.05$, compared with control. $^{\#}p<0$ with pcDNA3.1 lank.

Our its observed that, co pared with normal rectal helial NCM460 cells, DJ-1 mR was si icantly creased, while PTEN mRN viousl clined in SW480 and SW620 c dica that DJ-1 up-regulation ates l al cancer pathogenesis and redu PTEN ression. Lin et al[20] showed that pared with normal colon tissue, DJ-1 level ly increased in CRC tissues and ed to poor prognosis. Wu et al[21] reported that N level was obviously up-regulated in HCT-1 SW480 cells and CRC tissues, which inhibit CRC cells proliferation, promoted apoptosis, attenuated cell invasion, and restrained tumorigenicity in animals. Ke et al[22] revealed that PTEN expression was markedly declined in CRC tissues of patients and was associated with poor prognosis. Sun et al[23] 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[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[24] revealed that Cur treatment significantly up-regulated the expression of PTEN, inhibited [illegible] cell proliferation, and induced apoptosis, [illegible] siRNA PTEN treatment markedly attenua[illegible]he proliferation inhibition and apoptosis indu[illegible] impact of Cur. Yang et al[25] reported that Cur [illegible]alog inhibited the proliferation of [illegible]rostate canc[illegible] DU145 cells and induced apopt[illegible]nificantly up-regulating the expres[illegible]of P[illegible] via targeting miR-21. Gawde et [illegible]observ[illegible]hat Cur analog inhibited cell pr[illegible]tion a[illegible] apoptosis by significa[illegible]y e[illegible]he expression of PTEN. It w[illegible]owed t[illegible]r can affect the biological eff[illegible]of tumor ce[illegible]regulating PTEN. How[illegible]re is no repo[illegible]out the direct regula[illegible]of D[illegible] Cur. This study exhibited that Cur can down[illegible]ate the expression of DJ-1, [illegible]t the activity [illegible]EN/PI3K/AKT pathwa[illegible]nd ex[illegible]rt anti-therap[illegible]utic effects by inhib[illegible] CRC [illegible] proliferation and promoting ap[illegible]. Ho[illegible]er, the [illegible]tivity of DJ-1-PTEN/PI3K/[illegible]way a[illegible]e anti-tumor effect of C[illegible] in an[illegible]ere [illegible]ar.

Conclusions

[illegible]e showed that the Cur inhibited colorectal [illegible]er cell proliferation and promoted apoptosis b[illegible]n-regulating DJ-1 to regulate the activity of P[illegible]/PI3K/AKT pathway.

Conflict of Interest

The Authors declare that they have no confli[illegible]

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