Apoptosis of mouse myeloma cells induced by curcumin via the Notch3-p53 signaling axis

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Abstract. Resistance to apoptosis is a characteristic of cancer. Curcumin has become a potential anticancer drug for its pro-apoptotic effects, but the underlying mechanisms remain unclear. Furthermore, the Notch3-p53 signaling axis serves an important role in cell fate. The present study was designed to investigate the antitumor effect of curcumin by the Notch3-p53 axis in mouse myeloma P3X63Ag8 cells. The effects of curcumin on the viability of P3X63Ag8 cells were evaluated using an MTT assay. Quantitative expression of the Notch3-p53 signaling axis-associated genes was measured by reverse transcription-quantitative polymerase chain reaction, and western blot analysis was used to investigate the expression of proteins. Additionally, flow cytometry was used to measure the ratio of apoptosis. The results demonstrated that curcumin could significantly inhibit cell viability. No significant pro-apoptotic effect was observed when the concentration of curcumin was <30 μM. At 30 μM, curcumin-treated cells exhibited an apoptotic phenomenon, and the ratio of late apoptosis increased with the concentration of curcumin, and reached 28.4 and 51.8% in the medium- and high-dose groups, respectively. Curcumin inhibited the expression of Notch3, while the middle- and high-dose groups promoted p53. The expression of Notch3-responsive genes Hes family BHLH transcription factor 1 and Hes-related family transcription factor with YRPW motif 1 were notably promoted. Curcumin treatment significantly downregulated B-cell lymphoma-2 (Bcl-2) at the mRNA and protein levels, but upregulated Bcl-2-associated X. These data indicated that curcumin exhibited antitumor

effects in mouse myeloma cells with induction of apoptosis by affecting the Notch3-p53 signaling axis.

Introduction

Curcumin is the primary active component of turmeric and is part of the ginger family (Zingiberaceae). Due to it having no deleterious effects and it having a broad-spectrum anticancer activity, curcumin has become one of the most promising anticancer phytochemicals that has been frequently studied for its molecular mechanism (1-4). Accumulating evidence has indicated that curcumin may significantly inhibit the proliferation of tumor cells and induce apoptosis (5-8). Additionally, a number of studies have determined that curcumin may trigger the intrinsic apoptotic pathway (9,10).

Multiple myeloma (MM) is a B-cell malignancy characterized by overabundance of monoclonal plasma cells in the bone marrow, which can cause osteolytic lesions (11). Previous studies demonstrated that direct contact between osteocytes and MM cells activates the Notch signaling pathway, increases Notch receptor expression in MM cells, particularly Notch3 and 4, and stimulates MM proliferation (12-14). The Notch signaling pathway is a highly conserved system that dictates cell fate and critically influences cell proliferation, differentiation and apoptosis (15). Notch3 is one of the four Notch receptors (Notch1-4) that are overexpressed in numerous cancer cells, including ovarian high-grade serous carcinoma (16), pancreatic cancer (17) and urothelial carcinoma (18). It has been reported that overexpression of Notch3 may be responsible for promoting tumor cell growth in human lung cancer types, including hepatocellular carcinoma and non-small cell lung cancers (19,20). A similar study determined that enforced expression of Notch3 could cause dysregulated hyperplasia of T cells (21). All these data indicated that Notch3 serves a crucial role in the anti-apoptosis of cancer cells. Components of the two families of basic helix-loop-helix transcription factors, Hes family BHLH transcription factor 1 (Hes1) and Hes-related family transcription factor with YRPW motif 1 (Hey1), are two of the main effectors of the Notch signaling pathway (22).

Tumor suppressor p53 is an important transcription factor that modulates cell death and survival (23). A previous study determined that Notch3 regulates p53 at the post-transcriptional

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level by controlling cyclin G1 expression and the cell signal transduction circuit in hepatocellular carcinoma (24). There are a number of potential mediators of p53-induced apoptosis, including murine double minute 2 (MDM2), first apoptosis signal receptor (Fas) and leucine repeat death domain containing protein (LRDD) (25). p53 has been demonstrated to function by direct interactions with, or activation of, B-cell lymphoma-2 (Bcl-2) family proteins in the mitochondria (26). The members of the Bcl-2 family regulate apoptosis by the permeabilization of the outer mitochondrial membrane (27).

Notch3 and p53 signaling pathways are important in cell fate (15,23). Curcumin is known to activate cell death signals and induce apoptosis in cancer cells by regulating multiple important cellular signaling pathways, including Notch (28); however, the effects of curcumin on Notch3 are less well studied. Therefore, the present study focused on the effect of curcumin on mouse myeloma cells and the underlying mechanisms mediated through the Notch3-p53 signaling pathway.

Materials and methods

Cell culture. Mouse myeloma P3X63Ag8 cells (Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin-streptomycin (cat. no., 15140122, Gibico, Thermo Fisher Scientific, Inc., USA) and incubated in a humidified incubator containing 5% CO_2 at 37°C. P3X63Ag8 cells were stimulated with curcumin (0, 30, 40 and 50 μM) for 24 h at 37°C with 5% CO_2.

MTT assay. The cells were placed into 96-well plates (Eppendorf, Hamburg, Germany) at a density of $4 \times 10^4/200$ μl/well in DMEM (Hyclone; GE Healthcare Life Sciences) supplemented with 10% FBS and 1% penicillin-streptomycin, and cultured overnight at 37°C. Medium was replaced with fresh DMEM containing different concentrations (0, 10, 20, 30, 40, 50, 60, 70 and 80 μM) of curcumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following a further incubation for 24 h at 37°C, 20 μl MTT (2 mg/ml) was added to each well followed by a 4 h incubation at 37°C. Viability was determined with formazan crystal pellets dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA), and the absorbance of the plate was determined with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm. The results are expressed as percentage viability, calculated using the following formula:

$$\%Viability = 100 \text{ (O.D Text Item/O.D of control)},$$
$$\%Activity = 100 - \%Viability.$$

Isolation of RNA and DNase treatment. Total RNA was extracted using TRIzol Reagent® (Sigma-Aldrich; Merck KGaA). All RNA extracts were treated with DNase using a RQ1 RNase-Free DNase kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Synthesis of complementary DNA (cDNA) was performed using the GoScript™ Reverse Transcription system (Promega Corporation), according to the manufacturer's protocols. Quantitative expression of the genes was conducted by qPCR using a CFX96 Touch Deep Well Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Target genes were amplified using the primers in Table I. β-actin was employed as the endogenous control. The reaction mixture contained 6.25 μl 2X GoTaq®qPCR Master mix (Promega Corporation), 1 μl cDNA, 0.25 μl upstream and 0.25 μl downstream PCR primers, and nuclease-free water up to a final volume of 12.5 μl. Each reaction was conducted in triplicate. The RT-qPCR reaction conditions were subjected to an initial pre-degeneration step of 95°C for 3 min, followed by 39 cycles of 95°C for 20 sec, followed by 60°C for 30 sec. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (29) and normalized to those of β-actin. Fluorescence data were collected and analyzed with the Bio-Rad CFX Manager software 3.0 (Bio-Rad Laboratories, Inc.). The melting curves were produced following qPCR.

Western blot analysis. P3X63Ag8 cells were stimulated with curcumin (0, 30, 40 or 50 μM) for 24 h at 37°C. Subsequently, the cells treated with indicated reagents were lysed in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with phenylmethylsulfonyl fluoride (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 min on ice. Following centrifugation at 16,000 x g for 20 min at 4°C, the protein concentration was determined using a Bradford Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Subsequently, 30 μg protein was subjected to 10% SDS-PAGE electrophoresis. The separated proteins were transferred to a nitrocellulose filter membrane using the Trans-Blot®SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocols. Membranes were blocked with 5% skimmed milk powder at 4°C overnight and incubated with primary antibodies [anti-Notch3 rabbit polyclonal antibody (dilution, 1:1,000; cat. no., ab23426), anti-Bcl-2 associated X (Bax) rabbit monoclonal antibody (dilution, 1:8,000; cat. no., ab32503) and anti-Bcl-2 mouse monoclonal antibody (dilution, 1:500; cat. no., ab692) (all from Abcam, Cambridge, UK); anti-P53 mouse monoclonal antibody (dilution, 1:5,000; cat. no., GTX70214; GeneTex, Inc., Irvine, CA, USA); and anti-β-Actin mouse monoclonal antibody (dilution, 1:8,000; cat. no., HC201; Beijing Transgen Biotech Co., Ltd., Beijing, China)] in 5% skimmed milk in TBS with Tween 20 (TBST) for 1 h at room temperature. Following three washes with TBST for 5 min each, the membrane was incubated with the appropriate secondary antibody [goat anti-mouse IgG (H+L) horseradish peroxidase (HRP)-conjugated (dilution, 1:3,000; cat. no., BA1050; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and goat anti-rabbit IgG (H+L) HRP-conjugated (dilution, 1:3,000; cat. no., BA1054; Wuhan Boster Biological Technology, Ltd.) secondary antibodies] at room temperature for 1 h, followed by washing three times with TBST for 10 min each time. The membrane was developed with a Western Bright enhanced chemiluminescent (ECL) kit (Advansta, Menlo Park, CA, USA).

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay. Cells were harvested by

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene symbol	NCBI Ref seq no.	Sequence (5'→3')	Product length (bp)
β-actin	NM_007393.4		149
Forward		TGAGAGGGAAATCGTGCGTGAC	
Reverse		GCTCGTTGCCAATAGTGATGACC	
p53	NM_001127233.1		179
Forward		ACAGGCAGACTTTTCGCCACAG	
Reverse		CCGTCCCAGAAGGTTCCCACT	
Bax	NM_007527.3		118
Forward		TGGAGATGAACTGGACAGCA	
Reverse		GAAGTTGCCATCAGCAAACA	
Bcl-2	NM_009741.4		131
Forward		CGATTGTGGCAGTCCCTTA	
Reverse		CCAGGATGAAGTGCTCAGGT	
Hes1	NM_008235.2		157
Forward		CAACACGACACCGGACAAAC	
Reverse		GGAATGCCGGGAGCTATCTT	
Hey1	NM_010423.2		164
Forward		TGCAGTTAACTCCTCCTTGCC	
Reverse		CGCCGAACTCAAGTTTCCATT	

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X; Hes1, Hes family BHLH transcription factor 1; Hey1, Hes-related family transcription factor with YRPW motif 1.

centrifugation at 1,000 x g for 5 min, and washed twice with 500 µl of PBS at room temperature. Annexin V-FITC/PI double staining kit (cat. no., KGA106; Jiangsu KeyGen Biotech Co., Ltd., Nanjing, China), according to the manufacturer's protocols, were employed to analyze apoptotic rate in cells. In brief, liquots of 1×10^6 cells were suspended in 500 µl binding buffer followed by the addition of 5 µl Annexin V-FITC and 5 µl PI at room temperature for 5 min. Following incubation in the dark at 37°C for 5 min, the cells were analyzed using a flow cytometer BD Accuri C6 Plus (BD Accuri™; BD Biosciences, Franklin Lakes, NJ, USA). A total of 10,000 events were measured per sample.

Statistical analysis. Differences between groups were analyzed using one-way analysis of variance and multiple comparison between the groups was performed using Tukey's post hoc test. Experimental data are expressed as the mean ± standard error of the mean of three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed with SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA).

Results

The effect of curcumin on the proliferation of P3X63Ag8 cells. To determine the effect of curcumin on cell viability, cells were treated with different concentrations of curcumin for 24 h, and viable cells were measured using an MTT assay. As depicted in Fig. 1, increasing concentrations of curcumin resulted in a significant decrease ($P<0.01$) in cell viability compared with the 0 µM group. Notably, curcumin treatment resulted in a
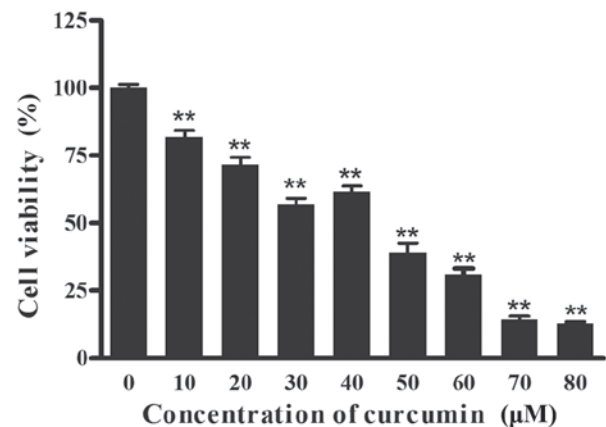

Figure 1. Effect of curcumin on the proliferation of P3X63Ag8 cells. The cells were treated with different concentrations of curcumin (0, 10, 20, 30, 40, 50, 60, 70 and 80 µM) for 24 h. The results are expressed as the mean ± standard error of the mean of eight independent experiments. **$P<0.01$ vs. the curcumin-untreated group.

reduced inhibition at 40 µM, compared with 30 µM. A total of 3 effective concentrations (30, 40 and 50 µM) were selected for all further mechanistic studies.

The effect of curcumin on the mRNA and protein levels of P3X63Ag8 cells. To determine the underlying molecular mechanisms of apoptosis of cells by curcumin, the mRNA levels of Notch3-p53 signaling axis genes were examined. Compared with the negative control group, the expression of Hes1 transcripts was significantly increased in
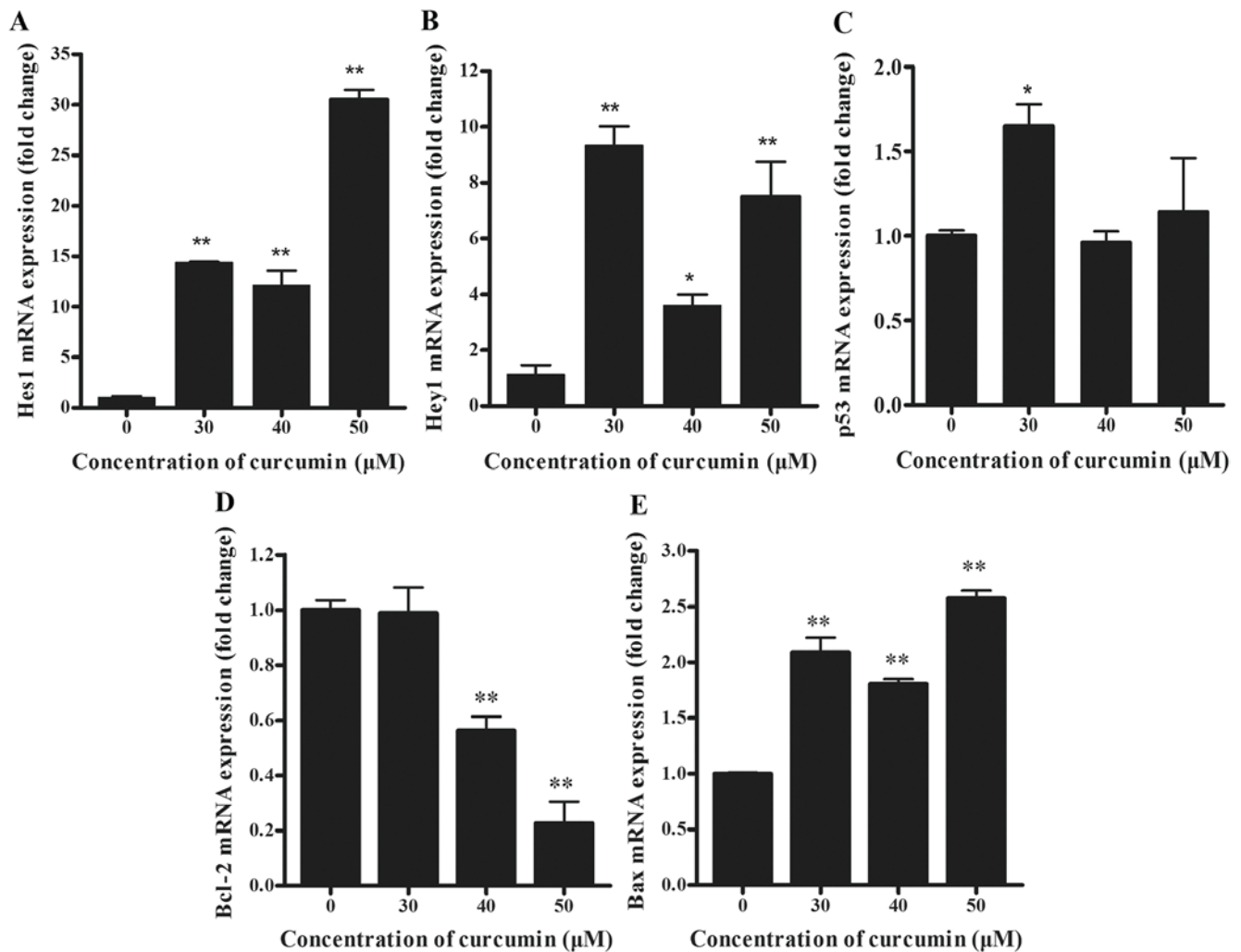
Figure 2. (A) Hes1, (B) Hey1, (C) p53, (D) Bcl-2 and (E) Bax mRNA levels of curcumin-treated (0, 30, 40 and 50 μM) P3X63Ag8 cells for 24 h by reverse transcription-quantitative polymerase chain reaction. The results are expressed as the mean ± standard error of the mean of eight independent experiments. *P<0.05 and **P<0.01, compared with the curcumin-untreated group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; Hes1, Hes family BHLH transcription factor 1; Hey1, Hes-related family transcription factor with YRPW motif 1.

curcumin-treated cells (Fig. 2A; P<0.01). Similarly, the expression of Hey1 mRNA copies was significantly increased in high-dose curcumin-treated cells compared with the negative control group (Fig. 2B; P<0.01). Furthermore, no significant differences were determined in the expression levels of p53 in 40 and 50 μM curcumin-treated cells, compared with the control, whereas levels increased significantly in 30 μM curcumin-treated cells (Fig. 2C; P<0.05). As depicted in Fig. 2D, Bcl-2 mRNA was expressed at significantly reduced levels in 40 and 50 μM curcumin-treated cells (P<0.01). By contrast, Bax transcripts were significantly overexpressed in curcumin-treated cells (Fig. 2E; P<0.01), compared with the control.

The activation status of the Notch3-p53 signaling pathways in curcumin-stimulated P3X63Ag8 cells was investigated using western blotting. The 90 kDa band for Notch3 was detectable in samples (Fig. 3). As predicted, Notch3 was overexpressed in P3X63Ag8 cells, compared with β-actin, and significantly decreased in curcumin-treated cells. The results demonstrated that Bcl-2 (26 kDa) and Bax (21 kDa) protein expression levels (Fig. 3) were similar to their mRNA levels, while the p53 (53 kDa) expression level was not similar to its mRNA levels.
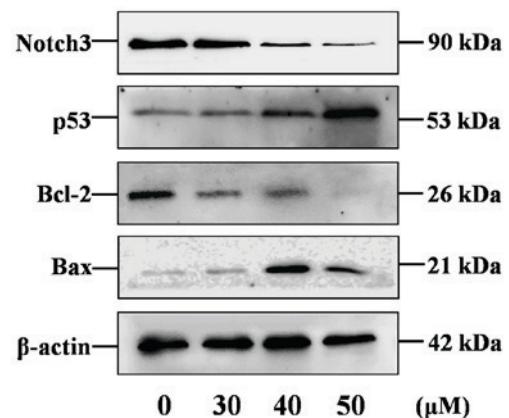

Figure 3. Western blotting of Notch3, p53, Bcl-2, Bax and β-actin in P3X63Ag8 cells stimulated with 0, 30, 40 and 50 μM curcumin for 24 h. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

The effect of curcumin on the apoptosis of P3X63Ag8 cells. To examine whether curcumin could cause apoptosis, cells treated with different concentrations of curcumin (30, 40 and 50 μM) were stained with PI and FITC, and analyzed by
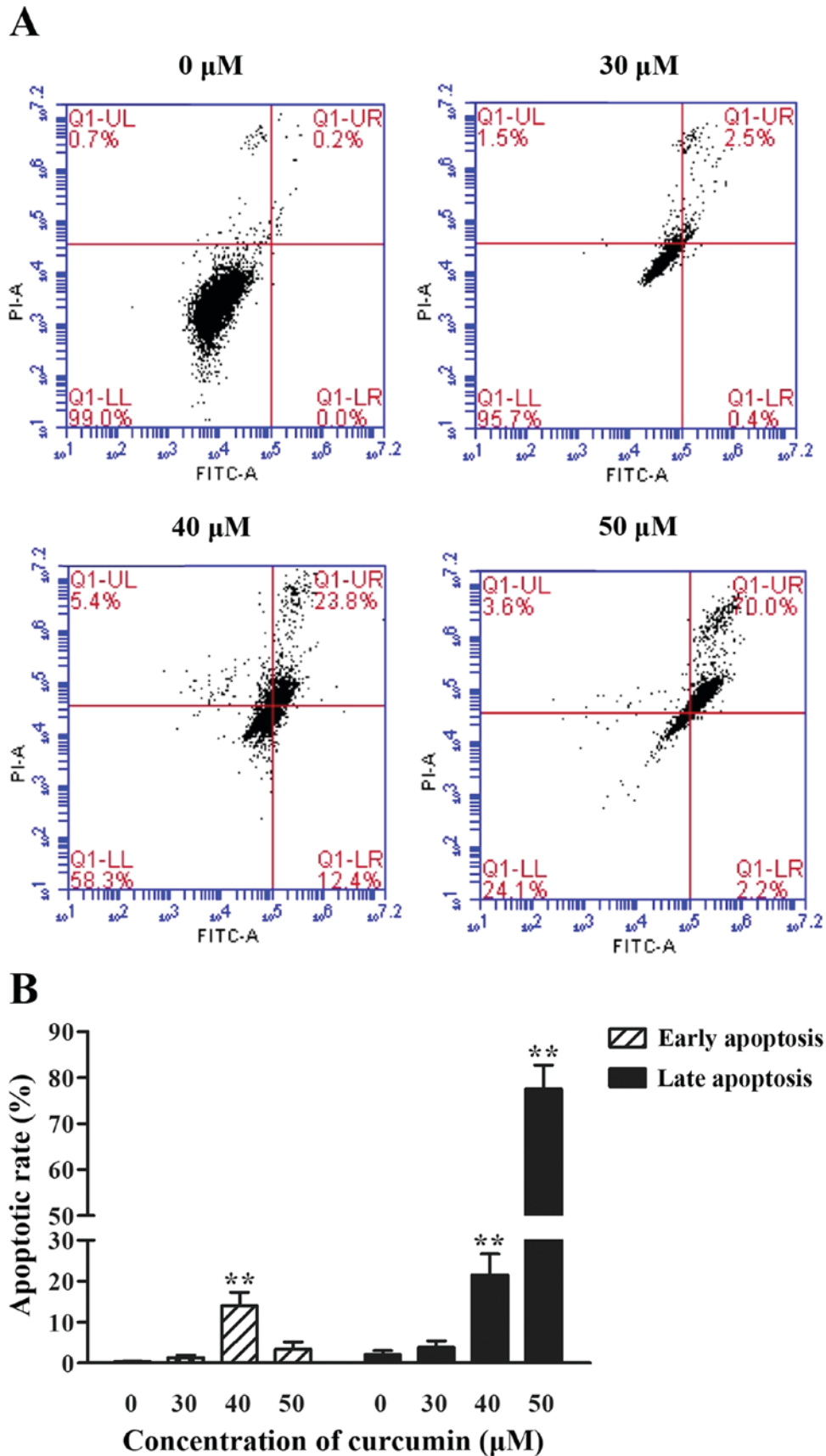
A

B

Figure 4. The effect of curcumin on the apoptosis of P3X63Ag8 cells. (A) Flow cytometric dot plots of one representative experiment. Results are depicted as logarithmic fluorescence intensity on the x-axis (Annexin V-FITC) vs. y-axis (PI). A total of four quadrants represent necrotic cells (Q1-UL:AV-/PI+), late apoptotic cells (Q1-UR:AV+/PI+), early apoptotic cells (Q1-LR:AV+/PI-) and viable cells (Q1-LL:AV-/PI-). The numbers indicate the percentage of cells in each quadrant and a minimum of 10,000 events were read. (B) The histogram depicts the percentage of early and late apoptosis cells ± standard error of three independent experiments. **P<0.01 vs. the curcumin-untreated group. Cells were treated with 0, 30, 40 and 50 μM curcumin for 24 h. FITC, fluorescein isothiocyanate PI, propidium iodide.

fluorescence-activated cell sorting analysis. Curcumin induced apoptosis in a dose-dependent manner, with the exception in a decrease of early apoptotic rate at 50 μM. The percentage of late apoptosis cells was notably increased (P<0.01) compared with the 0 μM group. As depicted in Fig. 4, the percentage of early apoptosis cells reached the highest level when stimulated with 40 μM curcumin (P<0.01).

Discussion

Apoptosis is a multi-step, multi-pathway cell-death program that is inherent in every cell of the body. A characteristic of cancer cells is anti-apoptosis; therefore, cancer treatment is primarily dependent on inducing apoptosis (30). Curcumin has been frequently used as a spice, food additive and herbal medicine in Asia, and has not been demonstrated to cause any toxicity (31). A number of studies indicated anticancer effects for curcumin, and the mechanisms underlying these effects may be the modulation of multiple oncogenic signaling transduction elements (32-34). In the present study, it was determined that curcumin-induced mouse myeloma P3X63Ag8 cell apoptosis is mediated by modulation of the molecular targets p53, Bcl-2 and Bax. It was also demonstrated, with RT-qPCR and western blotting, that curcumin regulates the Notch3 signaling pathway. Additionally, it was observed that curcumin dose-dependently induces apoptosis in P3X63Ag8 cells, as indicated by Annexin V-FITC/PI staining.

Notch3 overexpression has been observed in a number of tumor types, including T-cell acute lymphoblastic leukemia (35), triple-negative breast cancer (36) and prostate cancer (37). Notch3 activation favors cell proliferation, resistance to apoptotic stimuli, metastatic capability and maintenance of stem cell-like features (38). The results of the present study demonstrated that the Notch3 protein was overexpressed in mouse myeloma P3X63Ag8 cells, as predicted. Furthermore, curcumin treatment significantly decreased Notch3 protein expression, which is associated with the induction of apoptosis. p53 is a tumor suppressor and transcription factor (39). When activated, p53 induces cell cycle arrest in G_1 or G_2, p53 also induces cell death by apoptosis (39). The association between Notch3 and p53 has been studied in hepatocellular carcinoma (24,40). The results of the present study demonstrated that curcumin significantly decreased Notch3 expression, promoting cell apoptosis, which is consistent with the anticancer effects of sorafenib and valproic acid (41). In the present study, the mRNA and protein expression level of p53 demonstrated a comparative trend, which indicated that p53 may depend on the post-transcriptional regulation of curcumin. The explanation of the post-transcriptional regulation of curcumin on p53 has been reported. Curcumin could upregulate scaffold/matrix attachment region 1, decreasing histone acetylation at H3K9, a lysine residue 9 on histone H3, and H3K18, a lysine residue 18 on histone H3, resulting in the inhibition of E6 promoter, which interrupts degradation of E6-mediated p53 in HPV18-infected cervical carcinoma (42). In the present study, the protein expression level of p53 is significantly increased in the middle- and high-dose groups, while the low-dose group demonstrated no significant difference. We hypothesized that the apoptosis caused by the low-dose group may not depend on p53 activation, which is different to the middle- and high-dose groups. This may be the reason for reduced inhibition at 40 μM, compared with 30 μM. From the flow cytometry results (Fig. 4), it was also speculated that the middle-dose group causes an early appearance of apoptotic cells, which is comparative to the other groups. The mechanism underlying this remains to be further investigated.

The present study indicated that Hes1 lies downstream of Notch3 and mediates Notch3 signaling-induced proliferation (43). Notably, Notch-mediated cell death has been associated with upregulation of Hes1 expression (44). Hey1, a well-known transcriptional target of Notch signaling, may be a pathway of Notch3 in sustaining mature vessel structure and vascular integrity (45). Furthermore, the expression of Hes1 and Hey1 could induce cell death through the Notch signaling pathway (15). The Bcl-2 family is able to promote or inhibit apoptosis by direct action on permeabilization of the mitochondrial outer membrane (27). Additionally, it is well known that p53 can directly regulate the expression of Bcl-2 and Bax (26,46,47). To define the role of curcumin on the impact of the Notch3-p53 signaling pathway on apoptosis, the p53-responsive genes Bcl-2 and Bax were examined at the mRNA and protein expression levels (Figs. 2 and 3). Additionally, the Notch3-responsive gene Hes1 and Hey1 mRNA expression levels were measured. Curcumin inhibited the expression of Bcl-2 while promoting the expression of Bax, Hes1 and Hey1, indicating that the apoptosis effects of curcumin on P3X63Ag8 cells depend on the Notch3-p53 signaling pathway.

In conclusion, the results of the present study indicated that curcumin exhibited antitumor effects in mouse myeloma cells by inducing apoptosis through effects on the Notch3-p53 signaling pathway. Further studies are warranted to investigate the involvement of the Notch3-p53 signaling pathway in the mechanism underlying curcumin suppressing MM. Furthermore, the effect of inducing cell apoptosis by curcumin with inhibition of the Notch3-p53 signaling pathway will be the focus of our future investigations.

Availability of data and materials

All data generated or analyzed during the present study are included in this article.

Authors' contributions

XH conceived and designed the experiments. Data collection and experiments were performed by YZ and XL. JZ, ZX, HD and YH analyzed the data. YZ, XL and YH contributed to the writing of the manuscript. All authors revised and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent to participate

Not applicable.

Competing interests

The authors declare no conflict of interest.

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