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Curcumin promotes apoptosis in A549/DDP multidrug-resistant human lung adenocarcinoma cells through an miRNA signaling pathway

Jian Zhang [a,1,**], Tao Zhang [b,1], Xinyu Ti [a,1], Jieran Shi [a], Changgui Wu [a], Xinling Ren [a], Hong Yin [c,*]

[a] Department of Respiratory Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China
[b] Department of Thoracic Surgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an 710038, China
[c] The Medical Image Center, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China

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ABSTRACT

Curcumin extracted from the rhizomes of *Curcuma longa* L. has been shown to have inhibitory effects on cancers through its anti-proliferative and pro-apoptotic activities. Emerging evidence demonstrates that curcumin can overcome drug resistance to classical chemotherapies. Thus, the mechanisms underlying the anti-tumor activities of curcumin require further study. In our study, we first demonstrated that curcumin had anti-cancer effects on A549/DDP multidrug-resistant human lung adenocarcinoma cells. Further studies showed that curcumin altered miRNA expression; in particular, significantly downregulated the expression of miR-186* in A549/DDP. In addition, transfection of cells with a miR-186* inhibitor promoted A549/DDP apoptosis, and overexpression of miR-186* significantly inhibited curcumin-induced apoptosis in A549/DDP cells. These observations suggest that miR-186* may serve as a potential gene therapy target for refractory lung cancer that is sensitive to curcumin.

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1. Introduction

Lung cancer is the most common malignant tumor worldwide, and it is one of the leading causes of human cancer-related deaths [1]. Following the use of cisplatin chemotherapy, many changes have been associated with the multidrug-resistance phenotype of tumor cells [2,3]. Cancer chemotherapy is limited by the development of drug resistance by cancer cells and the adverse effects of anti-tumor drugs. The search for novel anti-tumor agents that circumvent these limitations has turned to natural plants [4].

Curcuma longa L. is a plant that belongs to the Zingiberaceae family. Curcumin, which is extracted from the rhizomes of *C. longa* L. is the major component of this plant [5]. Diverse pharmacological effects induced by curcumin have been reported, including anti-inflammatory, antioxidant, and anti-tumor activities [6–8]. There is a wealth of experimental evidence suggesting that multiple mechanisms of action are likely responsible for the various pharmacologic effects of curcumin on cancer-related signaling molecules [9,10]. These include modulation of Jak/STAT, NF-κB, jun, extracellular signal regulated kinase and other key molecules involved in tumorigenesis. Recent results suggest that curcumin can inhibit the proliferation of pancreatic cancer cells through the regulation of specific miRNAs [11]. These emerging results clearly suggest that specific targeting of miRNAs by natural agents may open new avenues for the complete eradication of tumors by killing the drug-resistant cells and improve survival outcomes in patients diagnosed with malignancies [12].

The aim of this study was to probe the anti-cancer effects of curcumin on A549/DDP multidrug-resistant human lung adenocarcinoma cells. In addition, the related miRNA mechanism of apoptosis was also analyzed.

2. Materials and methods

2.1. Cell culture

The human lung adenocarcinoma cell line A549 and the multidrug-resistant human lung adenocarcinoma cell line A549/DDP (obtained from the Academy of Military Medical Science, Beijing, China) were cultured in our laboratory. To maintain drug resistance, A549/DDP cells were cultured with 6 μmol/L DDP and were further cultured in DDP-free RPMI 1640 medium two days before starting the experiment.

2.2. MTT assay

The growth inhibitory effect of curcumin (Sigma, St. Louis, USA) on A549 and A549/DDP cells was assessed using the MTT method

Abbreviations: miR, miRNA or microRNA; DDP, cisplatin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; FCM, Flow Cytometry; IC50, 50% inhibitory concentration; cur, curcumin; TUNEL, TdT-mediated dUTP nick end labeling..

* Corresponding author.
** Corresponding author. Fax: +86 29 84775421.
E-mail addresses: zhangjian197011@yahoo.com (J. Zhang), yinnhong@yahoo.com (H. Yin).

[1] The first three authors contributed equally to this work.

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[13]. All experiments were performed five times, and numerical data are presented as the mean ± SD. The inhibition ratio (%) was calculated by the following formula: inhibition ratio (%) = $[1 - (A - B)/(C - B)] \times 100\%$, where A is the average optical density in curcumin-treated cells, B is the average optical density of the blank controls (only culture medium without any cells), and C is the average optical density of the negative control (culture medium only containing DMSO). The concentration at which curcumin produced 50% inhibition of growth (IC50) was calculated using the relative survival curve.

2.3. Morphologic changes

Cells were plated onto cover slips in 6-well plates at a density of 1×10^4 cells/well. Twelve hours later, the nutrient liquid was discarded, and curcumin was added to the medium to a final concentration of 40 μmol/L. Cells were incubated for an additional 24 h, and staining was performed using the Hoechst 33258 kit according to the manufacturer's instructions. Control cells were processed using the same procedure but were not exposed to curcumin.

2.4. TUNEL assay

Cells on cover slips treated with different concentrations of curcumin were prepared according to the process described above, after which cells were washed with cold PBS buffer and fixed with freshly prepared 4% paraformaldehyde. TUNEL staining was performed using an *in situ* cell death detection kit (Roche, USA) according to the manufacturer's instructions. All the experiments were performed three times. TUNEL-positive (brown staining) cells were characterized as apoptotic cells, and 10 randomly selected microscopic fields in each group were used to calculate the ratio of TUNEL-positive cells.

2.5. miRNA transfection

A549/DDP cells were seeded into 6-well plates (3×10^5 cells/well) for 24 h. The miR-186* mimic, inhibitor and their controls were synthesized by Shanghai GenePharma Co. The mimic and inhibitor are small, chemically-synthetic and optimized nucleic acids designed to mimic and inhibit endogenous mature miR-186* expression, respectively. miR-186* mimic, inhibitor and their controls (200 pmol final concentration for each) were transfected into A549/DDP cells using siPORT neoFX (Ambion) according to the manufacturer's instructions. After 48 h, apoptotic cells were analyzed by Flow Cytometery (FCM).

2.6. Flow cytometric analysis

A549/DDP cells were seeded in culture dishes and incubated for 24 h with media containing curcumin at a final concentration of 0.5, 10, 20, 30, or 40 μmol/L. After treatment, cells in each group were collected and cell apoptosis was analyzed by FCM using the Annexin V-FITC staining kit according to the manufacturer's instruction.

In addition, to evaluate the anti-apoptotic effects of miR-186* in A549/DDP cells, cells were transfected with miR-186* mimic, inhibitor, or their controls in the presence or absence of curcumin for 48 h. Then, cells were collected and cell apoptosis was an alyzed by FCM.

2.7. miRNA microarray

miRNA-enriched total RNAs extracted from curcumin-treated and untreated A549/DDP cells were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The miRNA fraction was further purified using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA). The isolated miRNAs were then labeled with the Hy5™ and the Hy3™ fluorescent labels using the miRCURY™ Array Power Labeling kit (Cat #208032-A, Exiqon) and hybridized on a miRCURY™ LNA miRNA Array (v10.0, Exiqon 8.1). Microarray images were acquired using a Genepix 4000B scanner (Axon Instruments, Union City, CA, USA) and were analyzed with Genepix Pro 6.0 software (Axon Instruments), in which the median normalization was obtained and the results were saved as Microsoft Excel files. All the experiments were performed three times.

2.8. Quantitative real-time PCR

Total RNA was extracted from curcumin-treated or untreated A549/DDP cells using Trizol (Invitrogen) and quantified by measuring the absorbance at 260 nm. The expression of mature miRNAs was assayed using stem-loop RT followed by real-time PCR analysis as previously described [14]. All the reagents for stem-loop RT were obtained from Applied Biosystems (Foster City, CA, USA). PCR products were analyzed by 3% agarose gel electrophoresis. The relative amount of each miRNA was normalized to the amount of U6 snRNA. The fold change for each miRNA in curcumin-treated cells relative to the control (untreated cells) was calculated using the $2^{\Delta\Delta CT}$ method [15], where $\Delta\Delta Ct = \Delta Ct$ curcumin-treated – ΔCt untreated and ΔCt = Ct miRNA – Ct U6. PCR was performed in triplicate. The primers used for stem-loop RT-PCR for miR-186* are listed in Table 1.

2.9. Statistical analysis

Each experiment was repeated at least three times. Numerical data are presented as the mean ± SD. The difference between means was analyzed using Student's *t*-test. All statistical analyses were performed using SPSS 11.0 software (Chicago, IL). Differences were considered significant when $P < 0.05$.

3. Results

Curcumin could significantly inhibit A549 and A549/DDP cells growth in MTT assays, and the inhibition ratio was markedly increased in a dose- and time-dependent manner (Fig. 1A). The IC50 of curcumin at 48 h were 16.28 ± 1.4 μmol/L for A549 cells and 18.06 ± 1.9 μmol/L for A549/DDP cells (Fig. 1B), which were not significantly different ($P \geqslant 0.05$). These data demonstrate that A549/DDP cells are still sensitive to curcumin.

3.1. Curcumin induces A549/DDP cells apoptosis

Using fluorescence microscopy, we observed changes in the A549/DDP cells treated with curcumin, including partially

Table 1
The primers used to detect the expression of miR-186* by qRT-PCR.

Primer	Sequence
U6 forward	5′GCTTCGGCAGCACATATACTAAAAT3′
U6 reverse	5′CGCTTCACGAATTTGCGTGTCAT3′
miR-186* RT	5′GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCCAAA3′
miR-186* forward	5′CCCGATAAAGCTAGATAACC3′
miR-186* reverse	5′CAGTGCGTGTCGTGGAGT3′

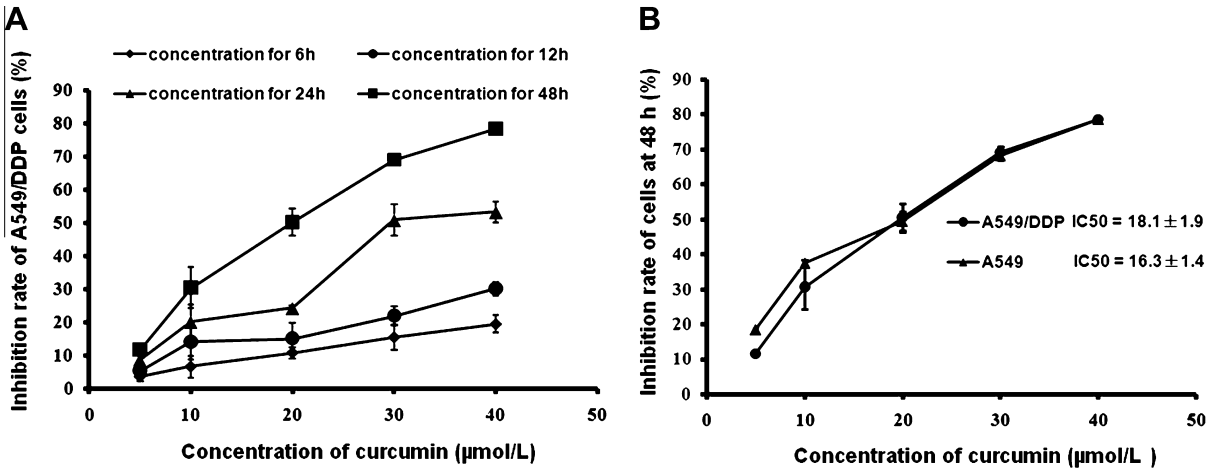

Fig. 1. The growth inhibitory effect of curcumin on A549 and A549/DDP cells at different concentrations and time points ($n = 5$, mean ± SD). (A) The inhibition ratio of A549/DDP cells treated with different concentrations of curcumin at different time points. (B) The inhibition ratio of A549 cells and A549/DDP cells treated with different concentrations of curcumin at 48 h. The IC50 of curcumin at 48 h were not significantly different ($P > 0.05$).

ruptured nuclei and cells with different sizes and shrunken or distorted nuclei showing conglomerated fluorescence that presented the appearance of grains. The typical apoptotic characteristics of curcumin-treated groups were more obvious as compared to the control group (Fig. 2A). Furthermore, the brown apoptotic nuclei of A549/DDP cells significantly increased with increasing concentrations of curcumin (Fig. 2B). FCM further proved the above results: the percentage of Annexin V^+ PI^- cells, which represents the early stage of apoptosis, increased with increasing curcumin concentration (Fig. 2C).

These data show that curcumin can induce apoptosis of A549/DDP cells.

3.2. Curcumin promotes apoptosis in A549/DDP cells through an miRNA signaling pathway

To determine whether miRNAs are involved in the curcumin-induced apoptosis of A549/DDP cells, we performed a comprehensive miRNA profiling of untreated A549/DDP cells and compared the results to those obtained for cells treated with curcumin. Four

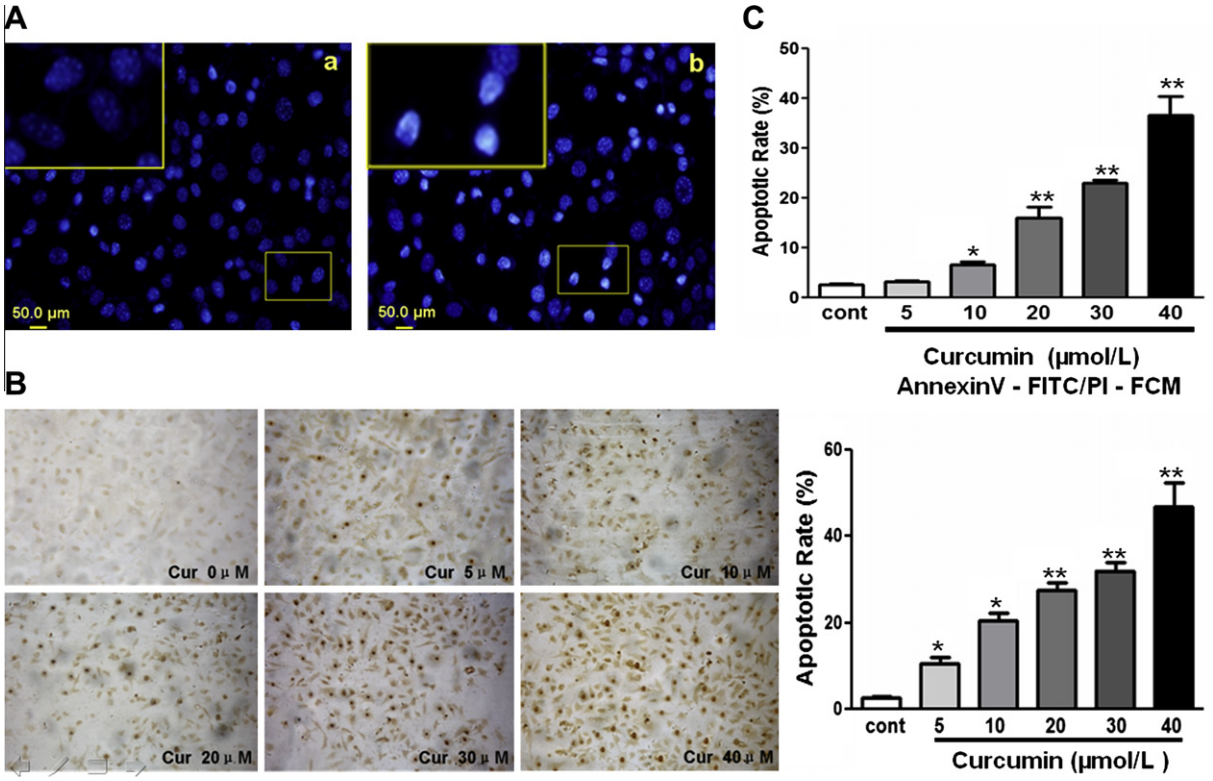

Fig. 2. Curcumin induces A549/DDP cells apoptosis. (A) Morphology of apoptotic cell nuclei was observed by Hoechst staining using a fluorescence microscope. (a) A549/DDP cells were incubated without curcumin for 24 h as control. The inserted enlarged micrograph shows the morphology of normal nucleus; (b) A549/DDP cells were incubated with 40 μmol/L curcumin for 24 h. The inserted enlarged micrograph shows the morphology of the apoptotic cell nuclei. (B) TUNEL was used to detect the rate of apoptosis of A549/DDP cells treated with different concentrations of curcumin for 24 h (TUNEL stain×100, $n = 3$, mean ± SD). Representative histograms are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control cells. (C) Percentages of apoptotic cells were analyzed using FCM ($n = 3$, mean ± SD). Representative histograms are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control cells.

of 342 human miRNAs examined were upregulated more than 2.5-fold in A549/DDP cells treated with curcumin compared to expression in the untreated group. In contrast, only two miRNAs (miR-186*, miR-136) were downregulated more than 2.5-fold compared to their levels in control cells. Specifically, the changes in miR-186* expression were statistically significant (Fig. 3A). The expression of miR-136 expression has been reported to be increased in lung cancer [16]. In contrast, there have only been a few reports about the role of miRNA-186* in tumorigenesis. We speculate that miR-186* may play an important role in curcumin-induced apoptosis. To verify the results obtained by microarray profiling, we performed stem-loop RT followed by real-time PCR analysis with the primers listed in Table 1 to examine miR-186* expression levels. In accordance with the microarray data, quantitative RT-PCR showed significantly decreased miR-186* expression in A549/DDP cells treated with different concentrations of curcumin compared to untreated cells (Fig. 3B).

We hypothesized that miRNA-186* may play an oncogenic role in human lung cancer cells. To confirm this, we further evaluated the anti-apoptotic effects of miR-186* in A549/DDP cells. A549/DDP cells were transfected with 200 pmol of the miR-186* inhibitor, inhibitor control, miR-186* mimic or mimic control for 48 h, and cell apoptosis was measured using FCM. The results demonstrated that overexpression of miRNA-186* inhibited cell apoptosis compared to that in the controls (Fig. 4A), and low expression of miR-186* increased cell apoptosis compared to that in the controls (Fig. 4B). These data show that miR-186* may act as an oncogenic miRNA (oncomir) in lung cancer.

To verify whether miR-186* plays a critical role during curcumin-induced A549/DDP cells apoptosis, the rate of apoptosis was detected using FCM in A549/DDP cells treated with curcumin, negative control, or curcumin combined with miR-186* mimic. The results show that apoptosis in the combination group was significantly decreased compared to that in cells treated only with curcumin (Fig. 4C).

Together, these findings reveal that curcumin induces A549/DDP cells apoptosis by downregulating miR-186*.

4. Discussion

The leading cause of chemotherapy failure is tumor resistance. Therefore, the search for new drugs that can evade drug resistance and adverse effects is very important in the clinic [17]. Since the Indian researcher Kuttan [5] reported that curcuma and curcumin may have anti-tumor effects, a large amount of research has proven that curcumin can induce apoptosis in many types of cancer cells [18,19] through inhibition of NF-κB, survivin/BIRC5, and bcl2 [20,21]. Although there is evidence for the anti-tumor activity of curcumin, there has been concern about the effects of curcumin on multidrug-resistant cells [22,23]. Labbozzetta et al. reported that the anti-tumor activity of curcumin is substantial in both MCF-7 and MCF-7R cells, a multidrug-resistant variant of the MCF-7 breast cancer cell line [24]. These emerging results clearly suggest that further in-depth research in different cancer cells is needed to fully appreciate the beneficial effects of curcumin.

In our study, we selected the multidrug-resistant human lung adenocarcinoma cell line A549/DDP to investigate the inhibitory and apoptosis-inducing effects of curcumin. First, our study showed that curcumin could inhibit A549/DDP cell proliferation in both a dose- and time-dependent manner, similar to the results observed for the parental A549 cells. Thus, compared to the parental cell line, the A549/DDP cell line is also sensitive to curcumin.

The imbalance between proliferation and apoptosis can lead to unlimited cell proliferation, which can ultimately develop into a tumor [25]. Thus, inducing apoptosis has become an important means to treat cancer. Our study further verified that curcumin suppresses A549/DDP cell proliferation through the induction of cell apoptosis. Using fluorescence microscopy, we found that A549/DDP cells treated with 40 μmol/L curcumin displayed shrunken or distorted nuclei, as observed with Hoechst 33258 staining. With increasing curcumin concentrations, the rate of apoptosis of A549/DDP cells also significantly increased. Taken together, these results demonstrate that curcumin has anti-cancer effects through the induction of cell apoptosis.

Recent studies have shown that natural agents, including curcumin, isoflavone, I3C, DIM, and EGCG, can alter the expression of specific miRNAs, which may lead to increased sensitivity of cancer cells to conventional agents and thereby inhibit tumor growth [26,27]. Sun et al. [11] reported that miRNA expression profiles are altered by curcumin in pancreatic cancer cells. That group found that curcumin upregulates miR-22 and downregulates miR-199a and that upregulation of miR-22 expression by curcumin or by transfection with pre-miR-22 in BxPC-3 pancreatic cancer cells suppressed the expression of target genes SP1 and ESR1. These emerging results clearly suggest that the specific targeting of miRNAs by natural agents may open new avenues for complete eradication of tumors by killing the drug-resistant cells and improve survival outcomes in patients diagnosed with malignancies. In our study, miRNA expression profiles were also altered by curcumin in lung cancer cells; in particular, curcumin inhibited the expression of miR-186* in A549/DDP cells. To date, this is the first report demonstrating that miR-186* is involved in the pathogenesis of drug-resistant tumor cell. miR-186* is located on chromosome 1p31.1. miR-186* and miR-186 originate from the same

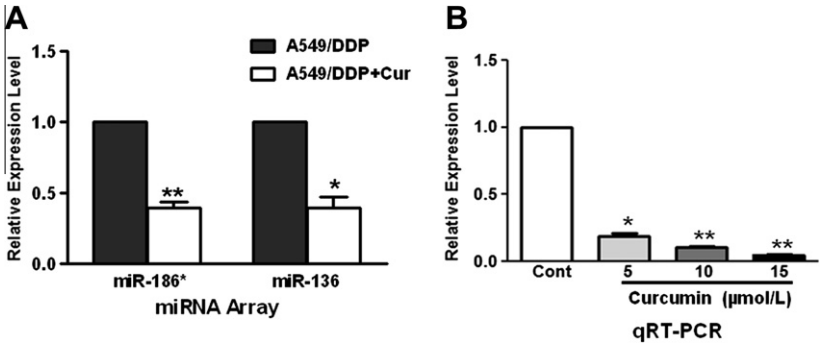

Fig. 3. Validation of microarray data using real-time RT-PCR ($n = 3$, mean ± SD). (A) The levels of miR-186* and miR-136 are downregulated in cells treated with curcumin compared to those in the control groups using miRNA microarray analysis. Data are shown as fold changes of miRNA levels in curcumin-treated groups relative to control groups, in which the level of expression was set as 1, $^{*}P < 0.05$, $^{**}P < 0.01$. (B) Real-time RT-PCR analysis of miR-186* was performed to validate the microarray results. Triplicate assays were performed for each RNA sample, and the relative amount of each miRNA was normalized to U6 snRNA. Data are shown as fold changes of miRNA levels in curcumin-treated groups relative to control groups, in which the level of expression was set as 1, $^{*}P < 0.05$, $^{**}P < 0.01$.

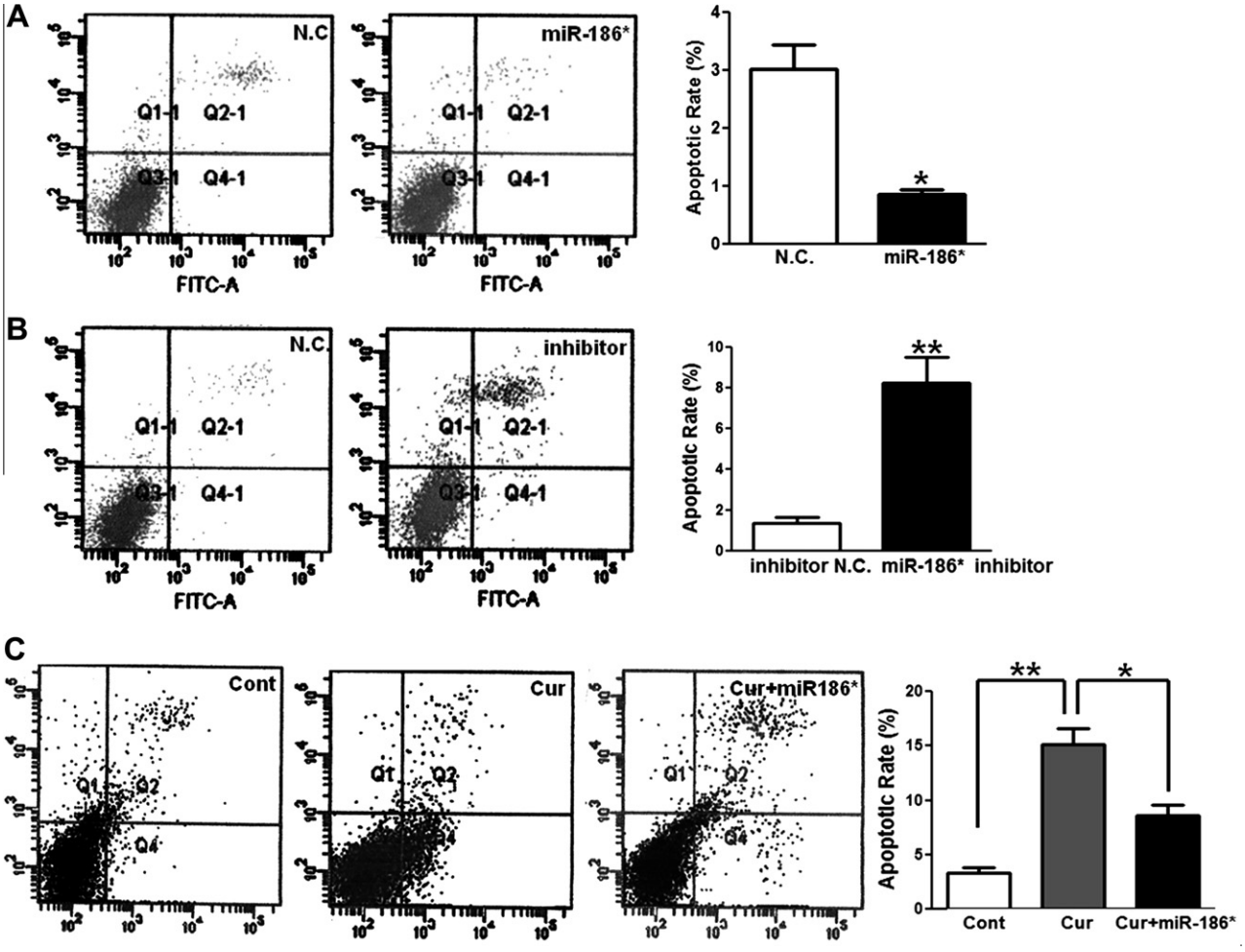

Fig. 4. Curcumin induces A549/DDP cells apoptosis via downregulation of miR-186* ($n = 3$, mean ± SD). (A) A549 cells were transfected with 200 pmol miR-186* mimic or mimic control. Percentages of apoptotic cells were analyzed by FCM. Representative histograms are shown. * $P < 0.05$, ** $P < 0.01$ compared to control cells. (B) A549 cells were transfected with miR-186* inhibitor or inhibitor control for 48 h. Percentages of apoptotic cells were analyzed using FCM. Representative histograms are shown. * $P < 0.05$, ** $P < 0.01$ compared to control cells. (C) A549/DDP cells treated with DMSO control, 15 μmol/L curcumin, or curcumin combined with miR-186*. The percentage of apoptotic cells was analyzed using FCM. Representative histograms are shown. * $P < 0.05$, ** $P < 0.01$.

pre-miRNA. The level of miR-186 was recently found to be increased in cancer epithelial cells compared to that in normal cells [28]. Interestingly, our results show that miR-186* was significantly downregulated in A549/DDP cells due to curcumin-induced apoptosis, although the function of miR-186* as an oncogenic miRNA still requires further elucidated. These results suggest that curcumin can inhibit the proliferation of lung cancer cells and induce apoptosis through the regulation of specific miRNAs.

In conclusion, A549/DDP multidrug-resistant human lung cancer cells are sensitive to curcumin, and curcumin can induce cell apoptosis via the downregulation of miR-186*. The findings reported here present the first evidence that miR-186* may serve as potential gene therapy target for refractory lung cancers that are sensitive to curcumin.

Acknowledgments

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