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NF-κB downstream miR-1262 disturbs colon cancer cell malignant behaviors by targeting FGFR1

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Abstract

Despite substantial advancements in screening, surgery, and chemotherapy, colorectal cancer remains the second most lethal form of the disease. Nuclear factor kappa B (NF-κB) signaling is a critical driver facilitating the malignant transformation of chronic inflammatory bowel diseases. In this study, deregulated miRNAs that could play a role in colon cancer are analysed and investigated for specific functions in vitro using cancer cells and in vivo using a subcutaneous xenograft model. miRNA downstream targets are analysed, and predicted binding and regulation are verified. miR-1262, an antitumour miRNA, is downregulated in colon cancer tissue samples and cell lines. miR-1262 overexpression suppresses colon cancer malignant behaviors in vitro and tumor development and metastasis in a subcutaneous xenograft model and a lung metastasis mouse model in vivo. miR-1262 directly targets fibroblast growth factor receptor 1 (FGFR1) and inhibits FGFR1 expression. FGFR1 overexpression shows oncogenic functions through the regulation of cancer cell proliferation, invasion, and migration; when cotransfected, lv-FGFR1 partially attenuates the antitumour effects of agomir-1262. NF-κB binds to the miR-1262 promoter region and inhibits transcription activity. The NF-κB inhibitor CAPE exerts antitumour effects; miR-1262 inhibition partially reverses CAPE effects on colon cancer cells. Conclusively, miR-1262 serves as an antitumour miRNA in colon cancer by targeting FGFR1. The NF-κB/miR-1262/FGFR1 axis modulates colon cancer cell phenotypes, including proliferation, invasion, and migration.
Keywords: colorectal cancer; miR-1262; fibroblast growth factor receptor 1 (FGFR1); NF-κB

Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed malignancies, with approximately 1 million new cases reported annually [1]. Despite substantial breakthroughs in screening, surgery, and chemotherapy, colon cancer remains a major public health concern in developed nations [2, 3].

MicroRNAs (miRNAs) are a class of small, noncoding RNA molecules ranging in size from 19 to 25 nucleotides. miRNAs affect RNA transcriptional or posttranscriptional regulation of gene expression [4, 5], regulating biological processes, including cellular growth, differentiation, proliferation, and apoptosis. Previous studies have reported the role of microRNAs in tumor biology and carcinogenesis [6-8]. In cancer cells, miRNAs are commonly deregulated; miRNAs show promise as tissue-based markers for the classification and prognosis of cancer [9-12] and therapeutic targets for cancer treatment regimens. Regarding colon cancer, the expression of several miRNAs has been reported to differ from those in matched normal tissues [13-17]. Some miRNAs, such as mir-21 [16, 18], miR-31 [16, 19], and miR-429 [20], are proposed as potential markers for CRC prognosis [16, 21], whereas others, such as miR-126 [22] and miR-150 [23], are characterized as biomarkers for response to chemotherapy. For instance, certain miRNAs have been identified as predictors of response to neoadjuvant chemoradiotherapy in rectal cancer [24, 25].
Identifying differentially expressed miRNAs in CRC that could influence the course of colon cancer could provide potential prognostic/diagnostic biomarkers and therapeutic targets for CRC treatment regimens.

Prior to the development of tumors, CRC risk factors include environmental and food-borne mutagenic agents, specific intestinal commensals and infections, and persistent intestinal inflammation [26, 27]. Colitis-associated cancer (CAC), a form of CRC connected to inflammatory bowel disease (IBD) [28] with a relatively high mortality rate, is currently incurable. Within 30 years of the onset of IBD, over 20% of individuals will develop CAC, and over 50% will suffer mortality [28]. Among dysregulated cytokines, NF-κB is a main regulatory factor in this complex phenomenon [29]. NF-κB expression and activation are significantly elicited in IBD patients' inflamed guts; notably, the cells in inflamed colon samples from IBD patients exhibited higher levels of nuclear p65 [30]. The level of NF-κB activation is related to the severity of inflammation [31], accompanied by increased production and secretion of TNF-α, IL-1, and IL-6 [32]. Moreover, NF-κB is a transcription factor that, when activated, regulates more than 200 genes associated with inflammation, cellular function, and survival [33]. Multiple miRNAs, such as let-7, miR-9, miR-21, miR-143, miR-146, and miR-224, are transcriptional targets of NF-κB [34]. Thus, NF-κB-targeted miRNAs could serve as key regulators in CRC.

Considering these previous findings, NF-κB might affect deregulated miRNAs and potential downstream targets, thereby modulating colon cancer cell phenotypes and colon cancer progression. Using The Cancer Genome Atlas Colon
Adenocarcinoma (TCGA-COAD), differentially expressed microRNAs in colorectal cancer (CRC) that may alter colon cancer progression were investigated in this work to validate this hypothesis. miR-1262 was selected. Reportedly, miR-1262 serves as a tumor-suppressive miRNA in numerous malignancies, including gastric cardia adenocarcinoma [35], lung adenocarcinoma [36], and breast cancer [37]. In addition, miR-1262, lncRNA-RP11-513I15.6, and RAB11A form a circulatory exosomal RNA-based biomarker panel, showcasing their abilities to be diagnostic and prognostic biomarkers for hepatocellular carcinoma [38]. However, the role and mechanism of miR-1262 in colon cancer remain unclear. Moreover, integrative bioinformatics analyses were performed, and possible binding between NF-κB and miR-1262 was predicted.

The specific functions of miR-1262 were investigated in colon cancer cell lines in vitro, a subcutaneous xenograft model and a lung metastasis model in mice in vivo.

The colon cancer cell lines SW620 [39, 40], SW480 [41, 42], HT-29 [43, 44], RKO [45, 46], and HCT 116 [47, 48] have long been used as cell models to investigate the etiology and carcinogenesis of colon cancer; therefore, these cell lines were used in this study. miR-1262 downstream targets were analysed, and fibroblast growth factor receptor 1 (FGFR1) was selected. The predicted binding was validated, and the dynamic effects of miR-1262 on colon cancer cells were investigated. The correlation and possible binding of NF-κB to miR-1262 and the dynamic effects were finally validated.
Materials and methods

Cell lineages and cell culture

A normal human colon epithelial cell line, FHC (CRL-1831), was procured from ATCC (Manassas, USA) and cultured in DMEM: F-12 medium (11320033; Gibco, Waltham, USA) supplemented with 10% FBS (Gibco). Colon cancer cell lines SW620 (CCL-227) and SW480 (CCL-228) were purchased from ATCC and cultured in Leibovitz’s L-15 medium (30-2008; ATCC) supplemented with 10% FBS. Colon cancer cell lines HT-29 (HTB-38) and HCT 116 (CCL-247) were purchased from ATCC and cultured in McCoy’s 5A medium (30-2007; ATCC) supplemented with 10% FBS. A colon cancer cell line, RKO (CRL-2577), was procured from ATCC and cultured in Eagle’s minimum essential medium (EMEM) (30-2003; ATCC) supplemented with 10% FBS. All cells were cultured at 37°C in 5% CO₂.

Clinical sampling

In this study, twelve tumor samples and paired adjacent normal samples were harvested from colon cancer patients with the approval of the Ethics Committee of Guangdong Provincial People’s Hospital (KY-Z-2021-153-01) and with the 1964 Helsinki Declaration. All samples were stained with hematoxylin and eosin (H&E) and analysed for histopathological characteristics before being frozen at -80°C for future use. All participants provided written informed consent, and the clinical characteristics are listed in Supplementary Table S1.

qRT-PCR

Total RNA was extracted from target samples using TRIzol (Invitrogen,
Carlsbad, USA) following the manufacturer’s protocols. A UV spectrophotometer (BD Diagnostics, Franklin Lakes, USA) was used to qualify the RNA concentration and purity. A reverse transcription kit (TaKaRa, Tokyo, Japan) was used to perform reverse transcription into complementary DNA. SYBR Premix Ex Taq TM II (TaKaRa) was used for PCR amplification on the ABI Step-one Plus Real-time PCR system (Applied Biosystems, Foster City, USA). GAPDH (mRNA expression level) and U6 (miRNA expression level) were used as internal controls to determine relative expression levels. The primer sequences for qRT-PCR are listed in Supplementary Table S2. Data were analysed with the $2^{-\Delta\Delta Ct}$ method.

**Hematoxylin and eosin (H&E) staining**

Tissues were fixed overnight in 4% paraformaldehyde (PFA), processed, and embedded in paraffin. Sections (5 μm in thickness) were produced and stained as directed by the manufacturer (Jiancheng Bioengineering Institute, Nanjing, China). A BX51 microscope was used to image the stained sections (Olympus, Tokyo, Japan).

**Cell transfection**

miR-1262 overexpression or inhibition was achieved in target cells by transfecting agomir-1262 or antagomir-1262 (GenePharma, Shanghai, China). Transfection was performed using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, USA). miR-1262 overexpression in model mice was induced by infection with lentivirus overexpressing miR-1262 (lv-miR-1262; GenePharma). FGFR1 overexpression was achieved in target cells by transducing lentivirus overexpressing FGFR1 (lv-FGFR1; GenePharma). The sequences of agomir-1262,
antagomir-1262, lv-miR-1262 and lv-FGFR1 are listed in Supplementary Table S2.

2 MTT assay detecting cell viability

Cells (3000 cells/well) were seeded in 96-well plates for 24, 48, and 72 h, and 10 μl MTT (Sigma-Aldrich, St Louis, USA) was added to each well at these time points. The medium was discarded following 4–6 h of incubation. Then, 100 μL of DMSO was added to each well, and the cells were incubated in the dark for 20 min. The absorbance was measured at 570 nm using a microplate reader (DNM9602; Prolong, Beijing, China).

9 Transwell assay

A Transwell chamber (Corning, Tewksbury, USA) with a base membrane pore size of 8 μm and Matrigel coating was utilized to detect cell invasion. The lower layer was filled with 600 μl of DMEM containing 10% FBS. A total of $5 \times 10^5$ colon cancer cells were plated into the upper chamber. The cells were cultured for 24 h at 37°C and 5% CO$_2$ in an incubator. The wells were subsequently fixed for 30 min in a mixture of methanol and glacial acetic acid (3:1), rinsed with PBS, stained with 0.1% crystal violet, and mounted. It was determined that stained cells were invasive, and representative photos were captured.

Wound healing assay

In a six-well plate, cells were plated and allowed to grow until 70% confluence. After 4 h, the medium in each well was replenished. After 24 h, a P200 tip was utilized to scratch the well’s center. The wound areas were photographed at 0 hours, and the medium was routinely replaced for up to 24 hours. ImageJ software (NIH,
Bethesda, USA) was used to measure and compare all wound areas.

### Immunoblotting analysis

RIPA buffer was used to lyse and extract total protein from cells. Fifty micrograms of protein samples were loaded onto 10% SDS-PAGE, transferred to PVDF membranes, and blocked with 5% Blotto for 2 h. The membranes were subsequently incubated with primary antibodies against PCNA (10205-2-AP; Proteintech, Wuhan, China), N-cad (ab76011; Abcam, Cambridge, USA), E-cad (20874-1-AP; Proteintech), FGFR1 (ab10646; Abcam), and vimentin (10366-1-AP; Proteintech) and cultured at 4°C overnight. The following day, membranes were treated with a secondary antibody and detected using an enhanced chemiluminescence reagent.

### Colon cancer xenograft model

Male nude mice (BALB/c, 5–6 weeks old) were procured from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). The experimental protocol was approved by the Regional Ethics Committee for Animal Research at Guangdong Provincial People’s Hospital (KY-Z-2021-153-01). A subcutaneous colon cancer xenograft model was established in male nude mice by subcutaneous injection of HCT116 cells into the left flank of the mice. The mice were randomly allocated into two groups: the control group (n=6), in which mice received a subcutaneous injection of $2.5 \times 10^6$ HCT116 cells (logarithmic phase) transduced with lv-NC, and the lv-miR-1262 group (n=6), in which mice received a subcutaneous injection of $2.5 \times 10^6$ HCT116 cells (logarithmic phase) transduced with lv-miR-1262. The growth of
the tumor was monitored by palpation, and its volume was measured every week beginning on day 7 after cell injection. All mice were euthanized after 28 days, and tumors were excised, measured, and photographed. The tumor samples were subsequently treated for immunohistochemistry or qPCR.

**In vivo tumor metastasis assay**

To produce experimental metastasis, HCT116 cells transduced with lentiviral constructs carrying either miR-1262 or vector control were harvested, washed and resuspended in PBS. Five- to six-week-old BALB/c nude mice were procured from Hunan SJA Laboratory Animal Co., Ltd, and $1 \times 10^7$ cells suspended in 0.2 mL serum-free McCoy’s 5A medium were injected into the lateral tail vein of each mouse. Each tumor cell line was injected into six mice, and animals were maintained in a sterile animal facility. Animals were euthanized after six weeks, and their lungs were evaluated for metastases. Lung-derived tumor tissues were dissected and analysed histologically.

**Immunohistochemical (IHC) staining**

Tumor tissues obtained from patients or the mouse model were sliced into 4-μm-thick sections and paraffin-embedded. Sections were incubated with anti-PCNA (10205-2-AP; Proteintech) or anti-FGFR1 (ab10646; Abcam). The sections were incubated with a biotinylated secondary antibody followed by an ABC reagent (Vector Laboratories, Burlingame, USA). The signal was detected using DAB solution (Vector Laboratories). Finally, the sections were counterstained with hematoxylin, photographed, and evaluated for areas of positive staining.
Dual-luciferase reporter assay

For miR-1262 binding to FGFR1, wild- and mutant-type FGFR1 reporter vectors (wt-FGFR1 and mut-FGFR1) were constructed. 293T cells were cotransfected with wt-FGFR1/mut-FGFR1 and agomir-1262/antagomir-1262. The luciferase activity was determined using the Dual-Luciferase Reporter System (Promega, Madison, USA).

For NF-κB binding to the miR-1262 promoter, wild- and mutant-type miR-1262 promoter reporter vectors (pro-miR-1262, pro-miR-1262-mut1+2) were constructed. 293T cells were cotransfected with reporter plasmids and pcDNA3.1/NF-κB. The luciferase activity was determined using the Dual-Luciferase Reporter System (Promega). The primers for vector construction are listed in Supplementary Table S2.

Statistical analysis

Each experiment was repeated at least thrice. Data were processed using GraphPad software (GraphPad Software Inc., San Diego, USA) and are shown as the mean ± SD. Before processing, all relevant data were examined for normal distribution and variance homogeneity. The Shapiro-Wilk test was utilized for data distribution analysis and the selection of a parametric or nonparametric statistical technique. The Brown-Forsythe test was applied for the comparison of group variances. The Kruskal-Wallis test was utilized for nonparametric statistical analysis. Student’s $t$-test was used to compare the differences between the two groups. If the data did not have equal variances and had a normal distribution, one-way ANOVA with Dunnett T3 was used to examine differences between two or more groups. If the variances of the data were equivalent, one-way ANOVA with Tukey’s post hoc test was utilized. A $P$
value of < 0.05 was considered statistically significant.

3 Results

4 miR-1262 is underexpressed in colon cancer tissues and cells

For selecting differentially expressed miRNAs that might affect colon cancer progression, we first downloaded the data of TCGA-COAD from the UCSC database of the University of California, Santa Cruz (https://xenabrowser.net/datapages/), containing the mature miRNA expression matrix in 253 colon cancer samples and 8 normal control samples; after difference analysis by the R language toolkit LIMMA, 740 downregulated miRNAs and 141 upregulated miRNAs were obtained (logFC > 0.56 or < −0.56, \( P < 0.05 \)) (Supplementary Figure S1A). TCGA-COAD also contains overall survival, disease-free survival, and time between recurrences of colon cancer patients. Multivariate Cox risk regression was used to analyse the correlation of miRNAs and prognosis in the TCGA-COAD dataset, and we found that 416 miRNAs were dramatically linked to colon cancer patients’ overall survival and served as risk or protective factors (hazard ratio (HR)>1 or <1, \( P<0.05 \)). After cross-checking for survival-related differential miRNAs, 24 downregulated genes were found to be related to survival (HR<1, \( P<0.05 \)), and 19 upregulated genes were related to survival (HR>1, \( P<0.05 \)) (Supplementary Figure S1B). Next, the GSE160432 dataset, containing 32 healthy control samples and 20 colon cancer samples, was further analysed to corroborate the differentially expressed miRNAs in colon cancer, and 30 downregulated miRNAs and 104 upregulated miRNAs were obtained (Supplementary
Figure S1C). Through cross-screening, 2 downregulated miRNAs (hsa-miR-1262, hsa-miR-194-3p) and 3 upregulated miRNAs (hsa-miR-193a-3p, hsa-miR-493-5p, hsa-miR-424-5p) were selected in the TCGA-COAD and GSE160432 datasets (Supplementary Figure S1D). Although the antitumour effect of miR-1262 on pulmonary cancer [36, 49], breast cancer [37], and gastric cardia adenocarcinoma [35] is clear, the biological functions of miR-1262 in colon cancer are still unclear and require further clarification (Figure 1A). Hence, the effect of miR-1262 on colon cancer was investigated. According to TCGA-COAD data, miR-1262 was downregulated in colon cancer tissue samples compared with normal noncancerous tissue samples (Figure 1B). Subjects from TCGA-COAD were assigned into high- and low-miR-1262 expression groups; higher miR-1262 expression was significantly related to better prognosis in patients with colon cancer (Figure 1C). miR-1262 expression was determined in the normal colon epithelial cell line FHC and five colon cancer cell lines, SW620, SW480, HT-29, RKO, and HCT116; similarly, miR-1262 expression was downregulated in colon cancer cell lines and more downregulated in RKO and HCT116 cells (Figure 1D). Clinical colon cancer and matched adjacent noncancerous samples were excised, and the histopathological characteristics were confirmed using H&E staining (Figure 1E). In collected colon cancer tissues, miR-1262 expression was significantly downregulated compared with that in adjacent normal healthy tissue samples (Figure 1F). These data indicated miR-1262 is underexpressed in colon cancer which was associated with poor prognosis.

miR-1262 affects colon cancer cell phenotypes
To investigate the role of miR-1262 \textit{in vitro}, agomir-1262/antagomir-1262 was transfected to achieve miR-1262 overexpression/inhibition in RKO and HCT116 cells, as confirmed by qRT-PCR (Figure 2A). After transfecting RKO and HCT116 cells with agomir-1262 or antagomir-1262, malignant cell phenotypes were analysed. miR-1262 overexpression inhibited cell viability (Figure 2B), cell invasion (Figure 2C), and cell migration (Figure 2D), whereas miR-1262 inhibition exerted the opposite effects on colon cancer cells (Figure 2A–D). Overexpression of miR-1262 decreased the protein levels of PCNA, N-cad, and vimentin but increased those of E-cad, whereas inhibition of miR-1262 had the opposite effect on these factors (Figure 2E). These data indicated miR-1262 inhibits colon cancer cell proliferation, invasion and migration ability.

\textbf{miR-1262 overexpression inhibits tumor growth and metastasis in a mouse model}

To investigate the effect of miR-1262 on colon cancer \textit{in vivo}, a colon cancer xenograft model was constructed in male nude mice by injecting lv-miR-1262-transduced HCT116 cells in the logarithmic phase, taking the mice injected with lv-NC-transduced HCT116 cells as controls; 28 days after injection, mice were sacrificed, and tumors were harvested (Figure 3A). qRT-PCR was used to confirm miR-1262 overexpression in tumor tissue samples (Figure 3B). The tumor volume was examined every 7 days from day 7 to day 28 of the modelling; Figure 3C shows that miR-1262 overexpression significantly reduced the tumor volume. On day
28, mice were sacrificed, and the tumor weight was determined; Figure 3D illustrates that miR-1262 overexpression significantly reduced tumor weight. H&E staining was subsequently performed to examine the histopathological characteristics of the tumors; Figure 3E shows that miR-1262 overexpression inhibited the necrosis of tumor tissues. miR-1262 overexpression lowered PCNA levels in tumor tissues, as demonstrated by IHC labelling and immunoblotting (Figure 3E,F). Consistently, miR-1262 overexpression downregulated N-cad and vimentin protein levels but upregulated E-cad levels in tumor tissues (Figure 3F). These data indicated miR-1262 plays antitumor effect on colon cancer xenograft mouse model.

To further investigate the activities of miR-1262 on tumor metastasis in vivo, the tumor metastatic potential of lv-miR-1262-transduced HCT116 cells was evaluated utilizing a colon cancer cell metastasis model in nude mice. As shown in Figure 4A,B, the incidence of lung metastases and the number of metastatic lung nodules in the lv-miR-1262 group were dramatically decreased compared with those in the lv-NC group. H&E staining indicated the macroscopic presence of lung metastases after implantation of HCT116 cells into the lateral tail vein of athymic nude mice (Figure 4C). These data indicate that miR-1262 suppresses colon cancer lung metastasis in mouse models.

FGFR1 is a direct downstream target of miR-1262

miRNAs posttranscriptionally modulate gene expression by targeting the 5'UTR, coding regions or 3'UTR of messenger RNAs (mRNA), repressing mRNA translation
or inducing mRNA degradation, subsequently exerting their biological functions [50, 51]. To identify miR-1262 targets, linkedomics (http://linkedomics.org/login.php) was used to screen genes related to miR-1262 expression; 357 genes were negatively related to miR-1262. The online tool miRDIP (https://ophid.utoronto.ca/mirDIP/) was then used to predict the target gene of miR-1262 (high score). After cross-checking for the 221 high-score genes, 7 genes were selected. Among them, FGFR1 has been recognized as a potential therapeutic target in colorectal cancer [52, 53] and was subsequently selected for further investigations (Figure 5A). In HCT116 and RKO cells, miR-1262 overexpression downregulated FGFR1 mRNA and decreased FGFR1 protein levels, whereas miR-1262 inhibition exerted the opposite effects (Figure 5B-C), indicating the negative regulatory role of miR-1262 in FGFR1 expression.

In contrast to miR-1262, FGFR1 was dramatically upregulated in colon cancer tissue samples relative to adjacent normal tissue samples (Figure 5D). Consistently, the protein levels of FGFR1 were evaluated in tissue samples using IHC staining; Figure 4E shows that FGFR1 protein levels were increased in colon cancer samples compared to adjacent normal tissue samples (Figure 5E). In tissue samples, miR-1262 was negatively related to FGFR1 (Figure 5F). To validate the predicted miR-1262 binding to FGFR1, wild- and mutant-type FGFR1 luciferase reporter vectors were processed based on psiCheck-2 plasmids and cotransfected into 293T cells with agomir-1262 or antagonim-1262. In wt-FGFR1 and agomir-1262/antagomir-1262 cotransfected cells, miR-1262 overexpression inhibited, whereas miR-1262 inhibition enhanced, luciferase activity; in mut-FGFR1 and agomir-1262/antagomir-1262
cotransfected cells, miR-1262 overexpression or inhibition caused no alterations in luciferase activity (Figure 5G). Thus, miR-1262 binds to the FGFR1 3′UTR and inhibits FGFR1 expression.

Dynamic effects of the miR-1262/FGFR1 axis

Given that miR-1262 targets FGFR1 to inhibit FGFR1, the dynamic effects of the miR-1262/FGFR1 axis on colon cancer cell phenotypes were investigated. Lentivirus overexpressing FGFR1 was transfected to achieve FGFR1 overexpression in HCT116 and RKO cells, as confirmed by qRT-PCR (Figure 6A). HCT116 and RKO cells were subsequently cotransfected with agomir-1262 and lv-FGFR1, and the protein levels of FGFR1 were assessed. Figure 5B shows that miR-1262 overexpression decreased, whereas FGFR1 overexpression increased, FGFR1 protein levels; FGFR1 overexpression partially reversed the effects of miR-1262 overexpression (Figure 6B). Regarding cell phenotypes, in contrast to miR-1262 overexpression, FGFR1 overexpression promoted cell viability (Figure 6C), cell invasion (Figure 6D), and cell migration (Figure 6E); moreover, FGFR1 overexpression partially mitigated the inhibitory effects of miR-1262 overexpression on colon cancer cell malignant phenotypes (Figure 6C-E). Additionally, FGFR1 overexpression increased the protein levels of PCNA, N-cad, and vimentin, while decreasing E-cad and partially reversing the effects of miR-1262 overexpression on these factors (Figure 6F). Thus, miR-1262 exerts its effects on colon cancer cells by targeting FGFR1.

NF-κB targets the miR-1262 promoter region and inhibits its expression
Colitis-associated carcinoma is a classic inflammatory-driven carcinoma in which NF-κB activation drives tumorigenicity [54, 55]. Given that NF-κB regulates the transcriptional activity of multiple factors, the correlation between miR-1262 and NF-κB expression in colon cancer tissues was determined according to data from TCGA-COAD (Figure 7A) and collected clinical tissue samples (Figure 7B). Figure 7A,B shows that miR-1262 and NF-κB expression within tissues was negatively correlated. In HCT116 and RKO cells stimulated with IL-1β or TNF-α, miR-1262 expression was dramatically downregulated (Figure 7C), indicating the negative regulatory role of NF-κB in miR-1262 expression. To validate NF-κB binding to miR-1262, wild- or mutant-type miR-1262 promoter-reporter plasmids were constructed (pro-miR-1262, pro-miR-1262-mut1, pro-miR-1262-mut2). These reporter plasmids were cotransfected with pcDNA3.1/NF-κB, and the luciferase activity was assessed. When cotransfected with miR-1262 and pcDNA3.1/NF-κB, NF-κB overexpression significantly inhibited luciferase activity; when cotransfected with miR-1262-mut1/miR-1262-mut2 and pcDNA3.1/NF-κB, NF-κB overexpression failed to alter luciferase activity (Figure 7D). Thus, NF-κB binds to the miR-1262 promoter region and inhibits transcriptional activity.

HCT116 and RKO cells were transfected with antagonir-1262, treated with CAPE (NF-κB inhibitor) or DMSO, and examined for cell phenotypes. miR-1262 inhibition promoted cell viability (Figure 7E), cell invasion (Figure 7F), and cell migration (Figure 7G), whereas CAPE treatment exerted the opposite effects on colon cancer cells; miR-1262 inhibition partially attenuated the effects of CAPE on colon
cancer cells (Figure 7E–G). miR-1262 inhibition consistently increased the protein
levels of PCNA, N-cad, and vimentin, whereas CAPE treatment had the opposite
effect on these proteins; miR-1262 overexpression partially mitigated the effects of
CAPE on these factors (Figure 7H). To ensure the rigor of the conclusion,
 supplementary experiments investigating whether miR-1262 could regulate the
nuclear translocation and expression of NF-κB were conducted. As shown in
Supplementary Figure S2, the miR-1262 antagomir alone failed to alter the
nuclear/cytoplasmic protein levels of NF-κB; however, the NF-κB inhibitor CAPE
significantly decreased nuclear NF-κB but increased cytoplasmic NF-κB, indicating
that miR-1262 inhibition alone could not alter the nuclear translocation and
expression of NF-κB. These findings indicate that NF-κB is upstream of miR-1262.

Discussion

This study recognizes miR-1262, which was downregulated within colon cancer
tissue samples and cells, as an antitumour miRNA. miR-1262 overexpression
inhibited the capacity of cancer cells to proliferate, invade and migrate in vitro and
tumor development in the subcutaneous xenograft model in vivo. miR-1262 directly
targeted FGFR1 and inhibited FGFR1 expression. FGFR1 overexpression showed an
oncogenic role by promoting cell proliferation, invasion, and migration; when
cotransfected, lv-FGFR1 partially attenuated the antitumour effects of agomir-1262.
NF-κB bound to the miR-1262 promoter region and inhibited transcription activity; in
addition, miR-1262 failed to alter the nuclear translocation and expression of NF-κB.
Finally, the NF-κB inhibitor CAPE served as an antitumour agent; miR-1262 inhibition partially reversed the effects of CAPE on colon cancer cells.

Deregulated miRNAs play a critical role in cancer tumorigenesis by regulating cancer cell phenotypes [56, 57]. Previously, exosomal miR-1262 expression was shown to be decreased in the serum of hepatocellular carcinoma patients; additionally, lncRNA-RP11-513I15.6 and RAB11A formed an exosomal competing endogenous RNA network biomarker panel as a biomarker tool for hepatocellular carcinoma diagnosis and prognosis [38]. As mentioned above, the antitumour functions of miR-1262 have been reported in lung malignancies [36, 49], breast cancer [37], and gastric cardia [35]. LRP8 accelerated the development of breast cancer by contributing to cellular aggression, which was inhibited by miR-1262 [37]. In gastric cardia adenocarcinoma, transcriptional regulation of miR-1262 by a genetic variation of the enhancer inhibits cancer cell malignancy by binding to the oncogene ULK1 [35]. Interestingly, miR-1262 downregulation was observed in colon cancer samples and cell lines, suggesting that miR-1262, as a deregulated miRNA, could exert effects on colon cancer tumorigenesis. As speculated, miR-1262 overexpression inhibited cancer cell viability, invasion, and migration and decreased proliferation and migration marker levels in vitro; in vivo, miR-1262 overexpression hindered tumor growth in a subcutaneous xenograft model in mice. These findings provide more evidence for the antitumour function of miR-1262.

Mechanically, miRNAs exert their functions by targeting downstream mRNAs; miR-1262 has been reported to function in cancers mostly by targeting downstream...
mRNAs [35, 37]. After validating the antitumour function of miR-1262 in colon
cancer, downstream target mRNAs were investigated, and FGFR1 was identified as
the best match. In contrast to miR-1262, the oncogenic nature of FGFR1 is well
known. A high proportion of human malignant tumors have been reported to have
amplified FGFR1, leading to abnormal activation of FGFR signaling, tumorigenesis
transformation, and tumor progression [58]. FGFR1 activation could enhance
epithelial-mesenchymal transition (EMT) and metastasis within prostate and breast
carcinomas [59, 60] and promote proliferation, EMT, migratory ability, and invasive
ability within FGFR1-amplified pulmonary carcinoma cells [61]. In this study,
FGFR1 overexpression was observed in colon cancer samples and cells. In contrast to
miR-1262 overexpression, FGFR1 overexpression promoted cancer cell proliferation,
invasion, and migration, which is consistent with data from other cancers.
Furthermore, FGFR1 overexpression inhibited the effects of miR-1262
overexpression on colon cancer cells, demonstrating that miR-1262 executes its
functions by targeting FGFR1. Given the oncogenic role of FGFR1 in colon cancer,
there are also relevant studies on inhibitors of FGFR1. Yin et al. reported that the
small molecule HCI-48 mainly targets PIM1 and FGFR1 kinases, thereby eliciting
antitumour effects on colorectal cancer growth [62]. In addition, anlotinib, a
multitarget tyrosine kinase inhibitor that is designed to primarily inhibit VEGFR2/3,
FGFR1-4 and so on, has significant anticancer effects on colon cancer [63, 64].

Although miR-1262 is an antitumour miRNA, its expression is considerably
downregulated in colon cancer, suggesting its potential benefits in decreasing cancer
cell malignancy. Investigating the process of its abnormal downregulation could yield strategies for restoring its expression. There is compelling evidence that inflammatory responses are one of the primary causes of colorectal cancer [65]. The NFκB transcription factor can regulate immune system component expression and has been reported to regulate proteins that promote inflammation and proliferation, which is associated with colon cancer [66]. Wang et al. demonstrated that NF-κB maintains the stem-like properties of colon cancer stem cells by negatively regulating miR-195-5p/497-5p [67]. Ryan et al. suggested that targeting NF-κB inhibits peritoneal tumor growth and metastasis of colon cancer [68]. Regarding why miR-1262 is underexpressed in colon cancer, NF-κB garnered academic attention due to its oncogenic role in colon cancer [54, 69]. Previously, NF-κB activation was shown to increase miR-16 and miR-224 expression by direct promoter binding [70] and reduce miR-199, miR-214, and miR-21 expression [71, 72], suggesting that NF-κB might also affect the transcription and expression of miR-1262. Herein, miR-1262 was negatively correlated with NF-κB. By targeting the promoter region of miR-1262, NF-κB inhibited miR-1262 expression; in addition, miR-1262 failed to alter the nuclear translocation and expression of NF-κB, suggesting that NF-κB is upstream of miR-1262 and modulates miR-1262 transcription and expression. Importantly, the NF-κB inhibitor CAPE [73] showed similar effects as miR-1262 overexpression on colon cancer cells, and miR-1262 inhibition partially attenuated the antitumour effects of CAPE, indicating that NF-κB causes miR-1262 downregulation in colon cancer cells.
In conclusion, miR-1262 is an antitumour miRNA in colon cancer cell and xenograft tumor models. The NF-κB/miR-1262/FGFR1 axis modulates colon cancer cell proliferation, invasion, and migration capacity. The axis may serve as a potential diagnostic/prognostic biomarker and therapeutic target, which will require future systematic investigation.

Supplementary Data
Supplementary data is available at Acta Biochimica et Biphysica Sinica online.

Funding
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Conflict of Interest
The authors declare that they have no conflict of interest.

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1  3-29


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factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa.

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17 proliferation, epithelial-mesenchymal transition, and metastasis in FGFR1-amplified lung
18 cancer. Oncogene 2018, 37: 5340-5354
20 targeting PIM1 and FGFR1 kinases inhibits colorectal cancer growth and patient-derived


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Figure legends

Figure 1. miR-1262 is underexpressed in colon cancer tissues and cell lines  (A)
A schematic diagram showing the process of selecting differentially expressed
miRNAs that might affect colon cancer progression. (B) miR-1262 expression in
colon cancer tissues and normal noncancerous tissues according to The Cancer
Genome Atlas-Colon Adenocarcinoma (TCGA-COAD). **P<0.01 compared to
normal noncancerous tissues. (C) Subjects from TCGA-COAD were assigned into
high- and low-miR-1262 expression groups (based on screening index median); the
correlation between miR-1262 expression and colon patients' overall survival was
analysed using the Surviver and Survminer packages in the R language. (D)
miR-1262 expression was determined in FHC, SW620, SW480, HT-29, RKO, and
HCT116 cells using qRT-PCR. *P<0.05, **P<0.01 compared to FHC cells. (E)
Clinical samples (colon cancer and matched adjacent noncancerous samples) were
collected and examined for histopathological characteristics using H&E staining.
Scale bar = 100 μm. (F) miR-1262 expression was determined in collected colon
cancer tissues (n=12) and adjacent noncancerous tissues (n=12) using qRT-PCR.
**P<0.01 compared to adjacent noncancerous tissues.

Figure 2. miR-1262 affects colon cancer cell phenotypes  (A) miR-1262
overexpression or inhibition was achieved in RKO and HCT116 cells by transfecting
agomir-1262 or antagonmir-1262; miR-1262 overexpression or inhibition was
confirmed using qRT-PCR. RKO and HCT116 cells were subsequently transfected with agomir-1262 or antagonmir-1262 and examined for cell viability by MTT assay (B) and cell invasion by Transwell assay (C), scale bar = 50 μm.; cell migration by wound healing assay (D), scale bar = 100 μm; the protein levels of PCNA, N-cad, E-cad, and vimentin by immunoblotting (E). *P<0.05, **P<0.01 compared to the agomir NC group; #P<0.05, ##P<0.01 compared to the antagonmir NC group.

Figure 3. miR-1262 overexpression inhibits tumor growth in a mouse model
(A) A colon cancer xenograft model was established in male nude mice by injecting lv-miR-1262-transduced HCT116 cells in logarithmic phase. The appearance of nude mice and tumors were photographed and shown. (B) miR-1262 overexpression in tumor tissues was confirmed using qRT-PCR. (C) Tumor volume was examined every 7 days from day 7 to day 28 of modelling. (D) All mice were sacrificed at day 28 of modelling, and the tumor weight was determined. (E) The histopathological characteristics of the tumors were examined by H&E staining (upper; scale bar = 100 μm), and the levels of PCNA in tumor tissues were examined using immunohistochemical (IHC) staining (lower; scale bar = 50 μm). (F) The protein levels of PCNA, N-cad, E-cad, and vimentin were detected by immunoblotting. **P<0.01 compared to the agomir NC group.

Figure 4. miR-1262 overexpression inhibits tumor metastasis in a mouse model
Lv-miR-1262-transduced HCT116 cells were injected into nude mice via the tail vein...
for an *in vivo* metastasis assay. Animals were killed 6 weeks after injection. (A) Images of the lung metastasis model from the different experimental groups. (B) Percentage of metastatic lung foci observed in different experimental groups. **P<0.01 compared to the lv-NC group. (C) Images showing representative H&E staining of lung tissue samples from the different experimental groups. Upper graph: scale bar = 100 μm; lower graph: scale bar = 50 μm.

**Figure 5. FGFR1 is a direct downstream target of miR-1262** (A) A schematic diagram showing the process of selecting the target gene of miR-1262. miR-1261 coexpressed genes were analysed by screening genes significantly associated with miR-1262 using Spearman's correlation analysis. (B-C) HCT116 and RKO cells were transfected with agomir-1262 or antagomir-1262 and examined for the mRNA expression of FGFR1 by qRT‒PCR and FGFR1 protein levels by immunoblotting. **P<0.01 compared to the agomir NC group; ##P<0.01 compared to the antagomir NC group. (D) The expression of FGFR1 was determined in colon cancer tissues and adjacent noncancerous tissues using qRT-PCR. **P<0.01 compared to adjacent noncancerous tissues. (E) The protein levels of FGFR1 in colon cancer tissues and adjacent noncancerous tissues were evaluated using immunohistochemical staining (IHC). Scale bar = 20 μm. (F) The correlation between miR-1262 and FGFR1 expression in tissue samples was analysed using Pearson's correlation analysis. (G) Wild- and mutant-type FGFR1 luciferase reporter vectors were constructed based on psiCheck-2 plasmids. The predicted miR-1262 binding site in FGFR1 was mutated in
a mutant-type FGFR1 reporter. Reporter plasmids were cotransfected into 293T cells
with agomir-1262 or antagonir-1262, and luciferase activity was determined.

**P<0.01 compared to the agomir NC group; ##P<0.01 compared to the antagonir
NC group.

Figure 6. Dynamic effects of the miR-1262/FGFR1 axis  (A) FGFR1
overexpression was achieved in HCT116 and RKO cells by transfecting
lentivirus-overexpressing FGFR1. FGFR1 overexpression was confirmed using
qRT-PCR. **P<0.01 compared to the lv-NC group. (B-F) HCT116 and RKO cells
were subsequently cotransfected with agomir-1262 and lv-FGFR1 and examined for
the protein levels of FGFR1 by immunoblotting (B); cell viability by MTT assay (C);
and cell invasion by Transwell assay (D), scale bar = 50 μm.; cell migration by
wound healing assay (E), scale bar = 100 μm; the protein levels of PCNA, N-cad,
E-cad, and vimentin by immunoblotting (F). **P<0.01 compared to the agomir
NC+lv-NC group; ## P <0.01 compared to the miR-1262 agomir+lv-FGFR1 group.

Figure 7. NF-κB targets the miR-1262 promoter region and inhibits its
expression (A,B) The correlation between miR-1262 and NF-κB expression was
analysed using Spearman's correlation analysis based on data from (A) TCGA-COAD
and (B) collected clinical colon cancer tissue samples. (C) HCT116 and RKO cells
were stimulated with IL-1β (10 ng/mL) or TNF-α (20 ng/mL) for 24 h and examined
for the expression of miR-1262. **P<0.01 compared to the control group. (D) Wild-
or mutant-type miR-1262 promoter-reporter plasmids (miR-1262, miR-1262-mut1,  
miR-1262-mut2) were constructed and cotransfected with pcDNA3.1/NF-κB1;  
luciferase activity was determined. **P<0.01 compared to the  
pro-miR-1262+pcDNA3.1 group. (E-H) HCT116 and RKO cells were transfected  
with antagomir-1262, treated with CAPE (200 μM) or DMSO for 24 h, and examined  
for cell viability by MTT assay (E); cell invasion by Transwell assay (F), scale bar =  
50 μm; cell migration by wound healing assay (G), scale bar = 100 μm; and the  
protein levels of PCNA, N-cad, E-cad, and vimentin by immunoblotting (H).  
**P<0.01 compared to the antagomir NC group; ##P<0.01 compared to the miR-1262  
antagomir +CAPE group.
For Peer Review

A. UCSC database
TCGA-COAD colon cancer

Risk or protective factors for overall survival
(HR > 1 or < 1, p < 0.05)

19 risk factors
24 protective factors

Colon cancer differentially expressed mRNA
(logFC > 0.56 or < -0.56, p < 0.05)

104 risk factors
30 protective factors

GSE160432

3 risk factors
hsa-miR-193a-3p
hsa-miR-493-5p
hsa-miR-424-5p

No literature reports of miRNA

2 protective factors
hsa-miR-1262
hsa-miR-194-3p

B. TCGA-COAD: hsa-miR-1262
Different expressions

C. TCGA-COAD

miR-1262 survival
HR = 0.4505, 95% CI = 0.2691 to 0.7540
p value = 0.0045

D. Relative miR-1262 expression
(Fold change)

E. Adjacent tissue-HE (100 x)

Tumor tissue-HE (100 x)

136x173mm (300 x 300 DPI)
Screening for genes related to miR-1262
Predict the target genes of miR-1262
The most reported factor in the literature to promote cancer in colorectal cancer
miR-1262 agomir
miR-1262 antagomir

B

Relative FGFR1 expression

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C

HCT 116
RKO

D

Relative FGFR1 expression

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Adjacent tissue
Tumor tissue

F

Relative FGFR1 expression

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Position 375-381 of FGFR1 3' UTR

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175x155mm (300 x 300 DPI)
Supplementary Figure S1. Bioinformatics analysis of differentially expressed miRNAs in colon cancer progression (A) The differentially expressed miRNAs based on the TCGA-COAD dataset were analysed by the R language toolkit LIMMA (logFC > 0.56 or < -0.56, P < 0.05) and visualized in a volcano map (left) and heatmap (right). (B) Multivariate Cox risk regression analysis.
of the correlation of miRNAs and prognosis in colorectal cancer cases according to TCGA-COAD
based on the best screening index cut-off (HR>1 or <1, P < 0.01). (C) The differentially expressed
miRNAs in 32 healthy control samples and 20 colon cancer samples were analysed based on the
GSE160432 dataset (logFC > 1 or < -1, P < 0.05) and visualized in a volcano map (left) and heatmap
(right). (D) Two downregulated miRNAs (hsa-miR-1262, hsa-miR-194-3p) and 3 upregulated
miRNAs (hsa-miR-193a-3p, hsa-miR-493-5p, hsa-miR-424-5p) were cross-screened based on
TCGA-COAD and GSE160432 datasets.
Supplementary Figure S2. Effects of miR-1262 on NF-κB nuclear translocation and expression (A) HCT116 and RKO cells were transfected with antagomir-1262, treated with CAPE (200 μM) or DMSO for 24 h, and examined for nuclear NF-κB and cytoplasmic NF-κB using immunoblotting.
### Supplementary Table S1. Clinical Characteristics of the Colorectal cancer patients

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**Supplementary Table S2. The primer sequences for the study**

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Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies with a high incidence and mortality rate. Accumulated evidences indicate that microRNAs (miRNAs) play a crucial role in the progression and metastasis of CRC. Here, we show that miR-1262 serves as an anti-tumor miRNA in CRC by targeting FGFR1 and the NF-κB/miR-1262/FGFR1 axis modulates CRC cells phenotypes, including proliferation, invasion, and migration.

1. miR-1262 is down-regulated in CRC tissues and cell lines.
2. miR-1262 inhibits the capacity of CRC cells in vitro and CRC tumor development in vivo.
3. FGFR1 is a direct downstream target of miR-1262.
4. NF-κB targets the miR-1262 promoter region and inhibits its expression.