microRNA-365, down-regulated in colon cancer, inhibits cell cycle progression and promotes apoptosis of colon cancer cells by probably targeting Cyclin D1 and Bcl-2

Jing Nie1, Lin Liu1, Wei Zheng1, Lin Chen1, Xin Wu1, Yingxin Xu1, Xiaohui Du1 and Weidong Han* 1Department of Molecular Biology and 2Department of General Surgery, PLA General Hospital, 28 Fuxing Road, Beijing 100853, China

Abbreviations: 5-FU, 5-fluorouracil; miRNAs, microRNAs; NC, negative control; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; 3' UTR, 3' untranslated region.

These authors contributed equally to this work.

Introduction

Colorectal cancer is the third leading cause of cancer-related deaths worldwide (1). Its overall incidence is ~5% and the 5 years survival rate ranges from 40 to 60% (2). Its high incidence and poor prognosis mean that the mechanisms of colon cancer carcinogenesis and progression have been the subjects of much attention in recent years, and several factors have been identified as playing critical roles in its pathogenesis. Oncogenic activation of intracellular signaling pathways, such as epidermal growth factor receptor, Wnt, transforming growth factor-β and phosphoinositide 3-kinase pathways, have been shown to play substantial roles in maintaining the growth and progression of colon cancer cells (3). However, the detailed genetic and epigenetic abnormalities in colon carcinogenesis and cancer progression are still not completely understood and further extensive investigations are required.

MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs that inhibit gene expression through the 3' untranslated region (3' UTR) of their target messenger RNAs (4). Due to the wide control of gene expression, miRNAs play crucial roles in numerous biological processes, including cell growth, apoptosis, metabolism and transformation (5–7). Deregulation of miRNAs may thus contribute to carcinogenesis and cancer progression (8). Research has revealed that several miRNAs are up- or downregulated in colon cancer and may participate in the pathogenesis of colon cancer through controlling the expression of key signaling molecules. Increasing evidence has demonstrated that miRNAs act as tumor suppressors or oncogenes, suggesting their considerable potential as new therapeutic targets (9,10). However, elucidating the roles of miRNAs in cancer biology, especially in colon cancer, remains an ongoing process.

Although little is known about the function of miR-365, its levels in NIH3T3 cells were shown to be significantly increased by UVB irradiation, exerting some tumor-suppressive effect while the detailed mechanism is unknown (11). In addition, miR-365 was reported to be downregulated in growth arrest states of quiescence and senescence in lung fibroblast cells, indicating a possible role for miR-365 in regulating cell proliferation (12). On the other side, miR-365 was upregulated in breast cancer (13) and endometriosis (14) and downregulated when the potential tumor suppressor NGX6 was overexpressed in HT-29 cells (15), all indicating that miR-365 may act as an oncogene. Therefore, the function of miR-365 is complicated, displaying either pro-proliferative or pro-apoptotic roles under specific physiological conditions and in different types of cancers.

In this study, we explored the potential role of miR-365 in colon cancer development. miR-365 expression was examined in clinically resected human colon cancer tissues, and the correlation between miR-365 deregulation and colon cancer progression and prognosis was analyzed. Furthermore, the mechanisms underlying the role of miR-365 in colon cancer development were investigated. The results indicate that miR-365 plays an important role in cell cycle progression and apoptosis control in colon cancer. This suggests that miR-365 may have potential prognosis prediction and therapeutic application in colon cancer patients.

Materials and methods

Patients and tissue samples

Surgically removed paired colon cancer tissues and adjacent normal mucosa tissues were collected from 97 colon cancer patients at the General Hospital of PLA (Beijing, China) and were used for quantitative reverse transcription–polymerase chain reaction (qRT–PCR) and western blot analysis. Detailed information on the patients is shown in Supplementary Table 1, available at Carcinogenesis Online. Surgically resected tissues were quickly frozen in liquid nitrogen until analysis. All samples were obtained with the informed consent of the patients and the experiments were approved by the ethics committee of General Hospital of PLA, Beijing, China.

RNA extraction and qRT–PCR

Total RNA, including miRNA, was isolated using TRIzol reagent according to the manufacturer’s instruction. Real-time qRT–PCR was carried out as described previously (16). For miRNA analysis, the stem-loop RT primer for miR-365 was 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCTTGTGCCTGAAATCCAGATUTTGGGAUUAUT-3', and the qRT-PCR primers were 5'-CGTAATGCCCCCTAATAATAT-3' and 5'-GTGCAAGGTTCGAGGT-3'. In-cell control U6 primers for qRT–PCR were 5'-CTCGCTTCGGCAGCACAT-3' and 5'-AACGCTTCGAAATTTCGCT-3'. The relative expression level of miR-365 was normalized to that of the internal control U6 by using the 2−ΔΔCt cycle threshold method (17).

Cell culture and transfection

The human colon cancer cell lines HT29, LoVo, SW480 and SW620 were obtained from the American Type Culture Collection. HT29 and LoVo cells were cultured, seeded and transfected as described previously (18,19). Negative control (NC) RNA and miR-365 mimics were synthesized by GenePharma (Shanghai, China). siRNAs targeting human Cyclin D1 were 5'-GUUCUAUCUCAGAUCUUCU-3' and 5'-GGGGCAUGAUGGAAUGAAU-3'; siRNA targeting human Bcl-2 were 5'-CCCGGGAUAGUGAAUGAAU-3' and 5'-CUUCAUCACUAUCCCGGCU-3'.

Analysis of cell viability

The in vitro cell viabilities of HT29 and LoVo cells were assessed using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, as described previously (10). Briefly, spent medium was replaced with fresh medium containing 0.5 mg/ml MTT at the indicated time periods. Cells were incubated for 4 h and the absorbance was measured at 570 nm using a microtiter plate reader.

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were then incubated at 37°C for 1 h and resolved by dimethylsulfoxide (Sigma). The absorbance was measured at 570 nm.

Detection of apoptosis
HT29 and LoVo cells were transfected with NC or miR-365, respectively. At 48 h post-transfection, spent cell culture medium was replaced with fresh medium containing 5 μM 5-fluorouracil (5-FU) for the indicated time course. Cells were harvested, washed, resuspended in staining buffer and examined using an Annexin V FITC Apoptosis Detection Kit (Calbiochem). Stained cells were detected by FACSCalibur and data were analyzed with CellQuest software (Becton Dickinson). Annexin V-positive cells were regarded as apoptotic cells.

Cell cycle analysis
The cell cycle was analyzed by flow cytometry. HT29 and LoVo cells were trypsinized, fixed in 70% ethanol, washed and incubated in phosphate-buffered saline containing propidium iodide and RNase A (Sigma) for 30 min at 37°C. Samples were then analyzed for their DNA content by FACSCalibur.

Tumorigenicity assay in nude mice
All experiments involving animals were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the General Hospital of PLA, Beijing, China. The tumorigenicity assay was performed as reported previously (19). NC or miR-365 mimic-transfected HT29 cells (1 × 10⁵) were suspended in 0.1 ml phosphate-buffered saline and then injected subcutaneously into either side of the posterior flank of the same female BALB/c athymic nude mice at 4 weeks old. Tumor growth was measured daily using calipers, and tumor volume was calculated according to the formula: volume = length × width² × 0.5.

3' UTR luciferase reporter assay
The human B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) 3' UTR luciferase reporter plasmid, plasmid containing miR-365 target site deleted Bcl-2 3' UTR, human Cyclin D1 3' UTR luciferase reporter plasmid and plasmid containing miR-365 target site deleted Cyclin D1 3' UTR were constructed as described previously (19). All constructs were confirmed by DNA sequencing. Luciferase reporter assays were performed, as reported previously (19). Briefly, luciferase activities were measured at 48 h post transfection using a Dual-Luciferase Reporter Assay System (Promega), following the manufacture’s instructions. Data were normalized by dividing firefly luciferase activity by that of Renilla luciferase.

Western blotting
The lysed protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and then blotted, as reported previously (16). Antibodies specific to Cyclin D1 (2978), Bcl-2 (2870) and β-actin (4970) were purchased from Cell Signaling Technology. Densitometric analysis was performed using Labworks Image Acquisition and Analysis Software (UVP, Upland, CA). The background was subtracted, and the signals of the detected bands were normalized to the amount of loading control β-actin band.

Statistical analysis
Data are shown as mean ± SD. Statistical comparisons between experimental groups were analyzed by Student’s t-tests, and a two-tailed P value <0.05 was considered to be significant. Correlation between miR-365 expression and colon cancer stages was analyzed using Spearman’s rank correlation coefficient analysis with SPSS 16.0 with r and P values as indicated. Disease-free survival of colon cancer patients was analyzed using log-rank tests with SPSS 16.0 with P values as indicated. Correlation between miR-365 expression and Cyclin D1 or Bcl-2 protein levels were analyzed using Pearson’s correlation coefficient analysis with SPSS 16.0 with r and P values as indicated.

Results

miR-365 is downregulated in colon cancer
In order to gain an insight into the roles of miR-365 in human colon cancer development, we collected 97 pairs of human colon cancer tissues and adjacent normal mucosa tissues. According to qRT–PCR analysis, miR-365 expression was significantly decreased in 61 of 97 (63%) tumor samples, compared with the adjacent normal mucosa tissues (Figure 1A). Moreover, miR-365 expression was also decreased in the colon cancer cell lines HT29, LoVo, SW480 and SW620, compared with normal colon mucosa tissues (Figure 1B). These results suggest that miR-365 is downregulated in colon cancer cells that might be relevant to human colon cancer development.

Downregulated miR-365 correlates with cancer progression and poor survival in colon cancer patients
We investigated the correlation between downregulated miR-365 and cancer progression and the survival in colon cancer patients. Spearman’s rank correlation analysis identified a significant correlation between miR-365 expression in cancer tissues and colon cancer progression from stages I to IV in the 97 samples tested (Figure 2A), suggesting that decreased levels of miR-365 were related to colon cancer progression. Furthermore, Kaplan–Meier analysis revealed that low miR-365 levels in cancer tissues were significantly correlated with the reduced disease-free survival in the 76 colon cancer patients who underwent radical resection (Figure 2B). These results indicate important roles for miR-365 in colon cancer progression and prognosis.

miR-365 inhibits cell cycle progression and promotes apoptosis in colon cancer cell lines
We investigated the biological function of miR-365 in colon cancer. MTT assays showed that restoration of miR-365 reduced cell viability in the colon cancer cell lines HT29 and LoVo (Figure 3A), as well as resulting in significant accumulation of the G1 population in these cell lines (Figure 3B). This suggests that miR-365 could inhibit cell cycle progression. miR-365 restoration also promoted 5-FU-induced cell apoptosis in colon cancer cell lines (Figure 3C), further indicating its antitumor effect. These observations demonstrate that miR-365 can inhibit colon cancer cell cycle progression and promote cell apoptosis in vitro, suggesting that miR-365 may function as a tumor suppressor in colon cancer.
miR-365 suppresses tumorigenicity in vivo
To examine the potential role of miR-365 in tumorigenesis, we evaluated the effect of miR-365 restoration on the tumorigenicity and growth of colon cancer cells. Notably, injection of miR-365 mimic-transfected colon cancer cell line HT29 resulted in delayed tumor formation and a dramatic reduction in tumor size, compared with the injection of NC transfectants (Figure 4). This result demonstrates that miR-365 inhibits the tumorigenicity of colon cancer cells in vivo, further suggesting a tumor-suppressive effect.

miR-365 targets Cyclin D1 and Bcl-2
Based on the fact that miRNAs function mainly through the inhibition of their target genes via binding to the 3’ UTR, we identified the targets of miR-365 to obtain insights into the molecular basis of its tumor suppressive effect in colon cancer development. Hundreds of predicted miR-365 target genes were retrieved from the TargetScan database (http://www.targetscan.org), and enrichment of the predicted targets was analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/). Interestingly, the KEGG pathway ‘colorectal cancer’ noted both Bcl-2 and Cyclin D1 as potential targets of miR-365 (Supplementary Figure S1 is available at Carcinogenesis Online).

Cyclin D1 is accepted as an important proliferation-promoting molecule, with a conserved putative miR-365 target site. Bcl-2, a fundamental anti-apoptotic gene with a recognized role in cancer development, contains two conserved putative miR-365 target sites by TargetScan prediction (Figure 5A). Previous studies determined that both Cyclin D1 and Bcl-2 were upregulated in colon cancer and played important roles in cancer development (19–22), and we therefore suspected that miR-365 might exert its antitumor effect through targeting Cyclin D1 and Bcl-2. To confirm Cyclin D1 and Bcl-2 as direct targets of miR-365, we constructed luciferase reporter plasmids containing Cyclin D1 or Bcl-2 3’ UTR sequence or with deleted putative miR-365 target sites. As shown in Figure 5B, co-transfection of miR-365 suppressed the luciferase activity of the reporters containing wild-type Cyclin D1 or Bcl-2 3’ UTR sequence, but failed to inhibit that of the target site-deleted construct by dual-luciferase reporter assay. These results suggest that miR-365 can directly target the 3’ UTR sequences of both Cyclin D1 and Bcl-2.

The influence of miR-365 on the endogenous expression of Cyclin D1 and Bcl-2 proteins was examined. Cyclin D1 and Bcl-2 expression were significantly decreased in miR-365-transfected colon cancer HT29 and LoVo cells, compared with control transfectants (Figure 5C), further demonstrating that cyclin D1 and Bcl-2 are direct targets of miR-365. Correlation between miR-365 levels and the Cyclin D1 or Bcl-2 expression was examined in colon cancer tissues. By qRT–PCR and western blot detection shown in Figure 6, Pearson’s correlation coefficient analysis suggested that Cyclin D1 and Bcl-2 expression were both inversely correlated with miR-365 expression.
in colon cancer tissues. These data support the idea that miR-365 downregulation may contribute to the overexpression of Cyclin D1 and Bcl-2 in colon cancer.

Antitumor effect of miR-365 may be mediated through targeting Cyclin D1 and Bcl-2

To ascertain the roles of Cyclin D1 and Bcl-2 in miR-365-regulated cell cycle progression and apoptosis, we determined the knockdown of endogenous Cyclin D1 or Bcl-2 was able to mimic the effect of miR-365 restoration. As Cyclin D1 was known to promote cell cycle progression, we confirmed that Cyclin D1 knockdown inhibited cell cycle progression in colon cancer cell lines, possibly by be G₁-phase cell cycle arrest (Figure 7A). Knockdown of anti-apoptotic Bcl-2 in colon cancer cells also increased apoptosis upon 5-FU treatment (Figure 7B). These data suggest that the antitumor effects of miR-365 may be mediated by inhibition of its target genes, Cyclin D1 and Bcl-2.

Discussion

Current clinical treatments for colon cancer are relatively ineffective, and colon cancer thus remains one of the most common causes of cancer-related deaths. Investigations into the molecular mechanisms responsible for colon carcinogenesis and progression, especially the identification and exploration of miRNAs deregulated in colon cancer development, represent a popular and promising field of study. Several deregulated miRNAs, such as upregulated miR-21 and miR-135 and downregulated miR-143, miR-195 and let-7 members, have been reported and remarked in colon cancer development (19,23–26). However, there may be many other deregulated miRNAs with important roles in the process of colon tumorigenesis and progression.

The present study determined that miR-365 was downregulated in colon cancer tissues and cell lines, and that this downregulation was correlated with cancer progression and prognosis. However, the underlying mechanisms mediating miR-365 downregulation in colon cancer development are still elusive till now. Deregulated genetic factors, epigenetic factors, signaling pathways, transcriptional factors or maybe the combination of them may participate in the deregulation of miR-365 in colon cancer. We will focus on this issue in our future works. Restoration of miR-365 in colon cancer cell lines reduced cell viability, inhibited cell cycle progression, promoted cell apoptosis and suppressed tumorigenicity, suggesting a tumor suppressor activity. And Cyclin D1 and Bcl-2 were shown to be direct targets of miR-365 and their inhibition may account for the antitumor effect of miR-365. Here, we cannot exclude the possibility that other potential targets of miR-365 may govern additional cancer pathways and promote colon cancer development as a single miRNA is known to target multiple messenger RNAs (4). This presumption indicates the need for further studies to reveal the entire ‘targetome’ of miR-365 in colon carcinogenesis and progression.
of cell cycle progression and promotion of apoptosis in colon cancer. Together with the presumption that miR-365 may have multiple target genes, these different target genes may exert various functions of miR-365 in these biological processes, raising important questions for future research.

The recent identification of the molecular markers correlated with the prognosis of cancer patients has attracted much attention. The present study demonstrated that miR-365 expression was frequently decreased in colon cancer tissues and that its downregulation was significantly correlated with the disease-free survival of colon cancer patients, thus indicating its use as a potential marker for prognosis prediction in these patients. The combination of 5-FU and other new chemotherapeutic agents represents an important therapeutic approach for treating colon cancer patients (27), and methods of enhancing chemotherapy sensitivity and minimizing toxic side effects have become increasingly important. The current results showed that miR-365 could promote 5-FU-induced apoptosis in colon cancer cells, indicating that it might have considerable potential in enhancing the therapeutic effects of 5-FU treatment. Restoration of miR-365 expression in colon cancer cells may thus provide a new strategy for overcoming cancer cell resistance to chemotherapeutic drugs (28).

Supplementary material

Supplementary Table 1 and Figure S1 can be found at http://carcin.oxfordjournals.org/.

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References


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