ORIGINAL RESEARCH

MicroRNA-421 Inhibits Apoptosis by Downregulating Caspase-3 in Human Colorectal Cancer

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Purpose: Dysregulated microRNAs (miRNAs/miRs) have been reported to play significant roles in pathogenesis of colorectal cancer (CRC). Previous studies have demonstrated that miR-421 regulates apoptosis in some cancers. Caspase-3 plays a key role in apoptosis, but the relationship between miR-421 and caspase-3 in CRC has not been determined. In this study, we investigated the role of miR-421 in CRC and the relationship between miR-421 and caspase-3.

Methods: Expression of miR-421 and caspase-3 were detected in human paired CRC cancer tissues and corresponding paracancerous tissues. In situ detection of tissue, apoptosis was performed via the TUNEL assay. HCT116 and SW480 cell lines were subjected to several in vitro experiments to explore the relationship between miRNA421 and caspase-3 during apoptosis using miR421 mimics/antagomir and luciferase reporter assay. Apoptosis was measured by determining the levels and activity of caspase-3 as well as DNA fragmentation. Luciferase reporter assay was performed to determine the potential interaction of miR-421 with caspase-3.

Results: The results showed that the expression of miR-421 in cancer tissues was higher than that in corresponding paracancerous tissues. Inhibition of miR-421 induced apoptosis, as shown by the upregulation of caspase-3 activity and expression as well as DNA fragmentation, which were attenuated by miR-421 mimic. We further showed that miR-421 targeted and inhibited *CASP3* expression by targeting sites located in the 3′-untranslated region (3′-UTR) of *CASP3* mRNA.

Conclusion: This study demonstrated an anti-apoptotic role of miR-421 in CRC and identified caspase-3 gene as a direct target of miR-421. These findings provide a potential treatment strategy using miR-421 as a therapeutic target for CRC.

Keywords: microRNA-421, colorectal cancer, apoptosis, caspase-3

Introduction

It is estimated that more than 1.8 million new cases of colorectal cancer (CRC) and 881,000 deaths will occur in 2018, accounting for about 1 in 10 cancer cases and deaths. Overall, CRC ranks third in incidence but second in mortality. In the last decade, mortality and morbidity due to CRC in the Chinese population have increased.[1] Most patients are diagnosed at a late stage and have limited treatment options and poor prognosis. If diagnosed early, CRC is one of the most curable types of cancer, with cure rates as high as 90%. Despite advances in treatment and diagnosis, many patients with advanced and metastatic tumors will still succumb to

the disease.[2–8] It is necessary to understand the molecular mechanisms that regulate the initiation and progression of CRC so that novel diagnostic markers and therapeutic agents can be developed.

Deregulated expression of certain microRNAs (miRNAs) has been reported to be related to the occurrence and development of CRC.[9–13] MiRNAs are a class of short, single-stranded, conserved, non-coding RNAs that negatively regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated region (3′UTR) of target mRNAs. MiRNAs are enriched in certain cells and tissues and are implicated in the control of diverse biological processes, including development, differentiation, metabolism, apoptosis, and tumorigenesis.[14–20] Previous studies have demonstrated that miR-421 regulates apoptosis in cervical and gastric cancer cells, as well as nasopharyngeal carcinoma.[21–23] However, it remains to be determined whether miR-421 plays a role in CRC cell apoptosis. Caspase-3 is the main terminal cleaving enzyme in the process of apoptosis and involved in regulating CRC cell migration, invasion and metastasis. It has been reported that miR-421 inhibits the expression of *CASP3* in gastric cancer.[21,24] The aim of this study was to investigate the functional role of miR-421 and caspase-3 in CRC, focusing on their effects on apoptosis and underlying mechanisms.

Materials and Methods
Clinical Specimen Collection
All pairs of specimens (paired tumor and adjacent tissues) of CRC were obtained by surgical resection at The First Affiliated Hospital of Anhui Medical University and were preserved in RNAlater (Invitrogen, Carlsbad, CA, USA) at −80°C immediately after removal from patients to prevent degradation. All cases were diagnosed by histopathology and specimens from patients who underwent preoperative radiotherapy or chemotherapy or had autoimmune diseases such as diabetes and kidney disease were excluded. The study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University, China. All patients provided informed consent, and that this was conducted in accordance with the Declaration of Helsinki.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)
Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The relative expression level of miR-421 was determined by TaqMan miRNA assay kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) using RNU6 as the internal control. First, complementary DNA (cDNA) was synthesized by stem-loop primers, followed by a reverse transcription system using 10 ng of RNA as the template. The conditions were 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, and 72°C for 7 min. Next, TaqMan miRNA-specific primers were added into a 2× PCR TaqMan Universal mixture, along with 5 μL of cDNA in RNase-free water. The PCR parameters were 40 cycles each of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 60 s, followed by elongation at 72°C for 7 min in a fluorescent quantitative PCR equipment (ABI7500, Applied Biosystems, Foster City, CA, USA). The relative level of miR-421 was determined by $2^{\Delta Ct}$ method. All experiments were performed in triplicate.

Immunohistochemistry (IHC)
Tissue sections with diameters of 3 μm were placed on slides coated with 3-aminopropyltriethoxysilane. IHC was performed according to the instructions; briefly, the antigen was first retrieved, and then hydrogen peroxide was added dropwise to block the endogenous peroxidase, and the section was incubated with the polyclonal anti-caspase-3 antibody at a dilution of 1:400 at room temperature and finally incubated with the universal secondary antibody and color reaction.

TUNEL Assay
Apoptotic cells in the paired CRC tumor and corresponding paracancerous tissues were detected using a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) apoptosis detection kit (Zhongshanjinqiao, Beijing, China) according to the manufacturer's instructions. Sections were assessed under the microscope (Nikon, Tokyo, Japan). The number of TUNEL-positive cells (green) was counted and compared with the total number of cells.

Cell Culture and Reagents
The human CRC cell lines HCT116 and SW480 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Passages 3 to 4 of HCT116 and SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA) at 37°C in a humidified

atmosphere with 5% CO_2. All experiments were repeated at least three times. Transfection reagent Lipofectamine 2000 was purchased from Invitrogen. miR-421 mimics and antagomir and scrambled oligonucleotide were purchased from GenePharm Co. Ltd. (Shanghai, China).

Mimics and Inhibitor Transfection Assays

The HCT116 and SW480 cells were seeded in a 6-well plate with DMEM/F12 supplemented with 10% FBS and allowed to grow to 70% to 80% confluence. The cells were then transfected with miR-421 mimics, antagomirs, or controls using Lipofectamine 2000 according to the manufacturer's instructions. The cells were incubated at 37°C for 48 h.

Caspase-3 Activity Measurement

Caspase-3 activity was detected using a caspase-3 activity assay kit (Biomol Research Laboratories, Plymouth Meeting, PA) according to the manufacturer's protocol. The cells were lysed and total cellular protein extracts were quantified using a protein assay kit. Next, an equal amount of total protein extract was incubated at 37°C overnight with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for the caspase-3 assay. The amount of pNA released was estimated by reading at 405 nm on a microplate ELISA reader. The relative activity of caspase-3 was calculated as follows: caspase-3 activity = (mean experimental absorbance/mean control absorbance) × 100%.

Western Blot

After 48 h of transfection with miR-421 mimics or antagomir or scrambled oligonucleotide, the cells were harvested and lysed with 1× sodium dodecyl sulfate (SDS) lysis buffer containing 50 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS. Cell lysates were boiled for 10 min and then centrifuged at 15,871× g for 15 min at room temperature. Samples were separated by 12% SDS-PAGE and transferred to a polyvinyl difluoride membrane (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked in 5% bovine serum albumin (BSA) for 2 h, followed by a 4°C overnight incubation with primary antibodies. The primary antibodies against caspase-3 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Primary antibodies were detected with corresponding horseradish peroxidase-conjugated secondary antibodies (Zhongshanjinqiao) coupled with enhanced chemiluminescence reagents (Engreen, Beijing, China).

Measurement of Cellular DNA Fragmentation

DNA fragmentation was measured using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science, Greenfield, IN, USA). Cells were seeded at a density of 1×10^5 cells per well in 96-well plates. Following 24 h of growth, the medium was changed to serum-free medium, and cells were grown for an additional 24 h. To label the DNA, the medium was replaced with 10% FBS-DMEM, 10 μM 5-bromo-2′-deoxyuridine was added to each well, and cells were incubated for 24 h. Cells were treated with calcitriol for 4 h and then incubated with MIS for an additional 96 h. Cells were then lysed in 200 μL of incubation buffer, and soluble DNA fragments were quantified using the Cellular DNA fragmentation ELISA kit according to the manufacturer's instructions. All experiments were performed in triplicate.

Luciferase Reporter Assays

HCT116 cells were cultured for 24 h. Two hundred nanograms of plasmid DNA (wt-Luc-Caspase-3 or mu-Luc-Caspase-3) and miR-421 mimic, miR-421 antagomir, or a scrambled oligonucleotide were co-transfected by using the Attractene Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The pRL-CMV vector containing the CMV enhancer and early promoter elements to provide high-level expression of Renilla luciferase (Promega, Madison, WI, USA) served as an internal control. Luciferase assays were performed by using the dual-luciferase reporter assay system (Promega) 24 h after transfection.

Statistical Analysis

All data are reported as mean ± standard deviation. Analysis of variance followed by Newman-Keuls test was performed for multigroup comparisons. A value of $P<0.05$ was considered statistically significant. Fold changes refer to the values of other groups compared with the control group, assuming the control group as 1.

Results
Expression of miR-421 in CRC Tissues

To investigate the levels of miR-421 in CRC tissues, total RNA was extracted from pairs of cancer tissues and corresponding paracancerous tissues from 33 patients with CRC, and the expression levels of miR-421 were analyzed using qRT-PCR. The results showed that the expression of

miR-421 in the cancer tissues was higher than that in the paired paracancerous tissues (Figure 1A).

Expression of Caspase-3 in CRC Tissues

Data of IHC showed that the expression level of caspase-3 in cancer tissues was lower than that in corresponding paracancerous tissues (Figure 1C). The apoptosis of CRC cells were detected by TUNEL assay, the percentage of TUNEL-positive cells was higher in corresponding paracancerous tissues than in cancer tissues (Figure 1B).

miR-421 Treatment in HCT116 and SW480 Cells

To determine the efficacy of miR-421 treatment, we measured the expression of miR-421 in HCT116 and SW480 cells after treatment with miR-421 mimics, antagomirs, or controls. Compared with the group receiving control miR, levels of miR-421 were significantly increased after miR-421 mimic treatment in both HCT116 and SW480 cells (Figure 2A and C). In contrast, levels of miR-421 were decreased by more than 50% after miR-421 antagomir treatment in both HCT116 and SW480 cells (Figure 2B and D). These results indicate the successful transfection of miR-421 mimics and antagomir in CRC cells.

Overexpression of miR-421 Inhibited Apoptosis in HCT116 and SW480 Cells

To determine the role of miR-421 in apoptosis, caspase-3 activity and expression and DNA fragmentation were determined. As shown in Figure 3A–D, caspase-3 activity was significantly increased in both HCT116 and SW480 cells treated with miR-421 antagomir, which was attenuated by miR-421 mimic treatment. Similarly, miR-421 antagomir augmented and miR-421 mimic reduced the levels of caspase-3 in both HCT116 and SW480 cells (Figure 3E–H). DNA fragments were significantly increased after miR-421 antagomir treatment, which was attenuated by miR-421 mimics in both HCT116 and SW480 cells (Figure 3I–L). These results suggest that miR-421 reduced apoptosis in human CRC cells.

miR-421 Targeted and Inhibited Caspase-3

A dual-luciferase reporter was performed to clarify the relationships between caspase-3 and miR-421. TargetScan7.1 was used to identify the putative miR421 binding site in 3′-UTR of caspase-3. The putative binding sites are shown in Figure 4A. In addition, mutant 3′-UTR was designed to clarify the potential interaction between
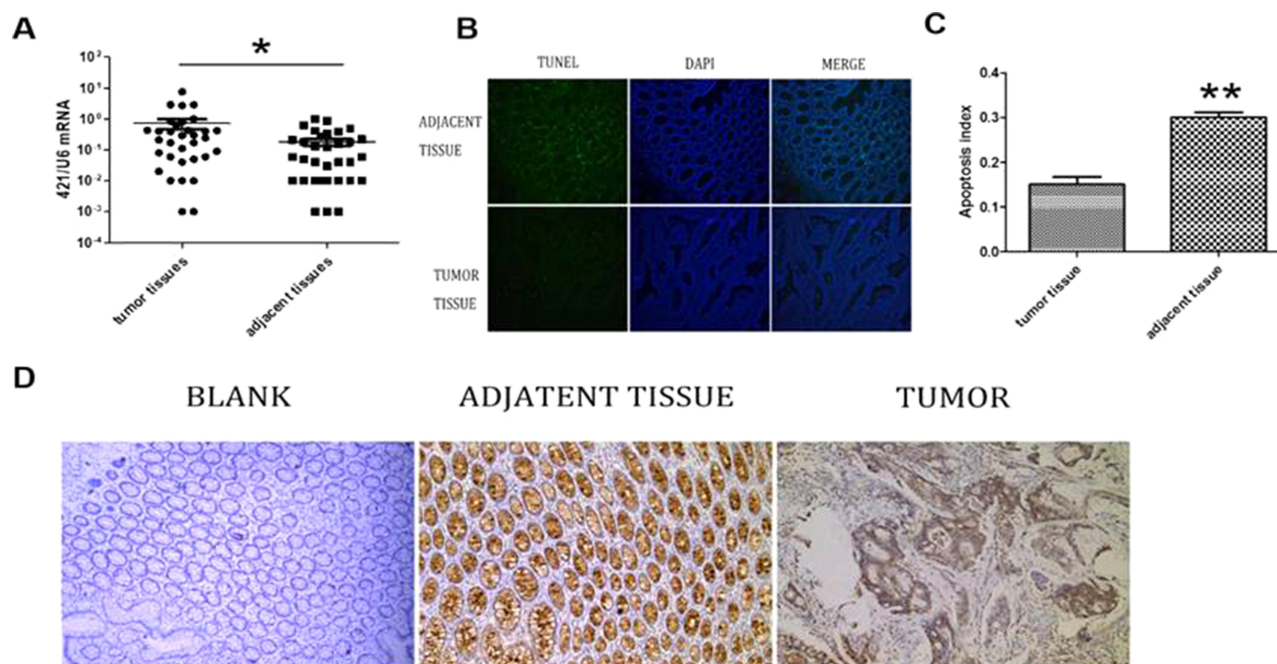

Figure 1 MiRNA-421 and caspase-3 may involved in inducing apoptosis of colorectal cancer. (**A**) MiR-421 expression in paired colorectal cancer and adjacent tissues (n=33, p=0.042). (**B**) Detection in situ of colorectal cancer tissue cell apoptosis by TUNEL assay. Magnification = ×100. (**C**) Bar graph of apoptosis index. (p=0.007) (**D**) Representative immunohistochemical data of caspase-3 expression in paired CRC cancer and the corresponding adjacent tissues. Magnification = 100×. *P<0.05 (p=0.042), **P < 0.01 (p=0.007).
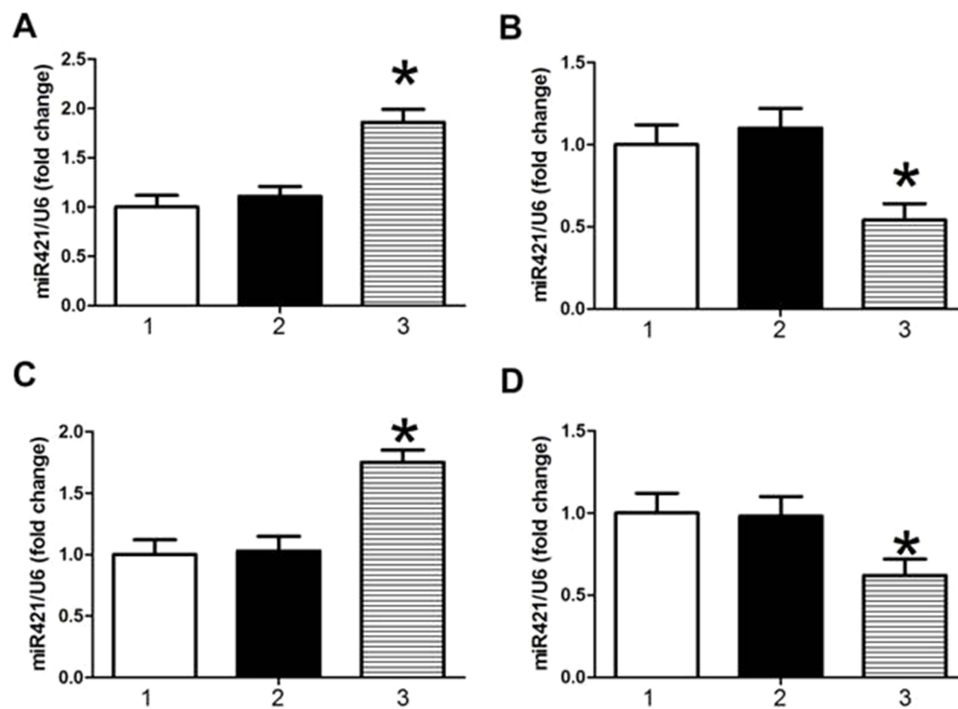
Figure 2 MicroRNA-421 expression in HCT116 cells and SW480 cells. Total RNA was extracted from HCT116 and SW480 cells using TRIzol reagent. Expression of miR-421 was determined using the miRNA plate assay kit, which was normalized to U6 snRNA as the internal control. (**A**) and (**B**) miR-421 expression in HCT116 cells. (p=0.027; p=0.034). (**C**) and (**D**) miR-421 expression in SW480 cells. A1, B1: HCT116 cells; A2, B2: HCT116 cells with control miRNA; A3: HCT116 cells with miR-421 mimics; B3: HCT116 cells with miR-421 antagomir; C1, D1: SW480 cells; C2, D2: SW480 cells with control miRNA; C3: SW480 cells with miR-421 mimics; D3: SW480 cells with miR-421 antagomir. *$P < 0.05$ vs control miRNA. (C: p=0.029; D: p=0.044).

caspase-3 and miR-421 (Figure 4B). Overexpression of miR-421 by its mimic-inhibited luciferase activity in wt-Luc-Caspase-3-transfected HCT116 cells. However, such phenomenon was completely reversed by the mutation of 3′-UTR of caspase-3 (Figure 4C). For further confirmation, we transfected HCT116 cells with wt-Luc-Caspase-3 or the mutated vector (mu-Luc-Caspase-3) and exposed these cells to miR-421 antagomir. The miR-421 antagomir clearly promoted luciferase activity in wt-Luc- Caspase-3 but not in mu-Luc-Caspase-3–transfected HCT116 cells (Figure 4D). The results showed that miR-421 is a direct target of caspase-3 and miR-421 inhibits caspase-3 expression. The protein levels of caspase-3 have been analyzed in miR-421 or miR-421 antagomir-transfected HCT116 cells (Figure 4E). It demonstrated the inhibitory effect of miR-421 on caspase-3 in vitro.

Discussion

This study indicates that miR-421 is highly expressed in CRC tissues and confirms that caspase-3 is a direct target of miR-421. miR-421 reversely regulates mRNA and protein levels of caspase-3. These results indicate the important role of miR-421 in apoptosis and reveal new signaling mechanisms in CRC.

Previous studies have shown that the expression of miR-421 was upregulated in various human cancers such as breast cancer, osteosarcoma, and non-small-cell lung cancer.[25–27] Recently, miR-421 was shown to be upregulated in fecal samples from patients with advanced CRC neoplasms.[28] In the present study, we found that the expression of miR-421 was increased in CRC tissues compared with adjacent noncancerous tissues.

MiR-421 is thought to play an important role in apoptosis regulation in breast cancer, lens epithelial cells, as well as gastric cancer.[24,26,29] Our data showed that the expression of caspase-3 was lower in cancer tissues than in adjacent tissues by IHC, and the percentage of TUNEL-positive cells was higher in corresponding paracancerous than in the tumor tissues. MiR-421 contributed to inhibiting apoptosis. Overexpression of miR-421 prevented apoptosis in CRC cells, whereas treatment with miR-421 antagomir enhanced apoptosis. This finding suggests that miR-421 may be an important factor in the development of CRC and may represent a new therapeutic target; however, this possibility needs to be examined in in vivo animal models in future studies.
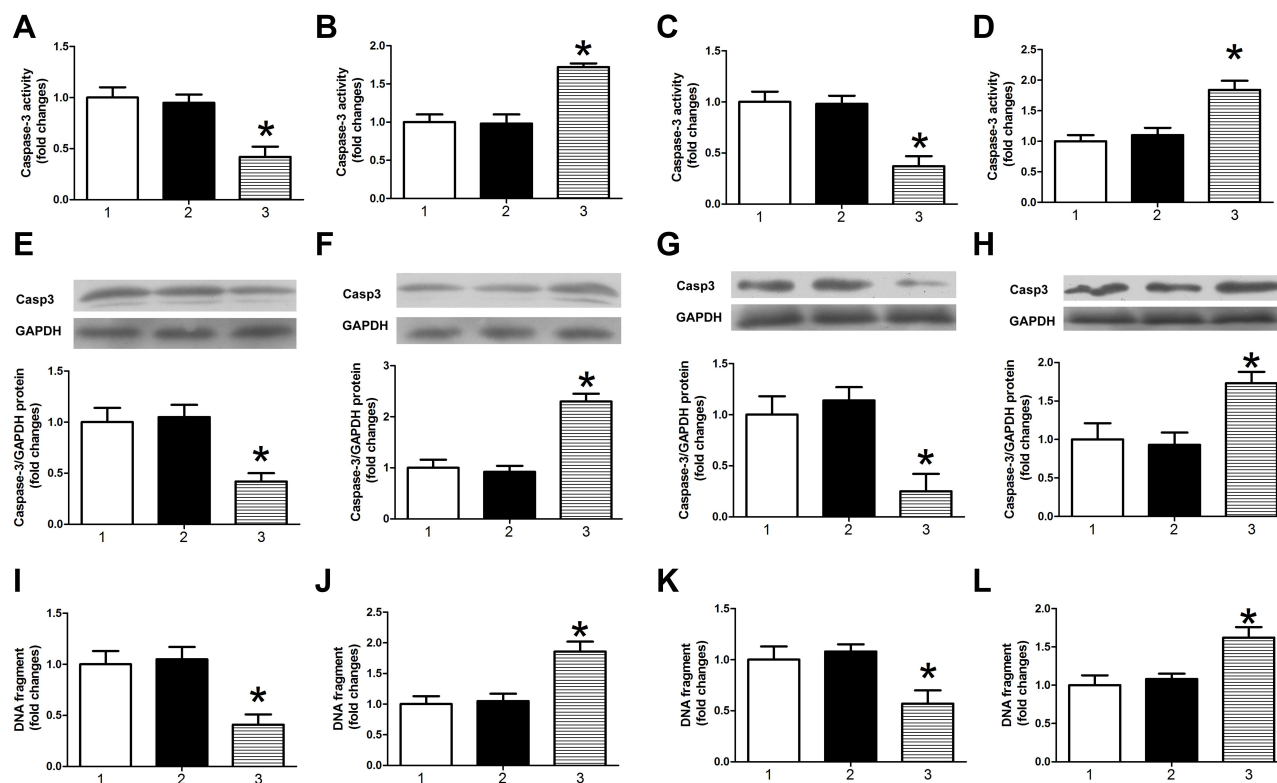
Figure 3 The effect of miR-421 on apoptosis of colorectal cancer (CRC) cells. (**A**) and (**B**) Caspase-3 activity in HCT116 cells. (**C**) and (**D**) Caspase-3 activity in SW480 cells. (**E**) and (**F**) Caspase-3 protein level in HCT116 cells. (**G**) and (**H**) Caspase-3 protein level in SW480 cells. (**I**) and (**J**) DNA fragments in HCT116 cells. (**K**) and (**L**) DNA fragments in SW480 cells. A1, B1, E1, F1, I1, J1: HCT116 cells; A2, B2, E2, F2, I2, J2: HCT116 cells with control miRNA; A3, E3, I3: HCT116 cells with miR-421 mimics; B3, F3, J3: HCT116 cells with miR-421 antagomir; C1, D1, G1, H1, K1, L1:SW480 cells; C2, D2, G2, K2, L2: SW480 cells with control miRNA; C3, G3, K3: SW480 cells with miR-421 mimics; D3, H3, L3: SW480 cells with miR-421 antagomir. *$P < 0.05$ vs control miRNA. (A: p=0.025; B: p=0.021; C: p=0.022; D: p=0.035; E: p=0.016; F: p=0.019; G: p=0.013; H: p=0.030; I: p=0.028; J: p=0.029; K: p=0.026; L: p=0.031).

Dysfunction of apoptosis-related genes is a key mechanism for cancer development, and apoptosis is considered as the most important type of cell death.[30–34] Caspases are a family of protease enzymes and are important mediators of inflammatory response and apoptosis. Dysregulation of caspases underlies many human diseases, including cancer and inflammatory disorders, and major efforts to design better therapies for these diseases seek to understand how the caspases work and how they can be controlled. It is generally believed that caspase-3 is the most important terminal cleaving enzyme in the process of apoptosis and a critical component of the killing mechanism of CTL cells.[35] However, the specific mechanism of caspase-3 in apoptosis is still unclear. Caspase-3 is necessary for apoptotic chromatin concentration and DNA fragmentation. Therefore, caspase-3 is essential for certain physiological processes related to the dismantling of the cell and apoptotic body formation.[34,36,37] It is known that caspase-3 is involved in regulating CRC cell migration, invasion and metastasis.

In the present study, we found that caspase3 was triggered by miR-421, that is, caspase-3 is a direct target

of miR-421. Luciferase reporter assay was performed to support this conclusion. MiR-421 mimic inhibited the luciferase activity of the reporter vector containing a luciferase gene followed by the 3′-UTR of the human caspase-3 mRNA while the luciferase activity was obviously reversed by the mutation of wt-Luc-Caspase -3. In addition, miR-421 induction correlated with a reduction in caspase-3 activity and expression. Furthermore, miR-421 antagomir upregulated and miR-421 mimic downregulated caspase-3 activity and expression in CRC cells. Importantly, these results were consistent in both types of CRC cell lines studied here. The miR-421-CASP3 targeting potential binding site studied in this article is consistent with a previous target examined in gastric cancer research.[24] This report found that overexpression of miR-421 inhibits the apoptosis of gastric cancer cells by targeting *CASP3*, consistent with the targeting relationship identified in the current study.[24] These data demonstrate the functional significance of miR-421 induction in the inhibition of caspase-3 protein expression for apoptosis.
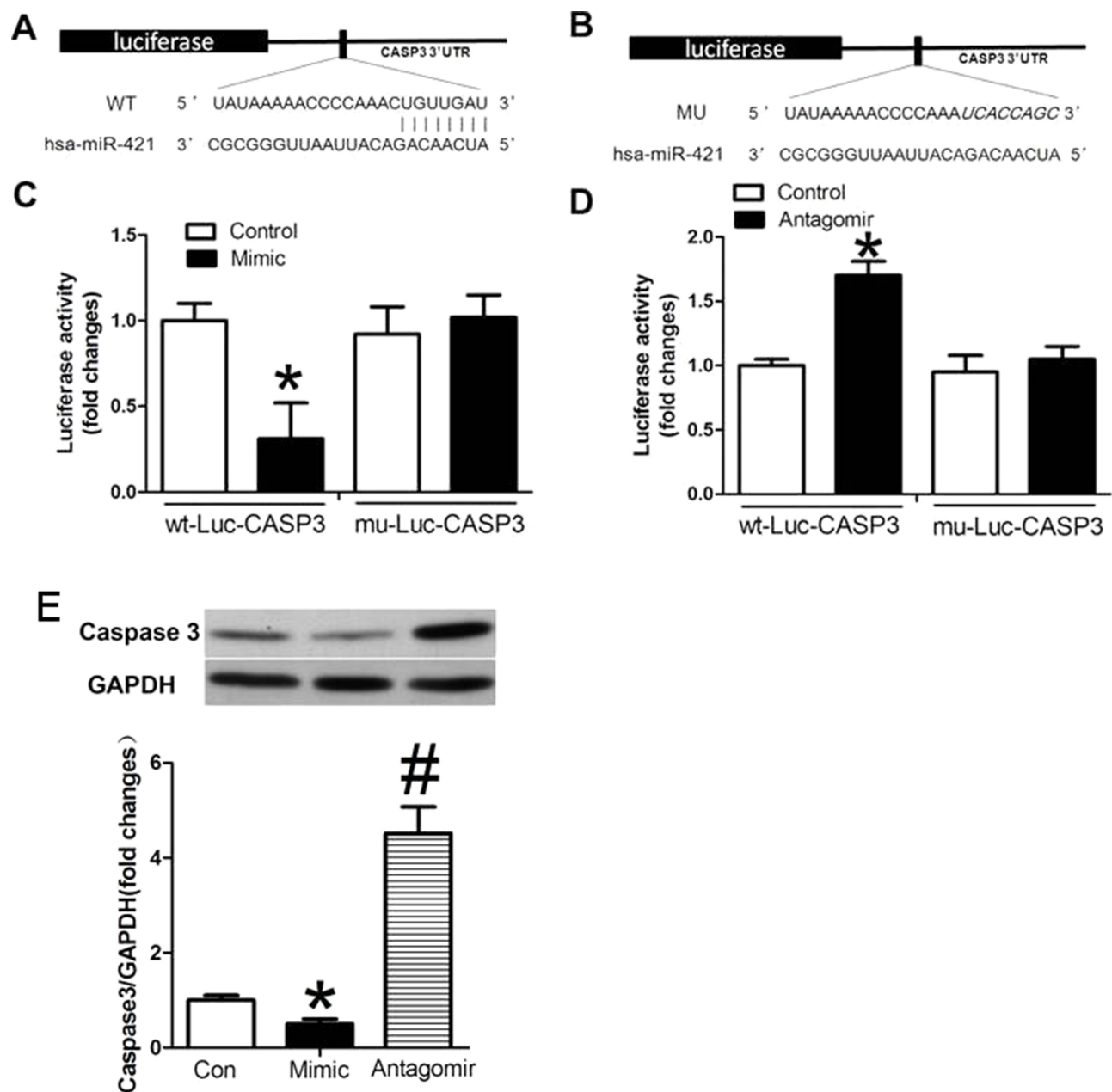
Figure 4 MicroRNA-421 targeted and inhibited caspase-3 expression. A dual-luciferase activity assay was performed. (**A**) A segment of caspase-3 3′-UTR was inserted downstream of the luciferase-coding sequence. Sequence alignment of miR-421 and the 3′-UTR of caspase-3 showed complementarity with the 5′ end of miR-421. (**B**) Sequence alignment of miR-421 and the mutated 3′-UTR of caspase-3 of showed no complementarity with the 5′end of miR-421. (**C**) HCT116 cells were co-transfected with the plasmid containing the wild-type 3′-UTR of caspase-3 (wt-Luc-Caspase-3) or the mutated 3′-UTR of caspase-3 (mu-Luc-Caspase-3) and miR-421 mimic or a scrambled oligonucleotide as a control. (**D**) HCT116 cells were transfected with either wt-Luc-Caspase-3 or mu-Luc-Caspase-3, and then incubated with the miR-421 antagomir. (**E**) The protein levels of caspase-3 in miR-421 or miR-421 antagomir-transfected HCT116 cells. *$P < 0.05$ vs control miRNA. #$P < 0.05$ vs control miRNA and miRNA mimic (p=0.027).

According to reports, miR-421 is regulated by HIF-1α, which can promote metastasis and inhibit apoptosis by targeting *CDH1* and *CASP3* in gastric cancer.[24] A previous study found that miR-421 inhibits EMT in colorectal cancer cells by increasing the expression of E-cadherin,[38] and other studies have found that miR-421 promotes EMT in head and neck squamous cell carcinoma[39] and breast cancer cells by

downregulating the expression of *CDH1*.[40] Therefore, the potential role of miR-421 in EMT in colorectal cancer remains to be studied.

Conclusion

Our results revealed a novel anti-apoptotic role of miR-421 in CRC, which probably functions by repressing

caspase-3–related apoptosis signaling, and we verified caspase-3 as a direct target of miR-421. The effect of miR-421 reduction on apoptosis is mediated by the down-regulation of caspase-3. Thus, miR-421 may be a new therapeutic target for colorectal cancer.

Author Contributions

Huaqing Zhu, Xiaowen Cheng and Zhengguang Wang contributed to conception and design of experiments. Yifan Zhou and Xiaowen Cheng drafted and critically revised the article. Yifan Zhou, Xiaowen Cheng and Tingting Chen completed the human tissues experiments. Yifan Zhou, Xiaowen Cheng and Yufeng Wan completed the cell experiments. Yifan Zhou, Xiaowen Cheng, Yufeng Wan and Qing Zhou contributed to the analysis. Huaqing Zhu and Zhengguang Wang gave final approval and agreed to be accountable for all aspects of work ensuring integrity and accuracy. All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors report no conflicts of interest in this work.

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