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# MiR-422a targets MAPKK6 and regulates cell growth and apoptosis in colorectal cancer cells

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ABSTRACT

The important role of miR-422a in tumor has been reported in several studies. Recent research discovered that the expression of miR-422a was significantly decreased in colorectal cancer tissues, providing miR-422a as a tumor suppressor in CRC. However, the concrete mechanism of miR-422a regulating CRC cell is still unclear. In this study, we demonstrated that miR-422a could inhibit CRC cell growth and promote cell apoptosis via in vitro analyses. Moreover, computational methods were adopted to identify the targets of miR-422a. We found MAPKK6 was the direct target of miR-422a. Consequently, we further elucidated that miR-422a inhibited CRC cell growth and induced cell apoptosis by inhibiting p38/MAPK pathway. Besides that, we established the tumor xenograft model using nude mice and the inhibitory effects on tumor volumes and weights by miR-422a mimic transfection were also detected. Taken together, these findings demonstrated miR-422a exerted anti-cancer activities on CRC, which could be potentially used for CRC prognosis prediction and treatment.

## 1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and the fourth leading cause of cancer mortality [1,2]. Although lots of researches made in diagnostic methods and treatment strategies, the prognosis of CRC patients remains poor, especially in patients with advanced CRC [3,4]. The multiple alterations of tumor suppressor genes and oncogenes are related with CRC carcinogenesis. However, it is still limited to use these genes as markers for early diagnosis, clinical prognosis and prediction. Nowadays, a growing number of evidence showed that miRNAs may manipulate about 30% of human genes and play a crucial role in many cellular processes including differentiation, proliferation, migration and apoptosis [5–9]. However, the functional mechanisms of miRNAs still remain unclear during CRC carcinogenesis and progression. Therefore, several efforts have been made to find dysregulated expression of miRNAs, which could be served as potential biomarkers for colorectal cancer diagnosis and prognosis [10–12].

MicroRNAs, which are approximately 21-nucleotide-long noncoding RNA, anneal in the 3′-UTR of protein-coding mRNAs and lead to repression of translational efficiency and/or decreased mRNA levels [13,14] (Filipowicz, 2008 #452). Deregulation of miRNAs contributes to human pathogenesis including cancer [15]. For instance, the expression of let-7, miR-15, miR-16, miR-17 and miR-21 and were aberrantly altered in different cancer cells [16]. That is probably caused by the amplification, deletion and rearrangement of miRNA genes located in the fragile sites in the genomic regions [17,18]. A theme is emerging that a miRNA could be considered either a tumor suppressor or oncogene depending on its targets in different cell types and tissues [19–21]. Identification of relevant targets or pathways controlled by miRNAs will ultimately provide insights into their biological functions.

Altered expression of miR-422a has been reported in different types of cancer. For example, miR-422a was supposed to target TGF-β and influence Wnt signaling pathways, thus influencing osteosarcoma cell proliferation [22]; miR-422a inhibited glioma proliferation and invasion by targeting IGF1 and IGF1R [23]; Mao et al. [24] proved that miR-422a suppressed MLH1 expression by targeting key mismatch repair protein (MutLα), leading to the instability of genome and tumorigenesis. According to the recent studies, miRNA arrays were adopted to identify new miRNAs that were related with CRC. MiR-422a was found to be deregulated in CRC patients and validated that had inhibitory effect on cell viability in colon cancer cell lines [25,26]. Besides that, miR-422a has been demonstrated to be a prognostic target and a potential tumor suppressor in CRC [27]. However, the underlying mechanisms of miR-422a regulating CRC progression remain unclear. In this study, we further confirmed the suppressive role of miR-422a in CRC via in vitro and in vivo experiments, and first found MAPKK6 was the target of miR-422a, indicating that p38/MAPK involved in the regulation of CRC cell apoptosis induced by miR-422a.

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**Table 1**
The expression level of miR-422a in colorectal cancer patients.

| Variables | n | miRNA-422a (mean ± SD) | *P* value |
|---|---|---|---|
| Age(years) | | | 0.151 |
| < 60 | 26 | 2.162 ± 1.39 | |
| ≧60 | 19 | 2.561 ± 1.62 | |
| Tumor size(cm) | | | 0.048* |
| ≧3.5 | 23 | 1.706 ± 1.35 | |
| < 3.5 | 22 | 2.417 ± 1.46 | |
| Gender | | | 0.165 |
| Male | 28 | 2.216 ± 1.13 | |
| Female | 17 | 2.607 ± 1.76 | |
| Invasion depth | | | 0.125 |
| T1-T2 | 20 | 2.104 ± 1.19 | |
| T3-T4 | 25 | 2.325 ± 1.32 | |
| Lymph node metastasis | | | 0.158 |
| N0 | 24 | 2.358 ± 1.15 | |
| N1-N2 | 21 | 2.153 ± 1.02 | |
| Distant metastasis | | | 0.038* |
| M0 | 27 | 3.583 ± 1.68 | |
| M1 | 18 | 2.172 ± 1.41 | |
| TNM stage | | | 0.042* |
| I, II | 24 | 3.475 ± 1.57 | |
| III, IV | 21 | 2.126 ± 1.33 | |

## 2. Materials and methods

### 2.1. Patients and tissue samples

The colorectal carcinoma tissues and normal tissues were recruited from 45 patients diagnosed with CRC at Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical University between 2012 and 2015. Informed consent was obtained from all patients, and all procedures were approved by the Ethics Committee of Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical University. The CRC patients were not taken the radiotherapy, chemotherapy or other treatment prior to surgery. All tissues obtained from surgical resection were immediately stored at −80 °C until RNA extraction. The clinicopathological information of the patients was shown in Table 1, which was obtained from the hospital.

### 2.2. Cell culture

The colorectal carcinoma SW620 and HCT116 cells were purchased from American type culture collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM medium (Sigma Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) at 37 °C with 5% $CO_2$ in a tissue culture incubator.

### 2.3. Cell transfection and vectors construction

$2 \times 10^5$ CRC cells were plated in six well plates until 40–60% confluence and then were transfected with 50 pmol miR-422a mimic or scrambled sequence control (GenePharma, Shanghai, China) by using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were then lysed and normalized for the following experiments. MAPKK6 overexpression plasmid (pcDNA3.1-MAPKK6) was constructed as previously described [28]. Lipofectamine 2000 reagent (Invitrogen) was used for cell transfection.

### 2.4. Cell proliferation assay

Cell proliferation was determined by cell counting kit 8 (#96992, Sigma Aldrich, USA) according to the protocol of manufacture. Briefly, SW620 or HCT116 cells were plated in 96-well plate at a density of 3000 cells per well. Following cells were transfected with miR-422a mimic or scramble control for different time points. 1/10 vol of the medium cell counting kit solution was added, and the cells were further incubated for another three hours in cell incubator. The number of living cells was measured by using the microplate reader at 450 nm wavelength. Cell growth inhibition rate = $(1 - A_{450,\ treated}/A_{450,\ control}) \times 100\%$.

### 2.5. Flow cytometry analysis of apoptotic cells

CRC cells were plated in 60 mm dishes at a density of $1 \times 10^6$ cells per dish, and then transfected with miR-422a mimic or control. Seventy-two hours after transfection, the cells were trypsinized, and apoptosis was measured using Annexin V-FITC and propidium iodide [22] (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. This assay enabled the identification of early apoptotic (Annexin-V-FITC + /PI − ) cells, late apoptotic/secondary necrotic (Annexin-V-FITC + /PI + ) cells, and primary necrotic (Annexin-V-FITC − / PI + ) cells. The experiments were repeated three times for each sample.

### 2.6. Predicted targets analysis of miR-422a

The predicted targets of the miR-422a were analyzed by online bioinformatics methods, which including TargetScan (http://www.targetscan.org/vert_61/) and microRNA.org (http://www.microrna.org/microrna/). The way of TargetScan and microRNA.org predict biological targets of miRNAs are via searching for the presence of conserved 8 mer, 7 mer, and 6 mer sites that match the seed region of each miRNA [29]. The predicting target genes of miR-422a were analyzed through two algorithms from this database.

### 2.7. 'UTR-luciferase reporter gene assay

The wild-type or mutant 3'UTR of MAPKK6 containing the predicted miR-422a binding sites was cloned into the pRL-TK vectors. 100 ng of pRL-TK-3'UTR-wt or mutant and 20 ng of pGL3.0 control vector were co-transfected in CRC cells planted in 24 well plate. Four hours after the transfection, 50 nM of miR-422a mimic or control was transfected into the cells respectively. After another 48 h' incubation, cells were lysed, and the luciferase activities in each group were analyzed by dual luciferase assay kit (Promega, Madison, USA).

### 2.8. Fluorescence analysis

SW620 and HCT116 cells were seeded on 24-mm sterilized coverslips, and then were co-transfected with MAPKK6-3'UTR-wt or mutant plasmids containing RFP expressing gene, and miR-422a mimic or control respectively. After 72 h' incubation, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with DAPI reagent. Images were captured by fluorescence microscopy (NIKON Instruments Inc., Tokyo, Japan) and analyzed with ImageJ software.

### 2.9. Real-time PCR analysis

Total RNA was isolated from cells with Trizol (Invitrogen) reagent, according to the manufacturer's instructions. The concentration and purity of isolated RNA was estimated using the ND-1000 microspectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed on BioAnalyzer 2100 using BioAnalyzer RNA 6000 Nano Lab Chip Kit (Agilent Technologies, Palo Alto, CA, USA). Then, 2 ug of RNA was utilized for cDNA synthesis using SuperScript II RNase H Reverse Transcriptase (Life Technologies). Following the reverse transcription reactions, real-time PCR was performed with SYBR Green (Thermo Fisher) detecting by Bio-Rad CFX