

Original Research Article

Curcumin inhibits viability and promotes apoptosis by modulating miR-17/caspase-9 pathway in colorectal cancer

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Abstract

Purpose: To investigate the mechanism of curcumin effect on colorectal cancer cells.

Methods: The miR-17-5p and caspase-9 were measured using quantitative real time polymerase chain reaction (qRT-PCR) and western blotting in colorectal cancer tissues or cells with or without curcumin treatment. The binding sites between miR-17-5p and caspase-9 were predicted by TargetScan and verified by luciferase assay. The miR-17-5p mimics were transfected into colorectal cells to determine its effects. The overexpressing miR-17-5p mimics and a caspase-9 plasmid were co-transfected into colorectal cells to explore the underlying mechanism. In addition, an *in vivo* experiment was performed in a mouse model after injection of HCT116 cells to determine the role of curcumin.

Results: MiR-17-5p was upregulated in colorectal cancer tissues and cells. Curcumin treatment inhibits viability and induces apoptosis of colorectal cancer cells. MiR-17-5p inhibits viability and induces apoptosis of colorectal cancer cells by regulating the expression of caspase-9. Mechanism studies showed that curcumin induced colorectal cell apoptosis by regulation of caspase-9 expression via miR-17-5p. Finally, the animal results demonstrated the anti-tumor activity of curcumin *in vivo*.

Conclusion: The findings of this study indicate that curcumin suppresses cell apoptosis and induce cell viability by regulating miR-17-5p and caspase-9 in colorectal cancer.

Keywords: Curcumin, Colorectal cancer cells, MiR-17, Caspase-9, Apoptosis

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INTRODUCTION

Colon cancer is a common gastrointestinal cancer ranking third in worldwide mortality. Although progress has been made in certain aspects of cancer therapy, the mechanisms of proliferation and spread of cancer cells are still not completely known. As a diketone extract of *Araceae*, curcumin has been extensively studied

both *in vitro* and *in vivo*, for the treatment of various tumors [1]. The effects of curcumin include inhibition of cancer cell proliferation, invasion, and/or angiogenesis. Curcumin also induces apoptosis and increases sensitivity to chemotherapeutic drugs [2], and regulates intracellular molecular signaling pathways [3,4].

In addition, curcumin exerts anticancer effect by regulating downstream genes and miRNA levels

[5]. For example, curcumin inhibits pancreatic cancer cell growth and invasion by upregulating miR-7 expression [6]. Curcumin also inhibits the growth of MCF-7 cells induced by bisphenol A by modulating the miR-19 and PTEN/AKT/p53 pathways [7]. Besides, curcumin inhibits non-small cell lung cancer proliferation and induces apoptosis by increasing miR-192-5p in the PI3K/Akt pathway [8]. In colorectal cancer, curcumin up-regulates miR-21 expression and inhibits cancer cell migration and invasion [9], and prevents the abnormal regulation of miR-34a and miR-27a levels [10]. Moreover, curcumin inhibits the proliferation of HCT-116 cells and promotes apoptosis by regulating miR-491 expression [11]. However, the pathogenesis of colorectal cancer is complex that the mechanisms need to be further studied.

The miR-17-5p is an oncogene that could promote tumor development [12]. In pancreatic cancer, it inhibits AIB1 mRNA transcription in breast cancer [13], and promotes cancer cell proliferation by disrupting the RBL2/E2F4 complex [14]. In gastric cancer, miR-17-5p inhibits SOCS6 expression and activates JAK-STAT pathway to promote cell proliferation [15]. However, miR-17-5p may be also regulated by multiple signaling pathways in colorectal cancer [16].

It has been reported that curcumin inhibits miR-17-5p in 3T3-L1 cells, which thus increases the downstream target gene, *Tcf712*, and regulate the WNT pathway [17]. It raises the possibility that curcumin could also regulate miR-17-5p in colorectal cancer. In addition, TargetScan has predicted that miR-17-5p binds to caspase-9, but this binding has not been experimentally confirmed. It seems that, in colorectal cancer, curcumin may cause the up-regulation of caspase-9 via miR-17-5p, thereby promoting apoptosis of cancer cells. The present study was performed to validate this assumption.

EXPERIMENTAL

Chemicals and cancer cell lines

Dulbecco's Modified Eagle Medium (DMEM), dimethyl sulfoxide (DMSO), TRIzol reagent, and fetal bovine serum (FBS) were obtained from Hyclone (Thermo Fisher Scientific, Waltham, MA, USA). Curcumin with a purity of >80% was obtained from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V-FITC/PI Apoptosis Detection kit and the caspase-9 assay were obtained from Beyotime (Shanghai, China). Western blot 3,3'-diaminobenzidine color development kit and bicinchoninic acid protein

assay (BCA) were obtained from Phygene™ Lifescience (Fuzhou, China).

293T and HCT116 cells were cultured in DMEM with 10 % FBS and 1 % penicillin/streptomycin. Cells were incubated at 37 °C in an atmosphere of 5 % CO₂. TargetScan prediction software (http://www.targetscan.org/vert_72/) was used for predicting the target of miRNA binding.

Sample collection

The study was approved by the ethics committee of Jinan University. An informed consent form was signed by every patient. Colorectal cancer tissues and adjacent normal tissues were collected during surgery.

Cell viability assay

SW480 and HCT116 cells at 5×10^3 cells per well were treated with curcumin at 0, 5, 10, 20, and 30 μ M for 24 h. For transfected cells, all groups were seeded with 5×10^3 cells per well, followed by addition of 20 μ M curcumin. All groups of transfected cells were cultured for 4 days. The MTT assay was then conducted at different times, MTT reagent (5 μ g/mL) was added and incubated for 4 h. The crystals were then dissolved with formazan, and 150 μ L DMSO (Sigma-Aldrich) was added for 20 min with shaking. The absorbance was measured at 490 nm using a plate reader (Thermo Fisher Scientific).

Flow cytometry

Cellular apoptosis was performed using flow cytometry (FCM). SW480 and HCT116 cells (1×10^6 cells) were cultured with curcumin (0, 5, 10, 20, and 30 μ M) for 1 day, and washed. For the apoptosis analysis, SW480 and HCT116 cells were treated with or without 20 μ M curcumin, and then washed and collected. Annexin V-FITC (5 μ L) was then added in the dark. Then, 10 μ L of propidium iodide was added to each well, and cellular apoptosis was immediately detected by FCM (Beckman Coulter, Pasadena, CA, USA) and analyzed by fluorescence-activated cell sorting.

qRT-PCR analysis

The tumor tissue or cells were homogenized in an ice bath and centrifuged to obtain a supernatant. One-fifth volume of chloroform was added to the supernatant, followed by vigorous shaking for 15 s. After 5 min, the samples were centrifuged, followed by addition of an equal volume of isopropanol, and incubated for 10 min.

After centrifugation at 12,000 × g, the supernatant was removed, and 75 % ethanol was slowly added and the sample was centrifuged to remove the supernatant containing RNA. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of RNA was performed with the following primers (prepared on ice, Table 1). Amplification was performed using the real-time detection system (Bio-Rad, Netherlands).

Table 1: Primer sequence used in this study

Primer	Forward	Reverse
Caspase-3	5'- TGGAACAAAT GGACCTGTTG ACC-3'	3'- AGGACTCAAATTC TGTTGCCACC-5'
Caspase-9	5'- GGCTGTCTAC GGCACAGATG GA-3'	5'- CTGGCTCGGGGT ACTGCCAG-3'
Mir-17-5p	5'- TGTC AAGTGCT TACAGTG-3'	5'- CACAGCTCGTAGA ACAGGAGG-3'
RARP	5'- ACGCACAATG CCTATGAC-3'	5'- CCAGCGGAACCTC TACAC-3'
GAPDH	5'- GAACGGGAAG CTCACTGGCA TGGC-3'	5'- TGAGGTCCACCAC CCTGTTGCTG-3'

Western blot analysis

For tumor tissue analysis, 50 mg of tumor tissue with 500 µL of RIPA lysate (Thomas Scientific, Swedesboro, NJ, USA) was incubated in an ultrasonic ice bath until the liquid was clear (eight times, 2 se per pulse). The samples were then centrifuged and the total protein extract was tested using the BCA assay. The samples were then centrifuged and a Tris-glycine gel (Bioswamp, Wuhan, China) was loaded with 50 – 100 µg of lysate. The membranes were blocked in 5 % bovine serum albumin for 1 h at 4 °C, and were then washed with TBST and incubated with primary antibodies, followed by washing five times with TBST. The immunoprecipitate was re-suspended and resolved.

The target protein was detected by the ChemiDoc™ XRS + gel imaging system (Bio-Rad, Hercules, CA, USA). The primary antibodies were against cleaved caspase-3 (Asp 175, 9661#), cleaved human specific poly(ADP-ribose) polymerase (PARP) (Asp214, #9541), or human specific caspase-9 (#9502), all provided by CST Biological Reagents Company Limited, Shanghai, China. The secondary antibody was horseradish peroxidase-labeled goat anti-human IgG (H + L) (A0201, Beyotime). The internal

reference protein was human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (402869, TaqMan™; Applied Biosystems, Foster City, CA, USA).

Cell transfection

The SW480 and HCT116 cells (1×10^5) were added to a culture well and incubated. 1 µg of miR-17-5p mimics or caspase-9 plasmid DNA were diluted with serum-free medium. MiR-17-5p mimics was co-transfected into 293T cells with wild type and mutant caspase-9, followed by mixing for 5 min. 4 mL of serum-free medium with cell was gently shaken at room temperature for approximately 24 h. After transient transfection, cell proteins or RNA were extracted after 48 h.

Animals

The protocol for animal use was approved by the Care Committee of Jinan University (approval no. 2017JN047). Male BALB/c nude mice were injected with HCT116 cells. The mice were randomly divided into two groups. The treatment groups were treated with 30 mg/kg of curcumin every day. At the end of 8 weeks, the mice were weighed, euthanized, and the tumor tissues were collected. The volume and weight of tumors were measured, and the tissues were analyzed using immunohistochemical staining and qRT-PCR.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance and unpaired *t*-tests with Bonferroni post-tests were used and analyzed using SPSS statistical software (version 17.0, SPSS, Chicago, IL, USA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

MiR-17-5p expression in colorectal cancer tissues and cells

Colorectal cancer tissues and adjacent normal tissues from 60 cases were collected, and miR-17-5p expression was shown in Figure 1 A. The expression of miR-17-5p in cancer tissues was upregulated than that of normal tissues. Particularly, there were 56 cases showing higher expression in cancer tissues than that in normal tissues. In addition, the relationship between miR-17-5p level and overall survival was evaluated (Figure 1 B). Patients with low expression of miR-17-5p were correlated with a longer overall survival than that of high

expression group ($p < 0.01$). Besides, the miR-17-5p level in colorectal cancer cell lines (HT29, SW480, HCT116 and Caco-2) was higher than HIEC-6 cell line (Figure 1 C). HCT116 and SW480 cells showed the highest expression of miR-17-5p, which were thus selected for further study. Totally, these results showed that miR-17-5p was upregulated in colorectal cancer tissues and cell lines, and the high expression of miR-17-5p was correlated with poor prognosis of patients.

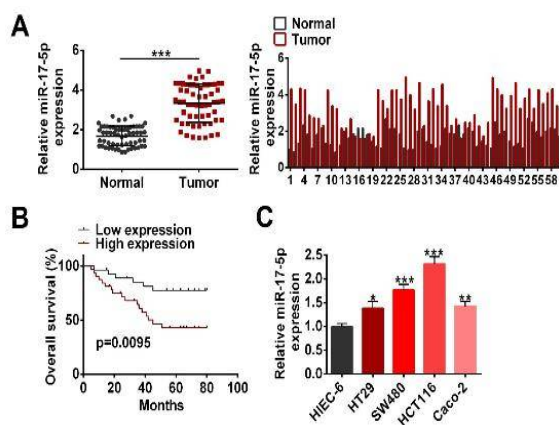


Figure 1: The miR-17-5p expression in colorectal tissues and cells. (A) The relative miR-17-5p expression in normal tissues (left) and colorectal cancer tissues (right) in 60 cases ($***p < 0.001$). (B) The overall survival rate of patients with high or low miR-17-5p expression samples over 80 months. (C) The relative miR-17-5p expressions in HIEC-6 cell line and colorectal cancer cell lines ($p < 0.05$; $**p < 0.01$; $***p < 0.001$)

Curcumin treatment inhibits viability and induces apoptosis in colorectal cancer cells

To explore the anticancer effects of curcumin on colorectal cells, the viabilities and apoptosis of SW480 and HCT116 cells were determined after curcumin treatment for 1 day using MTT assay and flow cytometry, respectively. The viability/apoptosis of SW480 and HCT116 cells were dramatically decreased/induced by curcumin treatment in a dose-dependent manner (Figure 2 A and B).

Poly ADP-ribose polymerase (PARP) is a cleavage substrate for caspase, and cleaved caspase-3 is an active fragment of caspase-3. Western blotting was conducted after curcumin treatment. The relative protein expressions of cleaved caspase-3 and cleaved PARP were also increased in SW480 and HCT116 cells (Figures 3A and 3B). However, expression of miR-17-5p was gradually decreased after curcumin treatment in SW480 and HCT116 cells.

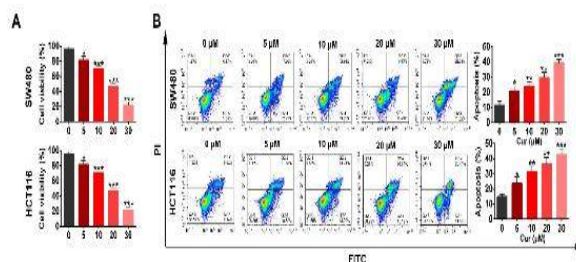


Figure 2: Curcumin treatment inhibits viability and induces apoptosis of colorectal cancer cells (A) The viability of SW480 and HCT116 cells after curcumin treatment was detected using the MTT assay. (B) The apoptosis of SW480 and HCT116 cells after curcumin treatment was detected using flow cytometry; $p < 0.05$; $*p < 0.01$; $***p < 0.001$ vs. control group (curcumin of 0 μ M)

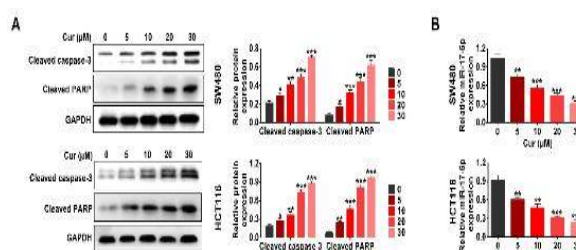


Figure 3: (A) the protein expression of cleaved caspase-3 and cleaved PARP in the SW480 cells (top) and HCT116 cells (bottom), using western blotting. (B) The expression of miR-17-5p using qRT-PCR; $p < 0.05$; $*p < 0.01$; $***p < 0.001$ vs control group (curcumin of 0 μ M)

MiR-17-5p inhibits viability and induces apoptosis of colorectal cancer cells by regulating the expression of caspase-9

The binding sites between miR-17-5p and caspase-9 were predicted by TargetScan (Figure 4 A), indicating that 2402-2408 of caspase-9 was a possible binding site with miR-17-5p. To verify it, miR-17-5p mimics were co-transfected into 293T cells with caspase-9-wt and caspase-9-mut. qRT-PCR was used to verify the transfection efficiency of miR-17-5p mimics (Figure 4 B). The results showed that the relative expressions of miR-17-5p after miR-17-5p mimics transfection was much higher than the control group ($p < 0.001$). As compared to the relative luciferase activity in cells that co-transfected with caspase-9-wt and miR-17-5p mimics had lower luciferase activity ($p < 0.001$) (Figure 4 C). Moreover, after miR-17-5p mimics was transfected into SW480 and HCT116 cells, the relative protein expression of caspase-9 was decreased in cells, when compared with the control group (cells without transfection (Figure 4 D).

In addition, the caspase-9 plasmid was transfected into SW480 and HCT116 cells, and the relative mRNA expression of caspase-9 was 20 times higher than the control group (cells without transfection; $p < 0.001$; Figure 4 E). Western blotting showed consistent results (Figure 4 F). MiR-17-5p mimics and caspase-9 plasmid were the co-transfected into SW480 and HCT116 cells. After miR-17-5p was overexpressed, the cell viability was increased (Figure 5 A and 5 B), and cell apoptosis was decreased. However, overexpression of caspase-9 and miR-17-5p resulted in a decrease in cell viability and caused an increase in cell apoptosis (Figure 5 A and 5 B). Caspase-9 overexpression also reversed the protein levels of caspase-9, cleaved caspase-3, and cleaved RAPA in SW480 and HCT116 cells caused by miR-17-5p mimics (Figure 5 C).

Curcumin induces colorectal cell apoptosis through regulating caspase-9 expression via miR-17-5p

SW480 and HCT116 colorectal cancer cells were treated with curcumin and caspase-9 level were measured. The expression of caspase-9 was increased gradually by curcumin (Figure 6 A). The cells were then treated with 20 μ M curcumin in SW480 and HCT116 cells that overexpressed miR-17-5p. MiR-17-5p overexpression reversed the enhancement in caspase-9 expression caused by curcumin treatment (Figure 6 B). MiR-17-5p overexpression also reversed the reduction in cell viability (Figure 7 A) and induction in cell apoptosis (Figure 7 B) caused by curcumin. Besides, miR-17-5p overexpression decreased the increased expression of cleaved caspase-3 and cleaved RAPA caused by curcumin (Figure 7 C). These results indicated that curcumin increased the expression of caspase-9 via inhibiting the expression of miR-17-5p, thereby promoting the apoptosis of colorectal cancer cells.

Anti-tumor activity of curcumin *in vivo*

To further confirm the results *in vivo*, BALB/c nude mice were subcutaneously injected with HCT116 cells. Curcumin (30 mg/kg) or saline was injected every day, and the tumor volumes were assessed on day 14. The data showed that curcumin caused a gradual decrease in tumor volume and weight (Figure 8 A). However, the body weight was not affected by curcumin treatment. In addition, the relative miR-17-5p expression after curcumin treatment was significantly decreased ($p < 0.001$ vs. the control group; Figure 8 B). Immunohistochemical staining (Figure 8 C) against Ki 67 revealed that

fewer proliferative cells and higher miR-17-5p expression were shown after curcumin treatment.

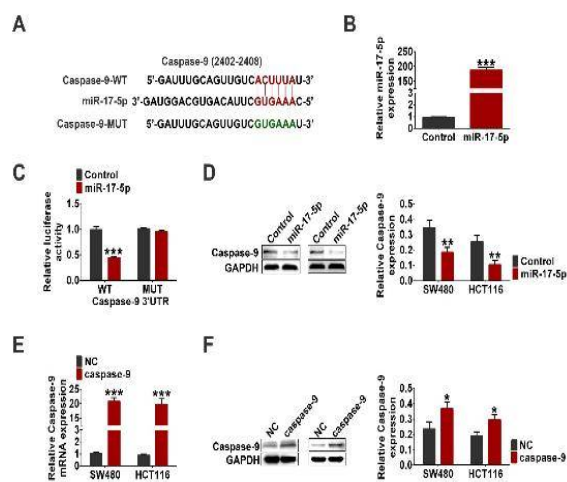


Figure 4: MiR-17-5p negatively regulated caspase-9 expression. (A) Binding sites between miR-17-5p and caspase-9 were predicted by TargetScan. (B) The overexpression efficiency of miR-17-5p ($***p < 0.001$). (C) Transcriptional activity of caspase-9-wt and caspase-9-mut 3'-UTR ($***p < 0.001$). (D) Relative caspase-9 protein expression in SW480 and HCT116 cells that transfected with miR-17-5p mimics ($p < 0.01$). (E) Relative caspase-9 mRNA expression in SW480 and HCT116 cells that transfected with caspase-9 plasmid ($***p < 0.001$). (F) Relative caspase-9 protein expression in SW480 and HCT116 cells that transfected with caspase-9 plasmid ($p < 0.05$ vs. the normal control)

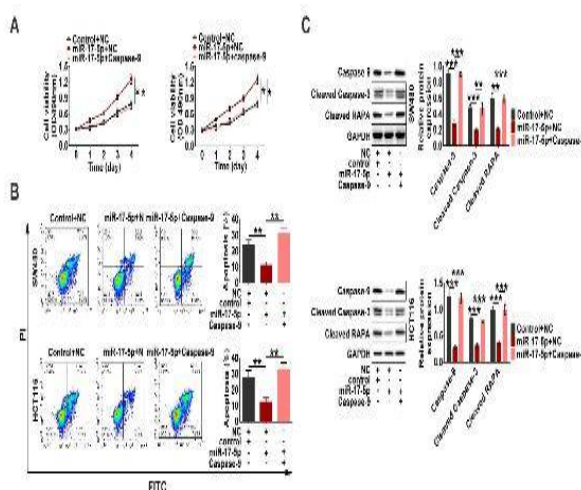


Figure 5: MiR-17-5p inhibits viability and induces apoptosis of colorectal cancer cells by regulating the expression of caspase-9. The viability of SW480 (left) and HCT116 (right) cells (A), apoptosis (B), and relative protein expressions (C) in SW480 and HCT116 cells that co-transfected with control + normal control, miR-17-5p + normal control, and miR-17-5p + caspase-9, respectively ($p < 0.05$; $**p < 0.01$; $***p < 0.001$).

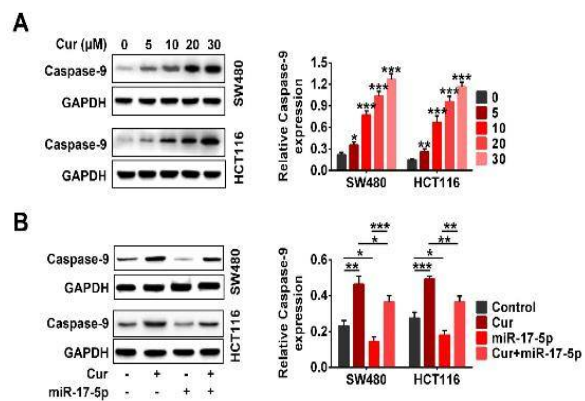


Figure 6: Curcumin regulated caspase-9 expression via miR-17-5p (A) Relative caspase-9 expression in SW480 and HCT116 after curcumin treatment ($p < 0.05$; $***p < 0.001$). (B) Relative caspase-9 expression in transfected SW480 and HCT116 cells with 20 μM curcumin treatment ($p < 0.05$; $**p < 0.01$; $***p < 0.001$)

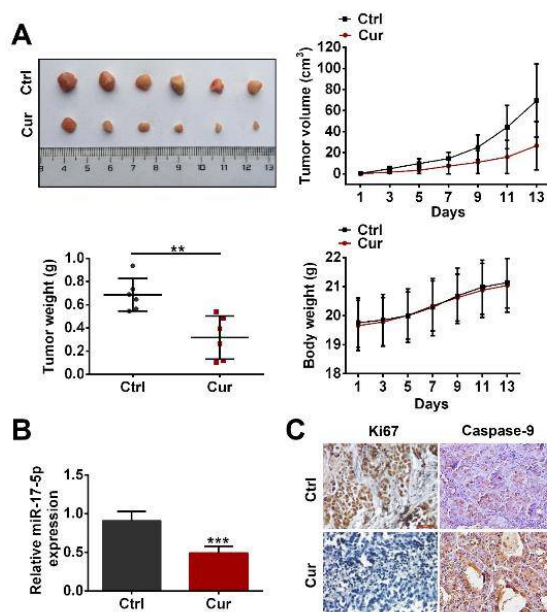


Figure 8: Anti-tumor effect of curcumin *in vivo*. (A) Tumor volume and tumor weight after curcumin and saline treatments ($p < 0.05$; $**p < 0.01$). (B) Relative miR-17-5p expression in the control and curcumin-treated samples ($p < 0.001$). (C) Immunohistochemical imaging of the control and curcumin-treated tissues

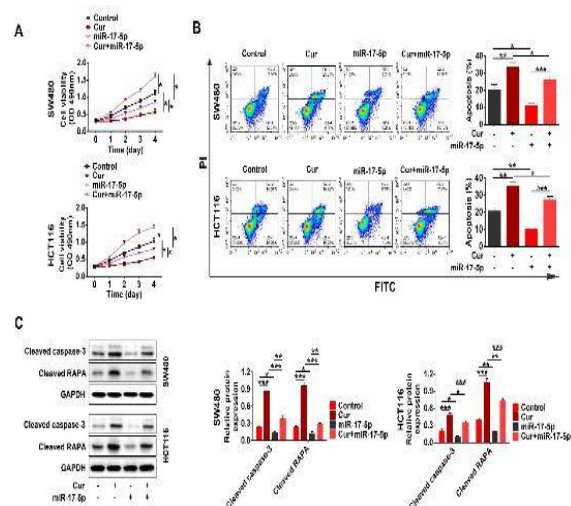


Figure 7: Curcumin induces colorectal cell apoptosis through regulating caspase-9 expression via miR-17-5p. (A) Transfected SW480 and HCT116 cell viabilities using the MTT assay ($*p < 0.05$). (B) The apoptosis of transfected SW480 and HCT116 cells using flow cytometry ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (C) Relative expression of cleaved caspase-3 and PARP in transfected SW480 and HCT116 cells ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$)

DISCUSSION

Curcumin is a promising anticancer drug widely in the treatment of various tumors. Previous results suggest that curcumin inhibits and reverses carcinogenesis through multiple molecular targets [18]. It has been shown that curcumin inhibits cancer, and may be a chemoprophylaxis agent [19]. Both *in vitro* and *in vivo* experiments have shown that curcumin affects molecular signal transduction pathways [20].

Curcumin modulates cell signaling pathways through pleomorphic effects, which may activate cell death signals and induce cell apoptosis. However, the role of curcumin in the modulation of miR-17-5p remains unclear. In this study, the mechanism of curcumin regulation of the miR-17/caspase-9 pathway was identified.

First, the correlation between miR-17-5p expression and clinical features was analyzed. MiR-17-5p expression in cancer tissues was higher than that in adjacent normal tissues. However, miR-17-5p expression was not significantly correlated with sex, age, local infiltration, and clinical tumor node metastasis staging. Survival analysis in colon cancer patients have revealed that high expression of miR-17-5p was associated with poor prognosis. MiR-17-5p expression in colorectal cancer cell lines was significantly higher than that in intestinal epithelial cell lines.

After SW480 and HCT116 cells were administrated with curcumin, the cell viability and level of miR-17-5p were decreased. However, cell apoptosis was increased by curcumin treatment. Caspase-3 plays a critical role in apoptosis [21]. Cleaved caspase-3 is a fragment of caspase-3 activated by cleavage, and its expression reflects the apoptosis levels of cells. The most important substrate for caspase-3 is PARP, which is involved in gene integrity monitoring. At the initiation of apoptosis, PARP

were cleaved into two fragments by caspase-3, between Asp216 and Gly217 [22]. The two zinc finger structures bound to DNA in PARP were separated from the catalytic region at the carboxyl terminus, resulting in inactivation. With the negative regulation of PARP, the activity of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease was increased. DNA of cells in the nucleosomes was cleaved to cause apoptosis, and curcumin treatment was thus induced the apoptosis of SW480 and HCT116 cells.

The role of miRNAs is to inhibit the translation of target genes, thereby regulates gene expression at the translational level. TargetScan is an important bioinformatics prediction tool. It was used to predict miR-17-5p and caspase-9 binding sites between 2402 and 2408. Overexpression of miR-17-5p was co-transfected into 293T cells with caspase-9-wt or caspase-9-mut. The transcriptional activities of caspase-9-wt and caspase-9-mut 3'-UTRs were then measured, which indicated that overexpression was effective.

The ability of miR-17-5p to inhibit apoptosis was attenuated by overexpression of caspase-9. The miR-17-5p bound to caspase-9 and negatively regulated caspase-9 expression, and thus reduced apoptosis in SW480 and HCT116 cells. Consistently, curcumin-treated SW480 and HCT116 cells were arrested in the G2/M phase, with increasing curcumin concentrations, and overexpression of miR-17-5p in SW480 and HCT116 cells accelerated apoptosis [23].

Furthermore, curcumin-upregulated caspase-9 expression was attenuated by miR-17-5p, indicating that curcumin inhibited miR-17-5p expression and then upregulated caspase-9 levels. Consistently, curcumin treatment also significantly inhibited the growth of HCT116 tumor cells in the BALB/c nude mice.

CONCLUSION

The findings of the present study show that miR-17-5p was upregulated in colorectal cancer tissues and cells, and that high miR-17-5p expression is associated with poor prognosis of patients. Curcumin upregulates the expression of caspase-9 via miR-17-5p, thereby promoting the apoptosis of colorectal cancer cells, and ultimately inhibit tumor growth. Although the results indicate that curcumin regulated miR-17-5p expression, further investigation is needed to unravel the mechanism of the signaling pathway. The results show that curcumin is a potential treatment for colorectal cancer.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. JT designed all the experiments and revised the manuscript. JY formed the experiments and wrote the manuscript.

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