microRNA-195 promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells

Lin Liu, Lin Chen, Yingxin Xu, Rong Li, Xiaohui Du *

Department of General Surgery, General Hospital of PLA, Beijing 100853, China

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ABSTRACT

Deregulated microRNAs and their roles in cancer development have attracted much attention. In the present study, we analyzed the roles of miR-195 in colorectal cancer pathogenesis, as its participation in some other types of cancer has been suggested by previous reports. By comparing miR-195 expression in 81 human colorectal cancer tissues and matched non-neoplastic mucosa tissues, we found that miR-195 was downregulated in cancer tissues. And restoration of miR-195 in colorectal cancer cell lines HT29 and LoVo could reduce cell viability, promote cell apoptosis and suppress tumorigenicity. Moreover, important antiapoptotic Bcl-2 was identified to be directly targeted by miR-195, and miR-195 was further suggested to exert its proapoptotic function mainly through targeting Bcl-2 expression. Taken together, our study provides important roles of miR-195 in colorectal cancer pathogenesis and implicates its potential application in cancer therapy.

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1. Introduction

microRNAs (miRNAs), an abundant class of 17–25 nucleotides small noncoding RNAs, posttranscriptionally regulate gene expression through directly binding to the 3′ untranslated region (3′UTR) of target mRNAs. Till now, about 1000 miRNA genes have been identified in mammals [1], but revealing their roles in physiology and pathology is still an ongoing process. Recently, miRNAs have been suggested to participate in the regulation of diverse biological processes [2], and their deregulation or dysfunction plays important roles in cancer carcinogenesis and progression [3,4]. However, deregulated miRNAs and their roles in cancer development remain largely illusive.

Colorectal cancer (CRC) is one of the most frequent cancers and a common cause of cancer-related death. Colorectal cancer development involves a multi-step process including both genetic and epigenetic changes, which leads to activation of oncogenes and inactivation of tumor suppressor genes in cancer cells [5]. Recently, emerging evidence has suggested that deregulated miRNAs are involved in the pathogenesis of CRC mainly by regulating the expression of oncogenes and tumor suppressors [6]. Deregulation of miRNAs in CRC can participate in cancer development if their target mRNAs are encoded by oncogenes or tumor suppressors. Both upregulation and downregulation of specific miRNAs have been described in CRC carcinogenesis and progression, such as upregulated miR-135, miR-21, miR-17–92, and miR-196a; and downregulated miR-101, miR-34, and miR-200 [7–13]. However, as only a few miRNAs were reported to be involved in CRC, we are still at the beginning of revealing the roles of deregulated miRNAs in CRC development.

Recently, miR-195 has been reported to be deregulated in some types of cancer, including upregulation in chronic lymphocytic leukemia and breast cancer but downregulation in hepatocellular carcinoma, adrenocortical carcinoma, and squamous cell carcinoma of tongue [14–19]. As miR-195 was suggested to be differentially deregulated in these malignancies, the roles of miR-195 in cancer development may be different in different types of cancer. However, whether miR-195 is deregulated in CRC and its roles in CRC carcinogenesis and progression are still elusive.

In the present study, we focused on the expression and roles of miR-195 in CRC development and found that miR-195 was downregulated in surgically removed human CRC tissues as well as in CRC cell lines. And miR-195 was further identified to function as a tumor suppressor in CRC, as restoration of miR-195 expression in CRC cell lines could reduce cell viability and promote cell apoptosis in vitro, and suppress tumorigenicity in vivo. Additionally, Bcl-2, an important antiapoptotic molecule, was proved to be directly targeted by miR-195, and the proapoptotic function of miR-195 is further suggested to be mainly through targeting Bcl-2 expression. Thus, our data suggest the important roles of miR-195 in CRC pathogenesis and implicate its potential application in cancer therapy.

2. Materials and methods

2.1. Patients and tumor samples

Surgically removed CRC tumor tissues and matched non-neoplastic mucosa tissues used for qRT-PCR and Western blot were

* Corresponding author. Address: Department of Surgery, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, China. Fax: +86 10 66938126.
E-mail address: geoffrey301@126.com (X. Du).
obtained from 81 CRC patients at General Hospital of PLA (Beijing, China), and the detailed information of the patients were shown in Supplementary Table 1. Human tissues were quickly frozen in liquid nitrogen after resection. All clinical samples were obtained with the informed consent of the patients and the investigation was approved by the ethics committee of General Hospital of PLA, Beijing, China.

2.2. RNA extraction and real-time quantitative RT-PCR

Total RNA, including miRNA, was isolated using TRIzol reagent according to the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) analysis was performed as we described previously [20,21]. For miR-195 analysis, stem-loop RT primer was 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG-3'; and PCR primers were 5'-CGT AGC AGC ACA GAA AT-3' and 5'-GTG CGT CCG AGG T-3' [22]. PCR primers for internal control U6 were 5'-CTC GCT TCG GCA GCA CA-3' and 5'-AAC GCT TCA CTA ATT TGC GT-3'. The relative expression level of miR-195 was normalized to that of U6 by 2^(-ΔΔCt) cycle threshold method [23].

2.3. Cell culture

Human CRC cell lines HT29 and LoVo were purchased from American Type Culture Collection (ATCC) and cultured as we described previously [20,21]. Bcl-2 stably transfected HT29 cells were selected in 600 μg/ml G418 for four wk and then proved by Western blot for Bcl-2 expression.

2.4. Transfection

1 × 10^4 or 1 × 10^5 HT29 or LoVo cells were seeded into each well of 96-well plate or 6-well plate, respectively and incubated overnight, then transfected with negative scramble control (NC) RNA or miR-195 mimic (Ribobio) using INTERFERin transfection reagent (Polyplus-transfection) at a final concentration of 50 nM.

2.5. Analysis of cell viability

The in vitro cell viability of HT29 and LoVo cells post transfection was measured using the MTT method [24]. In brief, in the indicated time points, spent cell culture medium was replaced with 0.1 ml fresh medium containing 0.5 mg/ml MTT. Cells were then incubated at 37 °C for one hr and resolved by 0.1 ml DMSO (Sigma). The absorbance was measured at 570 nm.

2.6. Detection of apoptosis

At 48 h post transfection of HT29 and LoVo cells, spent cell culture medium was replaced by fresh serum free DMEM. In the indicated time points post serum deprivation, cells were harvested and resuspended in the staining buffer, and stained using Vybrant Apoptosis Assay kit (Invitrogen). Stained cells were detected by FACSCalibur (Becton Dickinson) and data were analyzed using CellQuest software (Becton Dickinson). The Annexin V-positive cells were regarded as apoptotic cells.

2.7. Tumorigenicity assay

All experiments involving animals were examined 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 General Hospital of PLA, Beijing. The tumorigenicity assay was undertaken as reported [25]. In brief, NC or miR-195 mimic transfected CRC HT29 and LoVo cells (2 × 10^6) were suspended in 0.1 ml PBS and then injected subcutaneously into either side of the posterior flank of the same BALB/c athymic nude mice at four wk of age. Tumor growth was measured using caliper daily and tumor volume was calculated as reported [25].

2.8. 3'UTR luciferase reporter assay

The luciferase reporter plasmid containing human B-cell CLL/lymphoma 2 (Bcl-2) 3'UTR sequence was made by cloning amplified Bcl-2 mRNA 3' UTR sequence into the 3'UTR region of pmIR-Report construct (Promega). Reporter plasmid containing miR-195 target site deleted Bcl-2 3'UTR sequence was made by ligation of PCR fragments of Bcl-2 3'UTR luciferase reporter plasmid lacking the target site. 1 × 10^7 HT29 cells were seeded into each well of 96-well plate and co-transfected with 100 ng Bcl-2 3'UTR Firefly luciferase reporter plasmid, 20 ng pRL-TK-Renilla-luciferase plasmid, and NC or miR-195 mimic using jetPRIME transfection reagent (Polyplus-transfection). After 48 h, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Data were normalized by dividing Firefly luciferase activity with that of Renilla luciferase.

2.9. Western blot

Cells and grinded tissues were lysed and protein was extracted as we previously reported [20,21]. Equal amount of the extracts were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted. Antibodies specific to Bcl-2 and β-actin, and horseradish peroxidase-coupled secondary antibodies were obtained from Cell Signaling Technology.

2.10. Statistical analysis

Data are shown as mean ± s.d. Statistical significance was determined by Student's t test, with P-value of < 0.05 considered to be statistically significant.

3. Results

3.1. miR-195 is downregulated in CRC tissues and cell lines

In order to investigate the roles of miR-195 in human CRC development, we detected its expression level in 81 pairs of human CRC tissues and non-neoplastic mucosa tissues. As shown in Figure 1A, miR-195 expression is significantly decreased in 59 of 81 (73%) tumor tissues compared to that in their matched non-neoplastic mucosa tissues. Furthermore, miR-195 expression was also decreased in CRC cell lines HT29, LoVo, SW480, and SW620 (Figure 1B). These results suggest that miR-195 is downregulated in CRC, and its downregulation may participate in human CRC development.

3.2. Restoration of miR-195 in CRC cell lines reduces cell viability, promotes cell apoptosis and suppresses tumorigenicity

Silenced miR-195 expression prompted us to investigate whether miR-195 functions as a tumor suppressor in CRC. In CRC HT29 and LoVo, restoration of miR-195 reduced cell viability in both of the CRC cell lines (Figure 2A). Furthermore, restoration of miR-195 promoted cell apoptosis upon serum deprivation in HT29 and LoVo cells (Figure 2B). These results demonstrate that miR-195 promotes cell apoptosis of CRC cells in vitro.

We next investigate the effect of miR-195 restoration on tumorigenicity in vivo. miR-195 mimic transfected CRC HT29 and LoVo cells revealed a delayed tumor formation and a significantly reduced tumor size compared to that of negative control transfec-
S. tants (Figure 2C). This result demonstrates that miR-195 inhibits tumorigenicity of CRC cells and further suggests the tumor suppressive effect of miR-195 on CRC.

3.3. Antiapoptotic Bcl-2 is directly targeted by miR-195

In order to further investigate the mechanisms responsible for the apoptosis promoting effect of miR-195 on CRC, we next sought to identify the molecular targets of miR-195, as miRNAs function mainly through posttranscriptional inhibition of their target mRNAs via binding to the 3′ UTR. In the hundreds of predicted target genes in TargetScan (http://www.targetscan.org), we found that human Bcl-2, an important antiapoptotic molecule well-accepted for CRC development [26,27], contained conserved putative miR-195 target site by TargetScan prediction (Figure 3A). To determine whether Bcl-2 is directly targeted by miR-195 expression, we constructed luciferase reporter plasmids containing Bcl-2 3′ UTR, or bearing deletion of the putative miR-195 target site. By co-transfection with miR-195 mimic, we found that the luciferase activity of the full length Bcl-2 3′ UTR reporter was suppressed, while target site deleted reporter failed to be targeted by miR-195 co-transfection (Figure 3B). This result proves that human Bcl-2 3′ UTR is directly targeted by miR-195 expression. Additionally, endogenous protein expression of Bcl-2 was also inhibited by miR-195 expression in CRC HT29 and LoVo cells (Figure 3C). We further compared Bcl-2 expression in available protein samples of paired human CRC tissues and found that Bcl-2 protein level was also upregulated in miR-195 downregulated CRC tissues (Figure 3D), confirming that endogenously expressed Bcl-2 is regulated by miR-195 expression. Taken together, these data demonstrate that Bcl-2 is a direct target of miR-195 and further suggest that miR-195 may exert its apoptosis promoting effect through inhibition of Bcl-2 expression.

3.4. Proapoptotic effect of miR-195 is mainly through targeting Bcl-2 expression

As Bcl-2 expression is validated to be posttranscriptionally targeted by miR-195 expression above, we further sought to determine that the apoptosis promoting effect of miR-195 was mainly through targeting Bcl-2 expression. Bcl-2 expressing plasmids stably transfected HT29 cells were prepared, which transcribing Bcl-2 mRNA without its 3′ UTR. Bcl-2 expression was confirmed to be elevated by Western blot (Figure 4A). Since the transfected cells transcribed Bcl-2 mRNA without its 3′ UTR, miR-195 should no longer target Bcl-2 expression. As shown in Figure 4B, cell apoptosis promoted by miR-195 expression was significantly suppressed in Bcl-2 stably transfected HT29 cells. This result further proves that the apoptosis promoting effect of miR-195 is mainly through inhibition of Bcl-2 expression.
of tumor suppressors or oncogenes, we next will determine theregulated epigenetic status in cancer often mediates the deregulation of gene expression. Additionally, the underlying mechanisms mediating miR-195 may also reflect the diverse roles of miR-195 in different types of cancer. Furthermore, as miR-195 is reported to inhibit cell cycle progression in hepatocellular carcinoma [17], we found that cell cycle distribution is poorly affected by miR-195 expression in CRC HT29 and LoVo cell lines (data not shown). This controversial result may also reveal the diverse roles of miR-195 in different types of cancer. Additionally, the underlying mechanisms mediating miR-195 deregulation in cancer development are still elusive. As deregulated epigenetic status in cancer often mediates the deregulation of tumor suppressors or oncogenes [28], we next will determine whether the deregulation of miR-195 expression in cancer is mediated by epigenetic alterations, such as deregulated DNA methylation or histone modification.

The tumor suppressive roles of miR-195 in CRC are determined both in vitro and in vivo, which is restoration of miR-195 expression reduces cell viability, promotes cell apoptosis, and suppresses tumorigenicity. To date, reexpression of miRNAs has been suggested to hold considerable potential in cancer gene therapy [29], it is obviously suggested that restoration of miR-195 expression may be used for clinical treatment of CRC patients. This presumption may raise important and interesting topics in the future.

The molecular mechanism that mediates the apoptosis promoting effect of miR-195 in CRC is further identified to be its direct targeting to antiapoptotic Bcl-2 expression. However, as there are hundreds of predicted targets of miR-195 in TargetScan prediction and a single miRNA is proved to target multiple mRNAs to regulate gene expression [30], it is probable that other targets of miR-195 may also participate in CRC development and exert the proapoptotic effect of miR-195. And miR-195 may also target different molecules in different types of cancer. According to this presumption, interesting future work may be raised to identify the entire roles of miR-195 in cancer development.

4. Discussion

In the present study, we found that miR-195 is downregulated in CRC and further suggested the apoptosis promoting effect of miR-195 in CRC cell lines. Here, miR-195 is suggested to function as a tumor suppressor in CRC development, which is consistent with its roles in hepatocellular carcinoma and adrenocortical carcinoma [17,18]. However, in chronic lymphocytic leukemia and breast cancer, miR-195 expression is reported to be elevated in these malignancies [14–16]. Hence, deregulation of miR-195 may be different in different types of cancer, and the roles of miR-195 in carcinogenesis and progression can not be simply concluded as a tumor suppressor or oncogene. The roles of miR-195 upregulation in cancer development remain to be further investigated. Furthermore, as miR-195 is reported to inhibit cell cycle progression in hepatocellular carcinoma [17], we found that cell cycle distribution is poorly affected by miR-195 expression in CRC HT29 and LoVo cell lines (data not shown). This controversial result may also reveal the diverse roles of miR-195 in different types of cancer. Additionally, the underlying mechanisms mediating miR-195 deregulation in cancer development are still elusive. As deregulated epigenetic status in cancer often mediates the deregulation of tumor suppressors or oncogenes [28], we next will determine whether the deregulation of miR-195 expression in cancer is mediated by epigenetic alterations, such as deregulated DNA methylation or histone modification.

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5. Conflicts of interest statement

Nothing to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.046.

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