

ORIGINAL ARTICLE

MicroRNA-24 regulates the growth and chemosensitivity of the human colorectal cancer cells by targeting RNA-binding protein DND1

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Summary

Purpose: This study was designed to investigate the role and therapeutic potential of miR-24 in colorectal cancer (CRC).

Methods: The CRC cell lines HCT116, RKO, SW480, SW48, and the non-cancer cell line CCD-18Co were used in the present study. The miR-24 expression was determined by qRT-PCR analysis. Cell viability was determined by MTT assay. Apoptosis was examined by acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) staining. Transfection was performed by Lipofectamine 2000. Protein levels were examined by western blot analysis.

Results: miR-24 was significantly downregulated in CRC cell lines. Ectopic expression of miR-24 caused significant decrease in the cell viability by initiating apoptotic cell death of colorectal SW48 cancer cells, indicative of its tumor suppressive role. Moreover, miR-24 overexpression also enhanced the chemosensitivity of SW48 cells to 5-fluorouracil (5-FU).

In silico analysis together with dual luciferase reporter assay indicated the RNA binding protein DND1 was the potential target of miR-24 in SW48 cells. Investigation of DND1 expression in CRC cell lines showed up to 5.3-fold upregulation of DND1. Nonetheless, ectopic expression of miR-24 in SW48 cells resulted in the downregulation of DND1 expression. Additionally, silencing of DND1 in the SW48 cells also caused inhibition of SW48 cell proliferation. Moreover, overexpression of DND1 could rescue the tumor suppressive effects of miR-24, indicating direct involvement of DND1 in the miR-24 mediated inhibitory effects on SW48 cell proliferation.

Conclusion: The miR-24 acts as a tumor suppressor and may prove essential in the treatment of CRC.

Key words: colorectal cancer, apoptosis, microRNA, proliferation, chemosensitivity

Introduction

Being the fourth leading cause of cancer related mortality, colorectal cancer (CRC) is one of the common types of cancers. It ranks the third common type of cancer and around 1.4 million new cases of CRC are reported each year [1]. Approximately, 0.7 million deaths were reported to be due to CRC across the globe in 2013 alone [2]. The incidence of CRC has shown a decline in the recent past to some extent but it is believed the incidence

of CRC will increase by 60% till 2030 [3]. Late diagnosis and lack of potent and safe chemotherapeutic drugs, unavailability of potent therapeutic targets form an obstacle in the treatment this disease [4].

MicroRNAs (miRs) include around 23 nucleotides long RNA molecules that regulate the expression of a number of genes in human and other organisms by binding to the mRNAs to enforce their post transcriptional repression [5]. Since, miRNAs

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modulate the expression of about 30% of the human protein coding genes, they are involved in different cellular and physiological processes such as cell cycle, proliferation and apoptosis to name a few [6]. There is sound evidence indicating that several miRs are aberrantly expressed in cancer cells and are considered to be prospective therapeutic targets/agents for the management of cancer [7]. Amongst all, miR-24 has been shown to be involved in the proliferation and metastasis of a number of cancers. For example miR-24 has been reported to inhibit the growth and metastasis of bladder cancer cells by modulating the expression of FOXM1 [8]. In another study, miR-24 has been reported to reduce the apoptosis threshold in cancer cells [9]. Similarly, miR-24 has been shown to regulate the growth of tongue squamous cell carcinoma by modulating the expression RNA binding protein, DND1 [10]. Nonetheless, the therapeutic potential of miR-24 in CRC is yet to be investigated thoroughly.

Herein, we report for first time the role of miR-24 as tumor suppressor in CRC. Herein, we report for the first time the role of miR-24 as tumor suppressor in CRC and we strongly believe that miR-24 may prove essential therapeutic target for CRC.

Methods

Colorectal cancer cell lines HCT116, RKO, SW480, SW48 and non-cancer cell line CCD-18Co were procured from American Type Culture Collection. All of these cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and 2 mM glutamine. The cells were cultured in incubator (Thermo Scientific, Waltham, Massachusetts, USA) at 37°C with 98% humidity and 5% CO₂. The miR-24 mimics, negative control (NC), si-DND1 and pcDNA-DND1 were procured from GenePharm (Shanghai, China). All transfection assays were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) as per the manufacturer's instructions.

cDNA synthesis and quantitative RT-PCR

Total RNA was extracted from all the normal and CRC cell lines by using TRIzol reagent (Invitrogen). Thereafter, reverse transcription and quantitative real time PCR (qRT-PCR) were performed using PrimeScript RT reagent Kit (TaKaRa, Dalian, China) and SYBR Premix Ex TaqII (Tli RNase H Plus) kit (TaKaRa), respectively. Finally the relative expression was determined by 2^{-ΔΔCT} method and actin was used for normalization.

MTT cell viability assay

Because SW48 cells showed the lowest expression of miR-24, these cells were used for further experimentation. The SW48 cells were transfected with miR-24 mimics, seeded in 96-well plates and incubated for 24 h

at 37°C in Dulbecco's modified Eagle's medium (DMEM). Following incubation, the cells were further incubated with MTT for another 4 h. After this, DMEM was removed and the colored formazan product was solubilized by 200 µl of dimethyl sulfoxide (DMSO). The viability of the transfected SW48 cells was then determined by taking the absorbance at 570 nm using spectrophotometer.

Apoptosis assay

The transfected CRC SW48 cells (0.6×10⁶) were seeded in 6-well plates and incubated for 12 h. Then, these cells were subjected to incubation for another 24 h at 37°C. As the cells were removed 10 µl of cell culture were put onto glass slides and stained with 0.5 µl solution of acridine orange (AO) and ethidium bromide (EB). The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining of the SW48 cells was performed as described previously [11].

Western blotting

The SW48 cells were firstly washed with ice-cold phosphate buffered saline (PBS) and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and exposed to primary antibody (anti-DND1) at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

SPSS software package v.9.05 (SPSS Inc.Chicago, IL, USA) was used for statistical analyses. Analyses were performed using Student's *t*-test with GraphPad prism 7 software. P value<0.05 was indicative of significant difference.

Results

miR-24 is downregulated in CRC cell lines

The miR-24 expression was examined in normal and CRC cell lines by qRT-PCR (Figure 1A). The results showed that miR-24 was significantly downregulated (p<0.05) in all the CRC cell lines. Furthermore, miR-24 was found to be downregulated in CRC lines up to 9-fold relative to the normal cell line CCD-18Co line. The lowest expression was observed in case of the SW48 cell line.

miR-24 suppresses the proliferation rate of CRC via induction of apoptosis

Following expression analysis, we sought know about the role of miR-24 in CRC. Consequently, the SW48 cells were transfected with either the miR-24 mimics or NC (negative control). The ectopic

expression of miR-24 in SW48 cells was validated by the qRT-PCR which showed that transfection of the SW48 cells with miR-24 mimics caused around 5.2-fold enhancement in the expression of miR-24 relative to NC transfected cells (Figure 1B). The proliferation rate of the NC and miR-24 mimics SW48 transfected cells was monitored at different time intervals by MTT assay. The results showed that the transfection of miR-24 mimics in the SW48 cells caused significant decline in the proliferation rate (Figure 1C). AO/EB staining of the NC and miR-24 transfected SW48 cells was performed to unveil the underlying mechanism and it was observed that miR-24 mimics overexpression led to activation of apoptotic cell death of the SW48 cells (Figure 2A). Additionally, annexin V/PI staining clearly showed that the apoptotic cell percent increased from 2.45% in NC transfected cells to about 28.99% in the miR-24 mimics transfected SW48 cells (Figure 2B). These results unequivocally indicate that miR-24 suppresses the SW48 cell proliferation by prompting apoptosis.

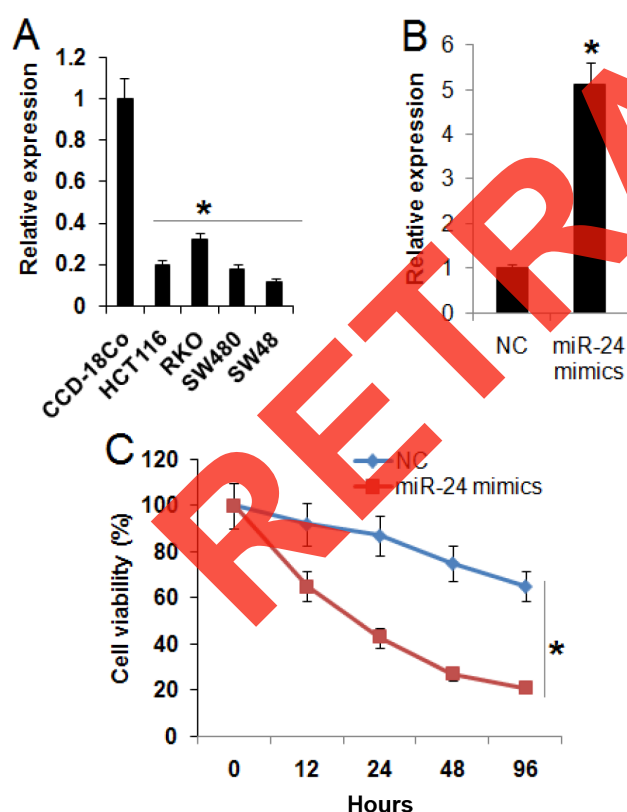


Figure 1. Overexpression of miR-24 inhibits the proliferation of colorectal cancer cells. **A:** Expression of miR-24 in normal and four different colorectal cancer cell lines. **B:** Expression of miR-24 in NC or miR-24 mimics transfected SW48 colorectal cancer cells. **C:** MTT assay showing the percent viability of NC or miR-24 mimics transfected SW48 colorectal cancer cells at the indicated time intervals. The experiments were performed in triplicate and expressed as mean \pm SD (* p <0.05).

miR-24 enhances the chemosensitivity of CRC cells to 5-fluorouracil

Next, we sought to know the effects of miR-24 overexpression on the chemosensitivity of the SW48 CRC cells. Henceforth, the NC or miR-24 transfected SW48 cells were treated with 2 μ M 5-fluorouracil (5-FU) and the cell viability was

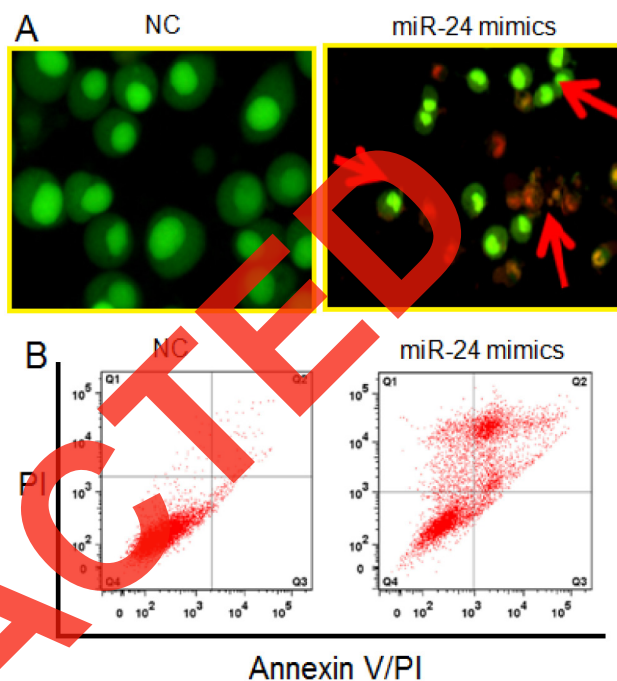


Figure 2. Overexpression of miR-24 triggers apoptosis in colorectal cancer cells. **A:** AO/EB staining showing induction of apoptosis in NC or miR-24 mimics transfected SW48 colorectal cancer cells (arrows show apoptotic cells). **B:** Annexin V/PI staining showing the apoptosis in NC or miR-24 mimics transfected SW48 colorectal cancer cells. The experiments were performed in triplicate.

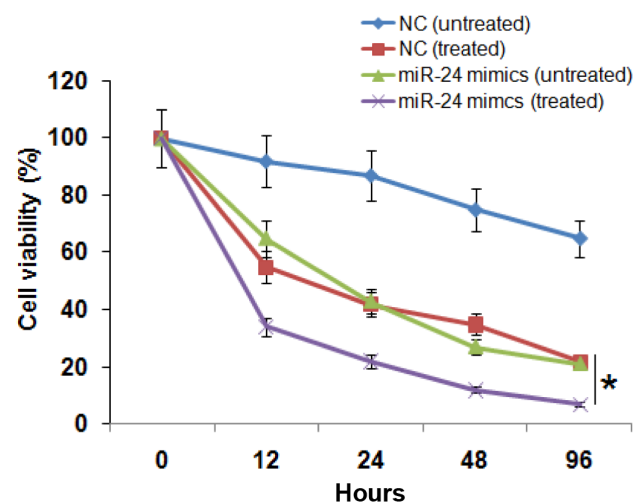


Figure 3. MiR-24 enhances the chemosensitivity of the SW48 colorectal cancer cells against the anticancer drug 5-FU. The experiments were performed in triplicate and expressed as mean \pm SD (* p <0.05).

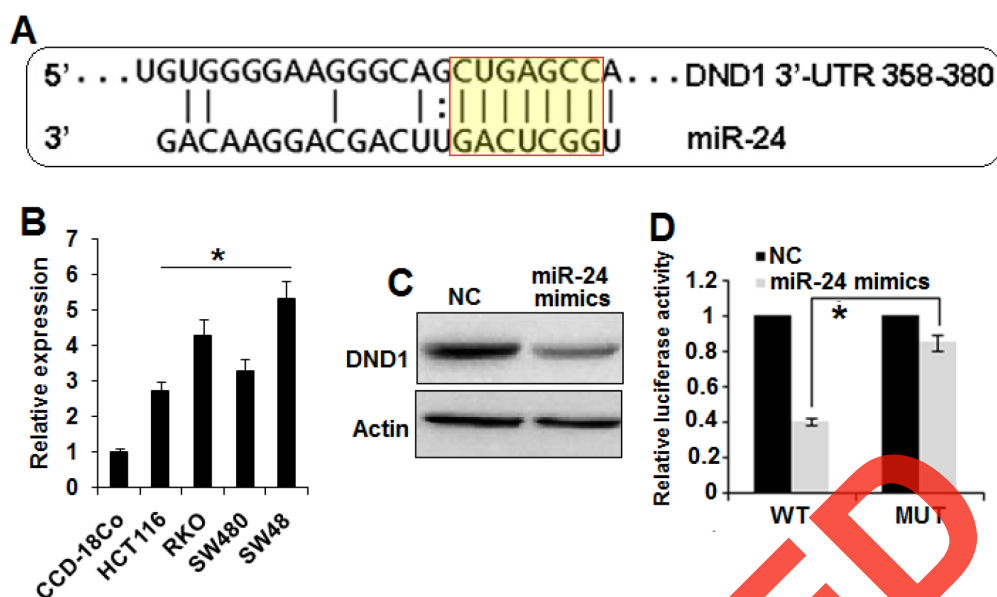


Figure 4. miR-24 exerts its effects by targeting DND1. **A:** TargetScan analysis showing DND1 as the target of miR-24. **B:** Expression of DND1 in normal CCD-18Co and four different colorectal cancer cell lines. **C:** Western blot analysis showing the expression of DND1 in NC or miR-24 transfected colorectal cancer cells. **D:** Dual luciferase reporter assay. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

monitored at different time intervals by MTT assay. It was found that the viability of the miR-24 mimics transfected plus 5-FU treated SW48 cells decreased significantly as compared to the NC (untreated), NC (treated) and miR-mimics transfected (untreated) SW48 cells (Figure 3). Taken together, these results indicate miR-24 overexpression increased the sensitivity of the SW48 cells to the anticancer drug 5-FU.

miR-24 targets DND1 in CRC

The target of miR-24 in CRC cells was identified by online TargetScan analysis. The RNA binding protein DND1 was identified as the potential target of miR-24 in SW48 CRC cells (Figure 4A) and therefore the expression levels of DND1 were investigated in all the CRC lines as well as the normal cell line. It was noted that relative to the expression of DND1 was significantly upregulated (up to 5.3-fold) in the CRC cell lines (Figure 4B). However, as the SW48 cells were transfected with the miR-24 mimics, the expression of DND1 was considerably downregulated as depicted by the western blot analysis (Figure 4C). The dual luciferase assay further confirmed DND1 to be the target of miR-24 (Figure 4D).

DND1 is essential for tumor suppressive effects of miR-24 in SW48 cells

The effects of the DND1 silencing on the proliferation rate of the CRC SW48 cells was also investigated. It was found that the silencing of DND1

expression (Figure 5A) caused significant decline ($p < 0.05$) in the viability of the SW48 CRC cells (Figure 5B).

Since miR-24 overexpression and DND1 silencing exhibited similar effects on the proliferation of the SW48 cells, we sought to know if DND1 overexpression could rescue the effects of miR-24 overexpression in SW48 cells. Interestingly, it was found that DND1 overexpression in the miR-24 mimics transfected SW48 cells, promoted the proliferation of the SW48 cells, indicating that the inhibitory effects of the miR-24 overexpression are directly due to DND1 suppression (Figure 5C).

Discussion

Colorectal cancer is a destructive malignancy and its frequency is expected to increase dramatically in the coming years [12]. The clinical outcome is unsatisfactory and treatment strategies have a number of flaws. The currently available chemotherapeutic agents create considerable adverse effects to the patients. Besides, the emergence of chemoresistance in cancer cells further makes it difficult to treat CRC [3]. Therefore, there is urgency to explore new drugs or to identify the effective therapeutic targets. miRs control the expression of the majority of human genes and are involved in a wide array of cellular processes [13]. Because of the importance of miRs in cellular and physiological processes, several studies have revealed their potential as therapeutic targets [14]. Herein,

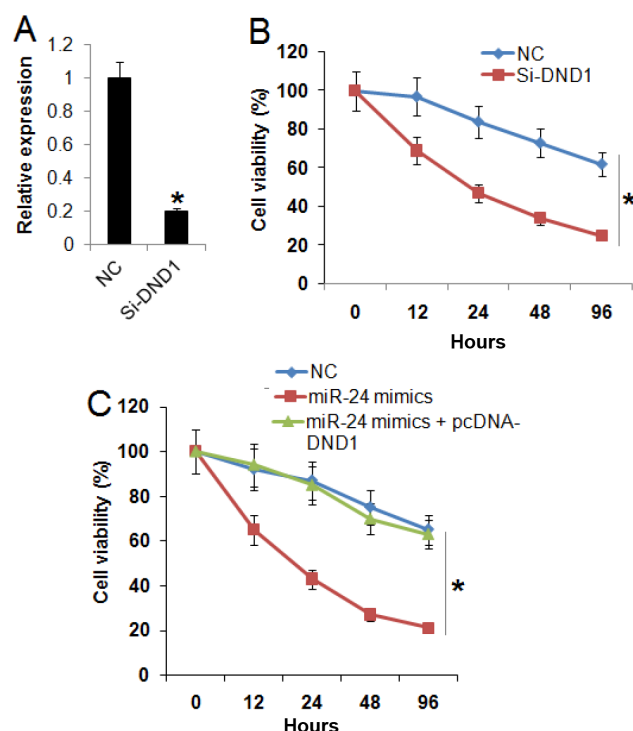


Figure 5. DND1 overexpression rescues the inhibitory effects of miR-24 overexpression on SW48 cells. **A:** Expression of DND1 in Si-NC or Si-DND1 transfected SW48 cells. **B:** MTT assay showing the viability of the Si-NC or Si-DND1 transfected SW48 cells. **C:** Effect of DND1 overexpression on the inhibitory effects of miR-24 overexpression on the proliferation of the SW48 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

the role and therapeutic potential of miR-24 was investigated in CRC and it was noted that it was aberrantly downregulated in the CRC cells. The miR-24 has also been shown to be a biomarker for CRC in a previous investigation [15]. In addition, miR-24 has been shown to be significantly downregulated in bladder cancer [16]. Overexpression of miR-24 caused significant reduction in the proliferation rate of the SW48 CRC cells via induction of apo-

ptotic cell death. An earlier study has also shown that miR-24 suppresses the proliferation of lung cancer cells by targeting PKC- α [17]. However, some studies have shown that miR-24 promotes the proliferation of lung cancer cells by targeting NAIF1 [18]. The present study also indicated that miR-24 enhances the chemosensitivity of the CRC SW48 cells to the anticancer drug 5-FU. This was also supported by an investigation carried out earlier wherein miR-24 was shown to regulate the cisplatin resistance of the tongue squamous cell carcinoma [19]. *In silico* analysis together with dual luciferase indicated DND1 to be the potential target of miR-24. The RNA binding protein DND1 was reported to regulate the activity of miRs by binding to the sequences on the 3'UTR of the targeted mRNAs [20]. Studies have shown that DND1 is imperative for the survival of germ cells in zebrafish [21], and regulates the development of testicular germ cell tumors in mice [22]. Herein, we observed that DND1 is highly upregulated in CRC and miR-24 overexpression could suppress the expression of DND1. Additionally, DND1 silencing could also inhibit the growth of SW48 CRC cells similar to that of miR-24 overexpression. Furthermore, DND1 inhibition was found to be essential for the tumor suppressive effects of miR-24 on CRC cells.

Conclusion

Taken together, miR-24 is downregulated in human CRC cells. It inhibits their proliferation by inducing apoptosis and enhances their chemosensitivity to 5-FU. Henceforth, miR-24 acts a tumor suppressor in CRC and may prove as an essential therapeutic target for CRC.

Conflict of interests

The authors declare no conflict of interests.

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