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Curcumin Inhibits Cell Viability and Increases Apoptosis of SW620 Human Colon Adenocarcinoma Cells via the Caudal Type Homeobox-2 (CDX2)/Wnt/ β -Catenin Pathway

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Background: Curcumin is a polyphenol compound extracted from the root of the herb *Curcuma longa*, which is used in traditional Chinese medicine (TCM). Worldwide, colorectal carcinoma (CRC) is an increasing cause of morbidity and mortality. This study aimed to investigate the effects of increasing concentrations of curcumin on cell viability, proliferation, and apoptosis of SW620 human colonic adenocarcinoma cells cultured *in vitro*, and the signaling pathways involved.

Material/Methods: SW620 human colonic adenocarcinoma cells were cultured in curcumin at concentrations of 0, 4, 8, 16, and 32 $\mu\text{mol/l}$ for 48 hours. Specific small interfering RNA (siRNA) was transfected into SW620 cells to silence the expression of caudal type homeobox-2 (CDX2). Cell viability was measured using the MTT assay. Flow cytometry evaluated cell apoptosis. Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) were used to assess the nuclear translocation of β -catenin and the activation of Wnt signaling.

Results: Curcumin reduced cell viability and increased apoptosis of SW620 human colonic adenocarcinoma cells in a dose-dependent way, and increased the expression of CDX2 but decreased β -catenin nuclear translocation and the expression of Wnt3a, c-Myc, survivin, and cyclin D1. CDX2 silencing significantly reduced the effects of curcumin on SW620 human colonic adenocarcinoma cells. The nuclear translocation of β -catenin, and expression levels of Wnt3a, c-Myc, survivin, and cyclin D1 were significantly higher in CDX2-silenced SW620 cells.

Conclusions: Curcumin reduced cell viability and increased apoptosis in SW620 human colonic adenocarcinoma cells by restoring CDX2, which inhibited the Wnt/ β -catenin signaling pathway.

MeSH Keywords: **Apoptosis • Colorectal Neoplasms • Curcumin**

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Background

Worldwide, colorectal cancer (CRC) ranks as the fourth leading cause of cancer-related death and is an increasing cause of morbidity and mortality [1]. Patients with advanced-stage CRC have a poor prognosis due to the lack of effective treatments. Chemotherapy is still the main treatment for patients with advanced-stage CRC, but can be ineffective in patients with chemotherapy resistance and is associated with significant side effects. Therefore, there is a continued need to identify safe and effective agents to treat CRC.

There has been increasing interest in the use and development of agents extracted from natural products, including herbal compounds used in traditional Chinese medicine (TCM), because of their broad spectrum of biological activities [2]. Some herbal TCMs have been studied as anticancer drugs [3]. Curcumin (C₂₁H₂₀O₆) is a polyphenol compound extracted from the root of the herb *Curcuma longa*, which is used in TCM. Previous studies have shown that curcumin has anticancer activity in patients with breast cancer, lung cancer, and gastric cancer [4–6]. However, the molecular mechanisms associated with the effects of curcumin remain to be determined.

Caudal type homeobox-2 (CDX2) was first reported as a homeobox protein that plays a role in maintaining the intestinal phenotype, regulating gut homeostasis and development [7]. Recent studies have shown an association between CDX2 and CRC [8]. Inhibition of CDX2 in patients with CRC resulted in worse prognosis and a reduced response to chemotherapy [9]. Previous studies also showed that CDX2 was a mediator of the Wnt signaling pathway [10]. Curcumin was shown to inhibit the activation of the Wnt signaling pathway, which is highly regulated in cancer growth [11]. Therefore, the hypothesis that drove this study was that curcumin might affect Wnt signaling by regulating CDX2 in colonic carcinoma cells.

Therefore, this study aimed to investigate the effects of increasing concentrations of curcumin on cell viability, proliferation, and apoptosis of SW620 human colonic adenocarcinoma cells cultured *in vitro*, and the signaling pathways involved. In this study, CDX2 was knocked-down using specific small interfering RNA (siRNA), and the involvement of the Wnt signaling was also studied.

Material and Methods

Cell culture and treatment

SW620 human colonic adenocarcinoma cells were purchased from the Cell Bank of the Shanghai Institute of Cell Biology, the Chinese Academy of Sciences. SW620 cells were maintained

in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and antibiotics (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Cells were cultured in a humidified atmosphere with 5% CO₂ and 95% air at 37°C. SW620 cells were treated with curcumin at concentrations of 0, 4, 8, 16, and 32 μmol/l for 48 hours.

Transfection with small interfering RNA (siRNA)

Specific small interfering RNA (siRNA) was transfected into SW620 cells to silence the expression of caudal type homeobox-2 (CDX2) before curcumin treatment. Cells were cultured in a six-well culture plate until they reached 50% confluence at 24 hours before siRNA transfection. RNA interfering silencing of CDX2 was performed by using Silencer™ CDX2 siRNA (ThermoFisher Scientific, Waltham, MA, USA). A Silencer™ negative control was also used. All protocols were performed according to the manufacturer's instructions.

Cell viability

The MTT assay was used to evaluate the viability of SW620 human colonic adenocarcinoma cells. Briefly, 1×10⁴ SW620 cells were seeded into wells of a cell culture plate. Then, siRNA-transfected and control cells were treated with curcumin at various concentrations for 48 hours. The MTT solution (Sigma-Aldrich, St. Louis MO, USA) at a final concentration of 5 mg/ml was added, and the cells were cultured in each well for 4 hours. Dimethyl sulfoxide (DMSO) was added to dissolve the formed crystals. A microplate reader was used to detect the optical density (OD) at 490nm (A₄₉₀). The cell viability rate was then calculated using the formula:

$$\text{cell viability} = (\text{OD}_{\text{treatment}} / \text{OD}_{\text{control}}) \times 100\%.$$

Flow cytometry for cell apoptosis

Cell apoptosis was detected by flow cytometry. After treatment, cells were harvested and trypsinized. After washing, Annexin-V and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) were used to stain the cells in the dark for 30 min. A flow cytometer (Beckman Coulter, Brea, CA, USA) was used to evaluate the samples, and the apoptotic rate was calculated.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The relative expression levels of mRNA of c-Myc, survivin, and cyclin D1 were analyzed with a Stratagene Mx3005P system (Agilent, Santa Clara, CA, USA) and SYBR Green Real-time PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA) according to the protocols provided by the manufacturers. The primers used for c-Myc, survivin, and cyclin D1 are listed in Table 1. The specificity of the primer products was confirmed using

Table 1. Primers used in reverse transcriptase-polymerase chain reaction (RT-PCR).

Gene	Direction	Primer sequence
c-Myc	Forward	5'-CGCGGATCCCTGGATTTTTTCGGGTAGTG-3'
	Reverse	5'-CCGCTCGAGCGCACAAAGAGTTCCGTAGCT-3'
Survivin	Forward	5'-TGGACAAACAAGAGCCAAGAA-3'
	Reverse	5'-TAGAGCAAAGCCACAAAACCAA-3'
Cyclin D1	Forward	5'-CCGCCTCACACGCTFCCTCTC-3'
	Reverse	5'-TCCTCCTCGGCGGCTTGGGG-3'
GAPDH	Forward	5'-TGCACCACCAACTGCTTA-3'
	Reverse	5'-GGCATGGACTGTGGTCATGAC-3'

melting curve analysis. The $2^{-\Delta\Delta CT}$ method was used to determine the quantitative values of the relative expression levels.

Western blot for CDX2, Wnt3a, c-Myc, survivin, and cyclin D1

Harvested cells were lysed with RIPA cell lysis buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA) supplemented with 1% PMSF (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Total protein was extracted using the Total Protein Extraction kit (Beyotime, Shanghai, China). Protein concentration was evaluated by Lowry protein assay. The protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) or nitrocellulose (NC) membranes by the dry blotting method. Membranes were blocked with 5% dried skimmed milk powder in Tris-buffered saline Tween20 (TBST). Membranes were incubated with primary antibodies to CDX2 (1: 2000) (Abcam, Cambridge, MA, USA), Wnt3a (1: 2000) (Abcam, Cambridge, MA, USA), c-Myc (1: 2500) (Cell Signaling Technology, Danvers, MA, USA), survivin (1: 2500) (Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (1: 2500) (Cell Signaling Technology, Danvers, MA, USA), and GAPDH (1: 5000) (Invitrogen, Carlsbad, CA, USA) at 4°C for 12 hours. After washing in TBST, secondary antibodies conjugated with horseradish peroxidase (HRP) were incubated on the membranes for 2 hours. SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) was used to develop the membranes, which were visualized using X-ray analysis.

Immunoblot for nuclear translocation of β -catenin

The nuclear translocation of β -catenin was assessed by immunoblot. Nuclear and cytoplasmic proteins were extracted using the Nuclear Protein Extraction kit (Beyotime, Shanghai, China). The primary antibodies were to β -catenin and histone H3. Relative expression levels of β -catenin were detected in the cell nuclei. Histone H3 was used as the internal reference to measure the nuclear translocation of β -catenin.

Statistical analysis

Data were analyzed SPSS version 16.0 software (IBM, Chicago, IL, USA). Data were presented as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the differences between groups. A P-value <0.05 was considered to be statistically significant.

Results

Curcumin reduced the viability of SW620 human colonic adenocarcinoma cells, which was reversed by CDX2 silencing

The MTT assay was used to evaluate the viability of SW620 human colonic adenocarcinoma cells. As shown in Figure 1A, after incubation with curcumin, cell viability significantly decreased in a concentration-dependent manner ($P < 0.05$). As shown in Figure 1B, silencing caudal type homeobox-2 (CDX2) using small interfering RNA (siRNA) significantly increased cell viability in SW620 cells cultured in curcumin ($P < 0.05$). The results suggested the inhibitory effect of curcumin on SW620 human colonic adenocarcinoma cells was reverse by CDX2 silencing.

Curcumin increased cell apoptosis, which was reduced by CDX2 silencing

Flow cytometry was used to evaluate cell apoptosis. As shown in Figure 2A, curcumin treatment significantly increased apoptosis of SW620 human colonic adenocarcinoma cells in a concentration-dependent manner ($P < 0.05$). As shown in Figure 2B, compared with SW620 cells cultured with curcumin, CDX2 silencing significantly inhibited apoptosis in SW620 cells cultured with curcumin at 32 $\mu\text{mol/L}$ ($P < 0.05$). The results suggested the apoptosis-inducing effects of curcumin on SW620 human colonic adenocarcinoma cells was reduced by CDX2 silencing.

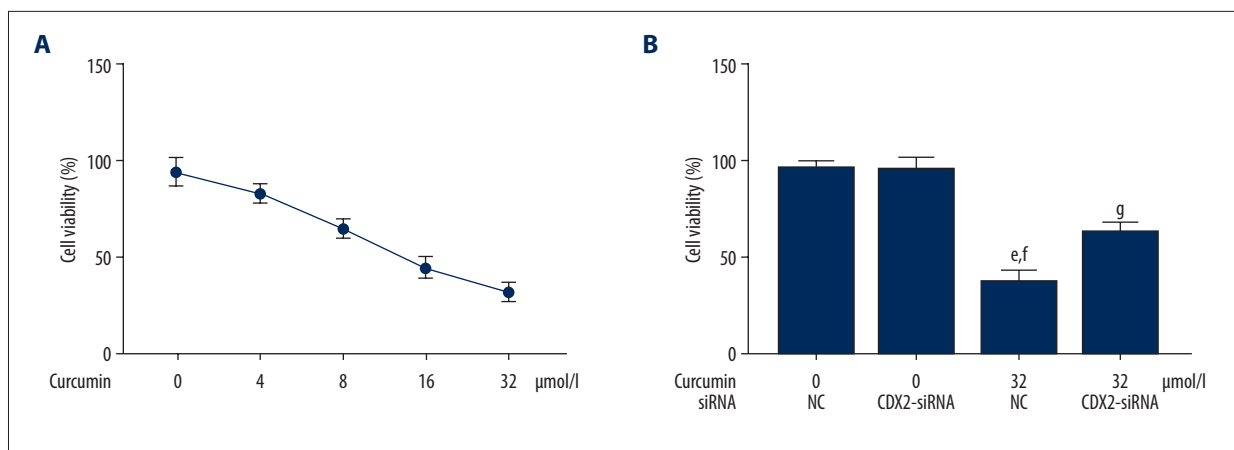


Figure 1. Cell viability of SW620 human colonic adenocarcinoma cells cultured with increasing concentrations of curcumin. **(A)** The line chart shows the cell viability of SW620 human colonic adenocarcinoma cells cultured with curcumin at concentrations of 0, 4, 8, 16, and 32 $\mu\text{mol/l}$ for 48 hours. **(B)** The columns indicate the cell viability of SW620 cells transfected with negative control (NC), small interfering RNA (siRNA), CDX2-siRNA, and curcumin at 32 $\mu\text{mol/l}$. ^a The significant difference compared with SW620 cells treated with curcumin at 0 $\mu\text{mol/l}$ ($P < 0.05$). ^b The significant difference compared with SW620 cells treated with curcumin at 4 $\mu\text{mol/l}$. ^c The significant difference compared with SW620 cells treated with curcumin at 8 $\mu\text{mol/l}$ ($P < 0.05$). ^d The significant difference compared with SW620 cells treated with curcumin at 16 $\mu\text{mol/l}$ ($P < 0.05$). ^e The significant difference compared with SW620 cells transfected with NC siRNA ($P < 0.05$). ^f The significant difference compared with CDX2 siRNA-transfected SW620 cells treated with curcumin at 0 $\mu\text{mol/l}$ ($P < 0.05$). ^g The significant difference compared with NC siRNA-transfected SW620 cells treated with curcumin at 32 $\mu\text{mol/l}$ ($P < 0.05$).

Curcumin significantly inhibited β -catenin nuclear translocation in SW620 cells, which was reversed by CDX2 silencing

The nuclear translocation level of β -catenin was assessed by Western blot of β -catenin in nuclear protein extracted from SW620 human colonic adenocarcinoma cells. The nuclear protein, histone H3, was used as an internal reference. As shown in Figure 3A, after incubation with curcumin, the nuclear translocation of β -catenin was significantly inhibited in SW620 human colonic adenocarcinoma cells. The inhibitory effect of curcumin was in a concentration-dependent manner ($P < 0.05$). However, as shown in Figure 3B, the nuclear translocation of β -catenin was increased in CDX2 silenced SW620 cells compared with the cells cultured with curcumin at 32 $\mu\text{mol/L}$. The results indicated that the inhibitory effect of curcumin on β -catenin nuclear translocation was reversed by CDX2 silencing.

Curcumin significantly suppressed Wnt/ β -catenin signaling activation, which was increased by CDX2 silencing

Both reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot were employed to determine the expression levels of targeted genes. As shown in Figure 4A, 4C, and 4D, the expression of CDX2 was significantly increased by curcumin treatment of SW620 human colonic adenocarcinoma cells in a concentration-dependent manner ($P < 0.05$). The expression of Wnt3a and the Wnt downstream signaling genes, including *c-Myc*, *survivin*, and *cyclin D1* were inhibited

by curcumin in a concentration-dependent manner ($P < 0.05$). As showed in Figure 4B, 4E, and 4F, CDX2 siRNA transfection significantly inhibited the expression of CDX2 in SW620 cells ($P < 0.05$). CDX2 silencing impaired the inhibitory effect of curcumin on Wnt3a, *c-Myc*, *survivin*, and *cyclin D1* in SW620 human colonic adenocarcinoma cells.

Discussion

Recent studies have shown an association between the expression of caudal type homeobox-2 (CDX2) and colorectal cancer (CRC) [12]. The findings from a previous study support those of the present study that loss of CDX2 in SW620 human colonic adenocarcinoma cells increased cell proliferation, and also promoted migration and drug resistance [13]. In 2016, Dalerba et al. showed that the expression of CDX2 was an independent prognostic indicator in patients with stage II and stage III colon cancer [12]. Previous studies reported that the down-regulation or knockdown of CDX2 facilitated the proliferation of colon cancer cells *in vitro* and the growth of tumor xenografts [14]. In the present study, CDX2 was highly expressed in SW620 human colonic adenocarcinoma cells, and silencing CDX2 expression with small interfering RNA (siRNA) significantly increased SW620 cell viability.

The findings from the present study also showed that CDX2 was a mediator of the Wnt signaling pathway, as CDX2 silencing increased the expression of Wnt3a, which further

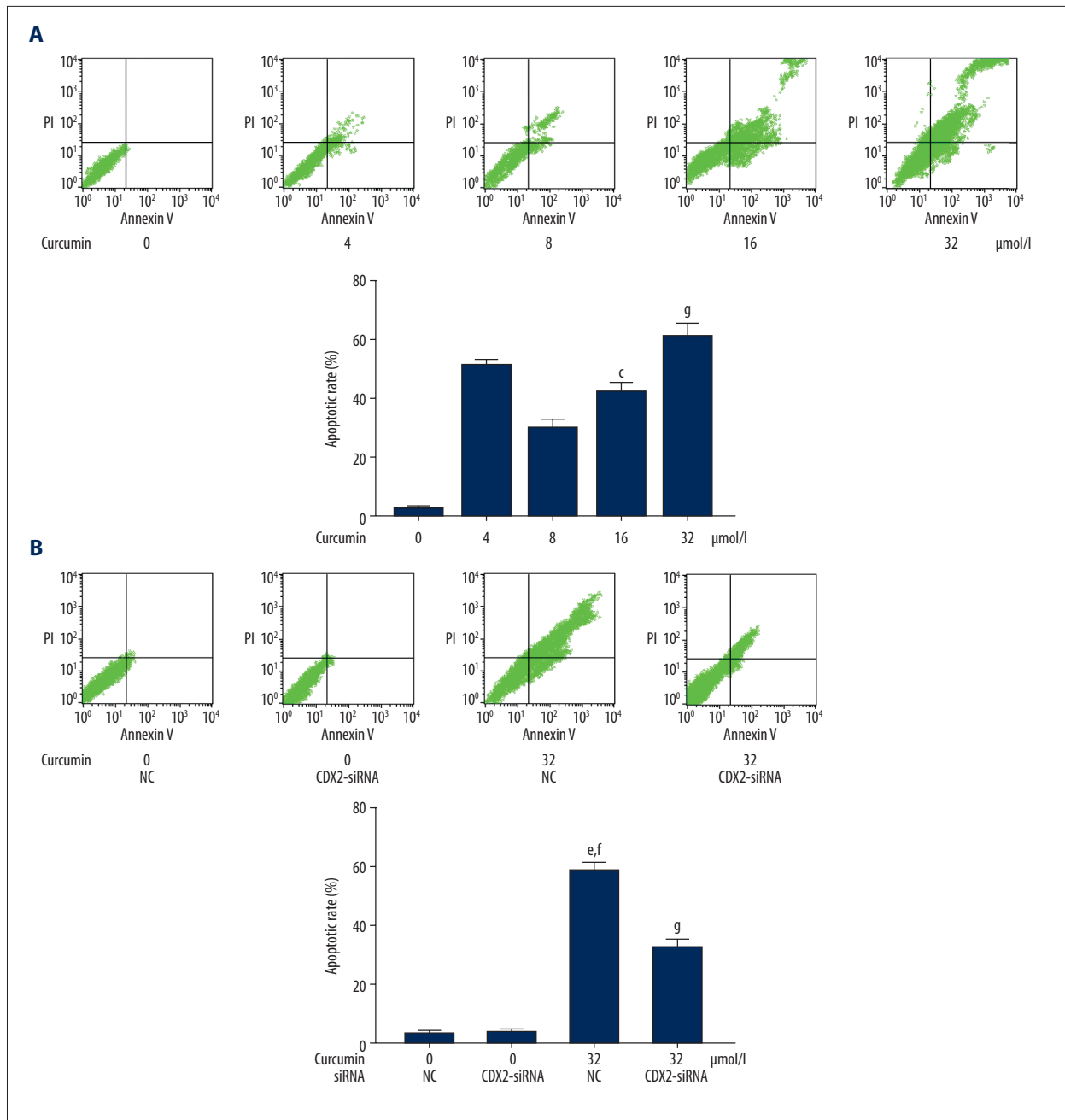


Figure 2. Apoptosis of SW620 human colonic adenocarcinoma cells cultured with increasing concentrations of curcumin. **(A)** The upper part of the chart shows the results of flow cytometry analysis of apoptosis of SW620 human colonic adenocarcinoma cells cultured with curcumin at concentrations of 0, 4, 8, 16, and 32 µmol/l for 48 hours. The columns in the lower part of the chart indicate the percentage of apoptotic cells. **(B)** The upper part of the chart shows the results of flow cytometry analysis of apoptosis of SW620 human colonic adenocarcinoma cells transfected with negative control (NC) siRNA, CDX2-siRNA, and curcumin at 32 µmol/l. Columns indicate the percentage of apoptotic cells. ^a The significant difference compared with SW620 cells treated with curcumin at 0 µmol/l ($P < 0.05$). ^b The significant difference compared with SW620 cells treated with curcumin at 4 µmol/l. ^c The significant difference compared with SW620 cells treated with curcumin at 8 µmol/l ($P < 0.05$). ^d The significant difference compared with SW620 cells treated with curcumin at 16 µmol/l ($P < 0.05$). ^e The significant difference compared with SW620 cells transfected with NC siRNA ($P < 0.05$). ^f The significant difference compared with CDX2-siRNA transfected SW620 cells treated with curcumin at 0 µmol/l ($P < 0.05$). ^g The significant difference compared with NC siRNA-transfected SW620 cells treated with curcumin at 32 µmol/l ($P < 0.05$).

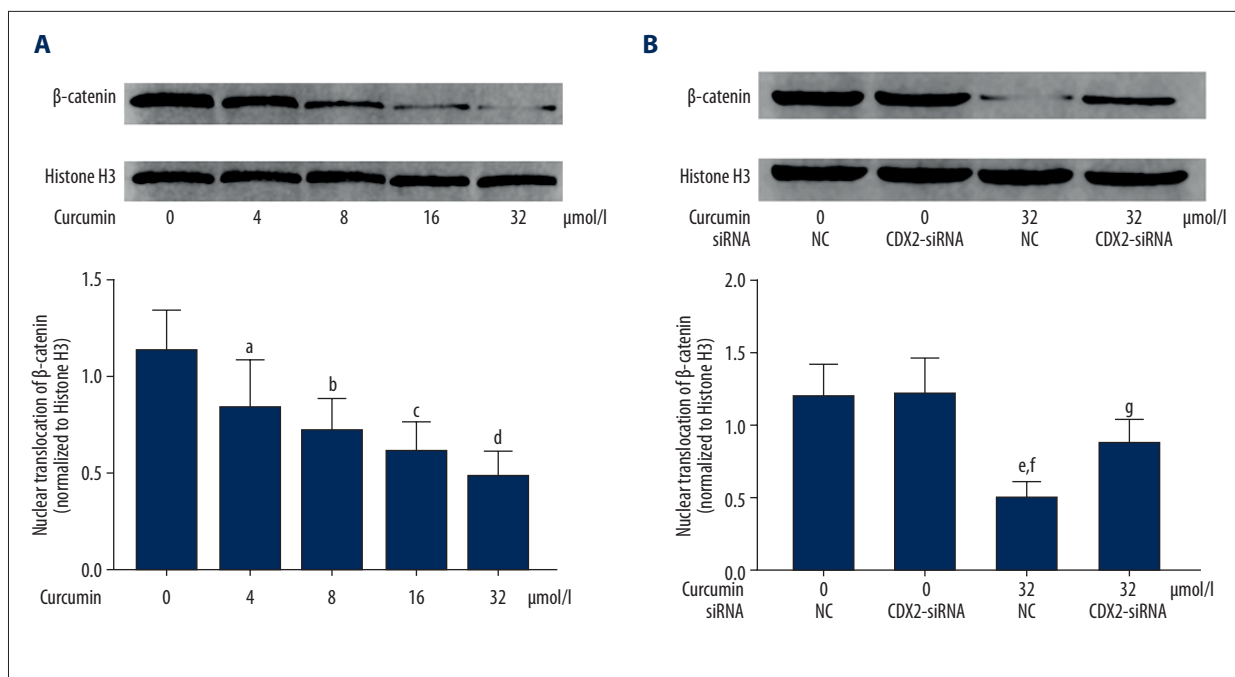


Figure 3. Western blot for the expression of nuclear β -catenin in SW620 human colonic adenocarcinoma cells cultured with increasing concentrations of curcumin. **(A)** Western blot of β -catenin and the internal reference, histone H3, are shown. The columns in the lower part show the relative expression levels of nuclear β -catenin in SW620 human colonic adenocarcinoma cells cultured with curcumin at concentrations of 0, 4, 8, 16, and 32 $\mu\text{mol/l}$ for 48 hours. **(B)** The upper part shows the Western blot of β -catenin and histone H3. Relative expression levels of nuclear β -catenin in SW620 human colonic adenocarcinoma cells transfected with negative control (NC) small interfering RNA (siRNA), CDX2-siRNA and curcumin at 32 $\mu\text{mol/l}$ are shown in the columns in the lower panel. ^a The significant difference compared with SW620 cells treated with curcumin at 0 $\mu\text{mol/l}$ ($P < 0.05$). ^b The significant difference compared with SW620 cells treated with curcumin at 4 $\mu\text{mol/l}$. ^c The significant difference compared with SW620 cells treated with curcumin at 8 $\mu\text{mol/l}$ ($P < 0.05$). ^d The significant difference compared with SW620 cells treated with curcumin at 16 $\mu\text{mol/l}$ ($P < 0.05$). ^e The significant difference compared with SW620 cells transfected with NC siRNA ($P < 0.05$). ^f The significant difference compared with CDX2 siRNA-transfected SW620 cells treated with curcumin at 0 $\mu\text{mol/l}$ ($P < 0.05$). ^g The significant difference compared with NC siRNA-transfected SW620 cells treated with curcumin at 32 $\mu\text{mol/l}$ ($P < 0.05$).

facilitated the nuclear translocation of β -catenin. The expressions of β -catenin targeted genes were found to be significantly increased in SW620 human colonic adenocarcinoma cells. The Wnt signaling pathway is evolutionarily conserved to maintain cell homeostasis [15]. The association between Wnt signaling and the progression of several types of human cancer have been demonstrated [16]. As one of the Wnt family members, Wnt3a, has key roles in regulating cell proliferation, migration, and differentiation [16,17]. Wnt3a binds to the low-density lipoprotein receptor (LDLR), which interact with the Frizzled (Fz) receptors that act as Wnt co-receptors, resulting in the stabilization of intracellular β -catenin levels [17]. The activation of Wnt signaling facilitates nuclear translocation of the nuclear factor, β -catenin. The genes for C-myc, survivin, and cyclin D1 are target genes for β -catenin [17]. These effectors are known cancer promoters that facilitate the proliferation, invasion, and metastasis of CRC [17]. The findings from the present study showed that the expression of c-Myc, survivin, and cyclin D1 were increased following silencing of

CDX2 expression in SW620 human colonic adenocarcinoma cells. These results indicated that CDX2 was a cancer suppressor that mediated its inhibitory effects in SW620 cells *in vitro* through the Wnt/ β -catenin signaling pathway.

The anticancer properties of curcumin and the proposed mechanisms of action have previously been reviewed [18]. *In vitro* cell studies have shown that curcumin inhibits cancer cell proliferation and induces cell apoptosis [19]. In 2017, Zheng et al. showed that curcumin inhibited the proliferation of gastric carcinoma cells *in vitro* and induced apoptosis via the Wnt/ β -catenin signaling pathway [20]. In the present study, the viability of SW620 human colonic adenocarcinoma cells was reduced by curcumin treatment, which also increased CDX2 expression and suppressed Wnt signaling, as shown by the reduced expression levels of Wnt3a, c-Myc, survivin, and cyclin D1, as well as the nuclear translocation of β -catenin. Silencing the expression of CDX2 with small interfering RNA (siRNA) reduced the effects of curcumin. Specifically, in CDX2 silenced SW620

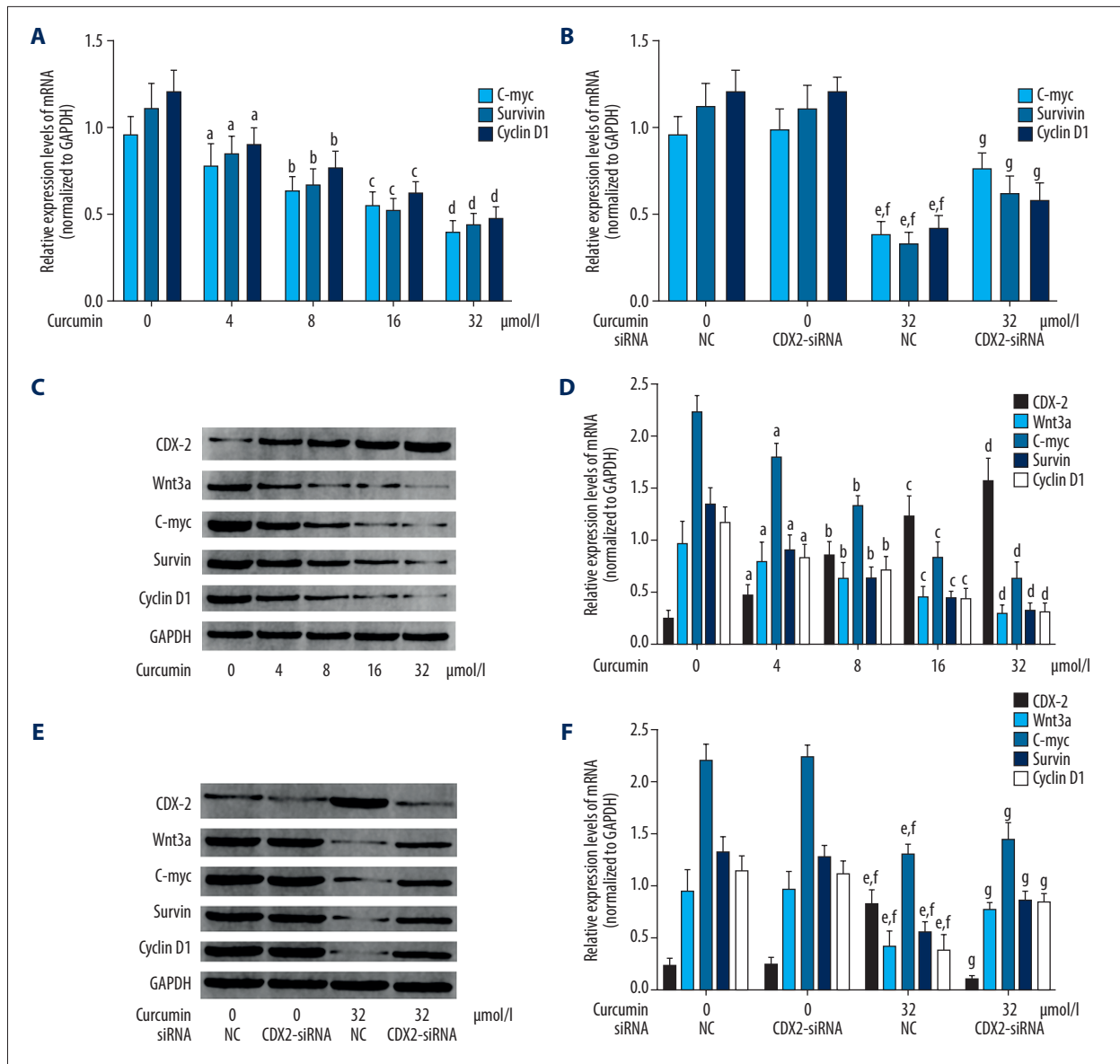


Figure 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) for the relative expression levels of mRNAs of the genes for c-Myc, survivin, and cyclin D1, and Western blot for CDX2, Wnt3a, c-Myc, survivin, and cyclin D1 in SW620 human colonic adenocarcinoma cells cultured with increasing concentrations of curcumin. **(A)** The columns indicate the relative expression levels of mRNAs of the genes for c-Myc, survivin, and cyclin D1 in SW620 human colonic adenocarcinoma cells cultured with curcumin at concentrations of 0, 4, 8, 16, and 32 μmol/l for 48 hours. **(B)** The columns indicate the relative expression levels of mRNAs of the genes for c-Myc, survivin, and cyclin D1 in SW620 human colonic adenocarcinoma cells transfected with negative control (NC) siRNA, CDX2-siRNA and curcumin at 32 μmol/l. **(C)** Western blot for CDX2, Wnt3a, c-Myc, survivin, cyclin D1, and GAPDH are shown. **(D)** The columns show the relative expressions levels of CDX2, Wnt3a, c-Myc, survivin and cyclin D1 in SW620 human colonic adenocarcinoma cells cultured with curcumin at concentrations of 0, 4, 8, 16, and 32 μmol/l for 48 hours. **(E)** Western blot for CDX2, Wnt3a, c-Myc, survivin, cyclin D1, and GAPDH are shown. **(F)** The columns indicate the relative expressions levels of CDX2, Wnt3a, c-Myc, survivin, and cyclin D1 in SW620 human colonic adenocarcinoma cells transfected with negative control (NC) siRNA, CDX2-siRNA and curcumin at 32 μmol/l. ^a The significant difference compared with SW620 cells treated with curcumin at 0 μmol/l ($P < 0.05$). ^b The significant difference compared with SW620 cells treated with curcumin at 4 μmol/l ($P < 0.05$). ^c The significant difference compared with SW620 cells treated with curcumin at 8 μmol/l ($P < 0.05$). ^d The significant difference compared with SW620 cells treated with curcumin at 16 μmol/l ($P < 0.05$). ^e The significant difference compared with SW620 cells transfected with NC siRNA ($P < 0.05$). ^f The significant difference compared with CDX2-siRNA transfected SW620 cells treated with curcumin at 0 μmol/l ($P < 0.05$). ^g The significant difference compared with NC siRNA-transfected SW620 cells treated with curcumin at 32 μmol/l ($P < 0.05$).

human colonic adenocarcinoma cells, curcumin failed to suppress the activation of Wnt signaling effectively. These preliminary *in vitro* findings, from the use of a single human colonic adenocarcinoma cell line, support the potential role CDX2 as a therapeutic targets of curcumin. However, these preliminary findings require further investigation, including studies with more colonic adenocarcinoma cell lines, and tumor xenograft models. Future well-planned clinical studies, possibly including the investigation of curcumin as adjuvant therapy, might include controlled studies of the effects of curcumin in patients with advanced-stage CRC.

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Conclusions

This study aimed to investigate the effects of increasing concentrations of curcumin on cell viability, proliferation, and apoptosis of SW620 human colonic adenocarcinoma cells cultured *in vitro*, and the signaling pathways involved. Curcumin reduced cell viability and increased apoptosis in SW620 human colonic adenocarcinoma cells by restoring the expression of caudal type homeobox-2 (CDX2), which inhibited the Wnt/ β -catenin signaling pathway.