

RESEARCH ARTICLE

MiR-150-5p Suppresses Colorectal Cancer Cell Migration and Invasion through Targeting MUC4

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Abstract

Growing evidence suggests that miR-150-5p has an important role in regulating genesis of various types of cancer. However, the roles and the underlying mechanisms of miR-150-5p in development of colorectal cancer (CRC) remain largely unknown. Transwell chambers were used to analyze effects on cell migration and invasion by miR-150-5p. Quantitative real-time PCR (qRT-PCR), Western blotting and dual-luciferase 3' UTR reporter assay were carried out to identify the target genes of miR-150-5p. In our research, miR-150-5p suppressed CRC cell migration and invasion, and MUC4 was identified as a direct target gene. Its effects were partly blocked by re-expression of MUC4. In conclusion, miR-150-5p may suppress CRC metastasis through directly targeting MUC4, highlighting its potential as a novel agent for the treatment of CRC metastasis.

Keywords: MiR-150-5p - colorectal cancer - metastasis - MUC4

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer related deaths worldwide (Jemal et al., 2009). Although improved treatment strategies involving surgery, chemo- and radio-therapy have increased the overall survival rates in early stages, metastasis and relapse are still the main cause of CRC death.

Over the past few years, a large number of miRNAs have been discovered to be correlated with tumor pathogenesis and progression, and miR-150 is one of the most widely researched miRNAs in various kinds of cancer (Lin et al., 2008; Wu et al., 2010; Watanabe et al., 2011). However, there are few researches about miR-150-5p in CRC was reported. Previous studies shown that miR-150-5p was low expressed in CRC tissues (Ma et al., 2012; Pizzini et al., 2013), and patients whose tumors had low miR-150-5p expression had shorter survival and a worse response to adjuvant chemotherapy than patients whose tumors had high miRNA expression (Ma et al., 2012), which indicated that miR-150-5p might act as a tumor suppressor gene in CRC. However, the role of miR-150-5p in CRC and its possible mechanism of the action remain to be further explored.

MUC4 is a high-molecular weight glycoprotein that belongs to the family of membrane bound mucins (Chaturvedi et al., 2008), which is expressed by epithelial cells in normal respiratory, gastrointestinal and genital tracts (Audie et al., 1993). In cancers originating from these epithelial cells MUC4 is frequently over-expressed

(Corfield et al., 2001), and MUC4 over-expression has been reported in a variety of carcinomas, including tumors of the lung, breast, colon, and ovary (Albrecht and Carraway., 2011). Expression analysis of MUC4 in CRC demonstrated CRC patients with increased MUC4 expression had short survival compared to low expressors (Shanmugam et al., 2010). However, there is still little known about the molecular mechanisms that regulate MUC4 expression in CRC.

In the present study, miR-150-5p was identified as a novel metastasis-inhibiting factor and miR-150-5p over-expression led to reduced migration and invasion in CRC cells. Furthermore, mechanical research revealed that miR-150-5p directly targeted the 3' UTR of MUC4 to suppress its expression, and miR-150-5p induced the reduced migration and invasion of CRC cells was partly blocked by MUC4 re-expression. Therefore, our study suggests tumor suppressive actions of miR-150 in CRC, and characterizes a miRNA-mediated mechanism of MUC4 regulation.

Materials and Methods

Cell lines and vector construction

The human CRC cell lines LoVo and HCT-116 were purchased from the American Type Culture Collection (ATCC, USA). HCT-116 was maintained in McCoy's 5a, and LoVo was maintained in F12-K. Media were supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂.

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The MUC4 cDNA is very large (29kb), therefore, it was difficult to clone the entire coding sequence into any available expression vector. Thus, we reduced the size of the repetitive sequence (by decreasing the number of tandem repeats) and generated an MUC4 expression plasmid, which encodes a protein of 320kDa (Moniaux et al., 2007)

The 3'UTR of MUC4 was amplified from human genomic DNA, and cloned into the pGL3-control vector (Promega, USA). Site-directed mutagenesis of the miR-150-3p target-site in the MUC4 3'UTR was carried out using site-directed mutagenesis kit (TaKaRa, Japan).

Oligonucleotide transfection

LoVo and HCT-116 cells were seeded at 5×10^4 and 8×10^4 cells per well in six-well plates respectively. After the cells reached 60-70% confluence, they were transfected with miR-150-5p mimics or control (miR-NC) mimics (RiboBio, China) at the concentrations of 50 nM using Lipofectamine 2000 (Invitrogen, USA).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNAiso Plus (TaKaRa, Japan). cDNA was synthesized with PrimeScript® RT-PCR Kit (TaKaRa, Japan). qRT-PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Japan) on ABI 7500 PCR System (Applied Biosystems, USA). Specific Bulge-Loop™ miRNA Primers were synthesized by RiboBio (Guangzhou, China). GAPDH and U6 small nuclear RNA were used as internal control for detection of mRNA and miRNA respectively. The primer sequence for mRNA qRT-PCR was displayed in Table S1.

Western blotting

Cells were lysed with 1×SDS-PAGE loading buffer. An equal amount of protein was separated on 8% SDS-PAGE gel and then transferred to the nitrocellulose membrane. After blocking with 5% milk, the membrane was probed with anti-MUC4 antibody (Santa, 1:1000) and anti-GAPDH antibody (Santa, 1:1000). GAPDH was used as loading control. The proteins were visualized using ECL reagents (Pierce, USA).

Luciferase assay

HEK 293T cells $1 \times 10^5/0.05$ ml were cultured in 24-well plates. For 3'UTR of MUC4 report system, HEK 293T cells were co-transfected with 200 ng WT or Mutant reporter plasmid, 25 nmol miRNA and 20 ng Renil using lipofectamine 2000 (Invitrogen, USA). After 24 h transfection, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, USA).

Migration and invasion assay

Transwell chambers (24-well insert; Corning) were used to analyze the ability of cell migration and invasion. For migration assay, after 48 h transfection, cells were suspended in culture medium with 1% FBS and added to the upper chambers (LoVo 5×10^4 cells/well, HCT-116 10×10^4 cells/well). At the endpoint of incubation (LoVo 18 h, HCT-116 24 h), cells on the upper membrane surface

were removed. The lower membrane surface was fixed by 4% formaldehyde, stained with Hoechst 33342 and counted under a fluorescence microscope. For invasion assay, the chambers were pre-coated with 0.05 ml matrigel (BD Biosciences, 50mg/ml 1:8) for 4 h, and the endpoint of incubation was 22h for LoVo cells and 48h for HCT-116 cells.

Cell proliferation assay

HCT-116 cells (3000/well) and LoVo cells (2000/well) were planted into 96-well culture plate. 16h later, miR-150-5p or miR-NC was transfected by lipofectamine 2000 (Invitrogen). Cell proliferation was measured at 24h, 48h and 96 h post-transfection respectively by using the Cell Counting Kit-8 (Dojindo, Japan) according to the manufacture's instruction.

Statistical analysis

Statistical analysis was performed using SPSS15.0. Values were expressed as mean±standard deviation (SD) for parametric data. Difference between groups was calculated using Student's T-test. A *p*-value less than 0.05 was considered statistically significant.

Results

MiR-150-5p suppresses CRC cell migration and invasion in vitro

Transwell chambers were performed to investigate the potential role of miR-150-5p in CRC cells. The transfection efficiency was confirmed by qRT-PCR 48 hours after transfection (Figure 1A). For migration assays, we observed that the number of migrated cells was significantly decreased in miR-150-5p transfected LoVo and HCT-116 CRC cells as compared with their respective controls (Figure 1B). For invasion assays, our data demonstrated also decreased invasiveness of the LoVo and HCT-116 CRC cells transfected with miR-150-5p as compared with the cells transfected with miR-NC (Figure 1C). In addition, miR-150-5p was found to have no effect on cell proliferation in LoVo and HCT-116 cells (data was not shown). Therefore, these findings indicate that miR-150-5p act as a metastasis suppressor in CRC cells.

MiR-150-5p directly targets 3' UTR of MUC4 in CRC cells

Previous studies have identified MUC4 as a target of miR-150-5p in pancreatic cancer cells (Srivastava et al. 2011), however, miR-150 might exercise distinct roles in different tissues or exercise the same roles through different mechanisms in a tissue-specific manners. Therefore, it is necessary to explore the regulatory role

Table 1. Sequence of the Primers Used in the Study

Primer	Sequence
miR-150-5p-F	5'-tcggcgTCTCCCAACCCCTTGAC-3'
miR-150-5p-R	5'-GTCGTATCCAGTGCAGGGTCCGAGGT-3'
U6-F	5'-CTCGCTTCGGCAGCACATATACT-3'
U6-R	5'-ACGCTTCACGAATTTGCGTGTGC-3'
MUC4-F	5'-GCCCAAGCTACAGTGTGACTCA-3'
MUC4-R	5'-ATGGTGCCGTTGTAATTTGTTGT-3'
GAPDH-F	5'-GGTGGTCTCCTCTGACTTCAACA-3'
GAPDH-R	5'-GTT GCTGTAGCCAAATTCGTTGT-3'

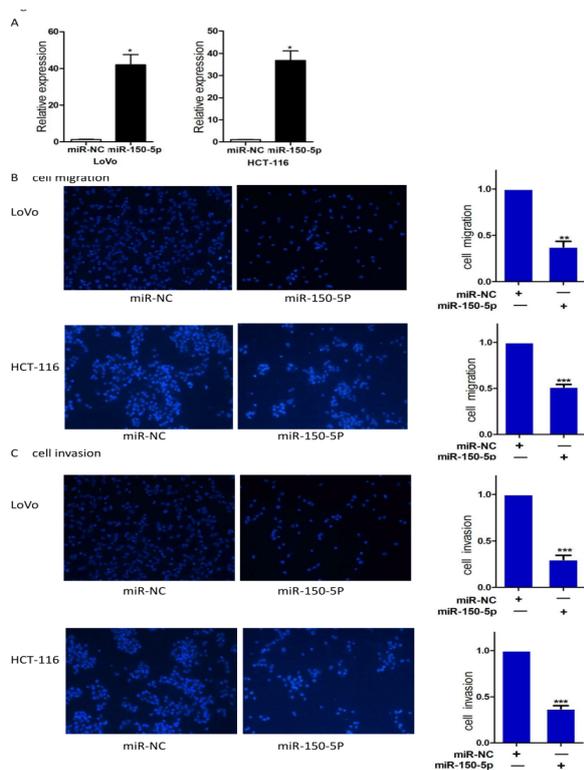


Figure 1. miR-150-5p Suppresses CRC Cells Migration and Invasion *in vitro*. LoVo cells (8×10^4) and HCT-116 cells (14×10^4) were seeded in 6-well plates. After 16 h, cells were treated with either miR-150-5p mimic or miR-NC mimic. 48 h later, cells were harvest and calculated the expression of miR-150-5p by qRT-PCR. (B) LoVo or HCT-116 cells transfected with either miR-150-5p mimic or miR-NC mimic were assayed for migration. (C) LoVo or HCT-116 cells transfected with miR-150-5p mimic or miR-NC mimic were assayed for invasion. Numerical data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n \geq 3$

of miR-150-5p in MUC4 expression in CRC. LoVo and HCT-116 cells were transiently transfected with miR-150-5p mimic or miR-NC, and the expression of MUC4 was examined. Our data showed significantly down-regulation of MUC4 expression at both the transcript level and the protein level (Figure 2A and 2B) in miR-150-5p mimic transfected cells. To further confirm whether MUC4 was a direct target of miR-150-5p, the 3'UTR of MUC4 was cloned into a luciferase reporter vector and then the predicted binding site of miR-150-5p in the MUC4 3'-UTR was mutated to evaluate the effect of miR-150-5p using luciferase assay (Figure 2C). It was found that ectopic over-expression of miR-150-5p significantly reduced the luciferase activity of pGL3-MUC4 3'UTR WT, whereas mutation of the binding site of miR-150-5p in the 3'-UTR of MUC4 abolished the effect of miR-150-5p (Figure 2D). Therefore, these results suggest that miR-150 directly regulated MUC4 in CRC cells.

MiR-150-5p suppresses CRC cells metastasis through regulation of the target gene MUC4

To further examine whether miR-150-5p induced inhibition of CRC cell metastasis was dependent on MUC4, LoVo and HCT-116 cells were transfected with miR-150-5p with or without MUC4 in the transwell migration and invasion assays. It was found that the

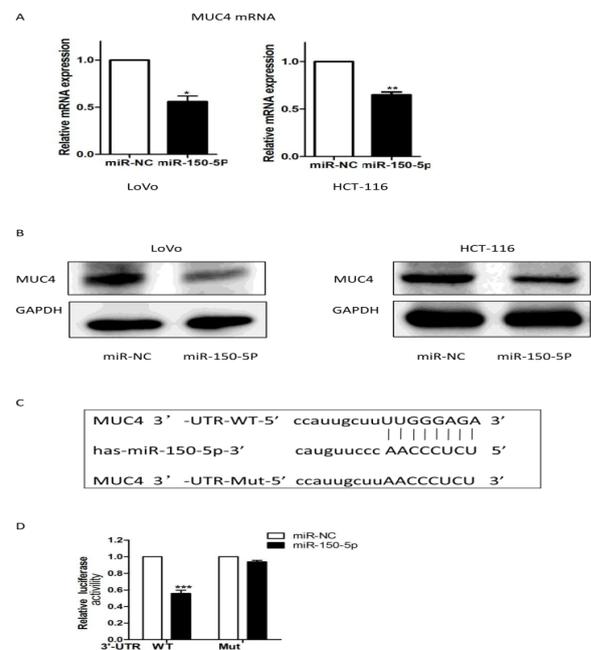


Figure 2. MUC4 is a Direct Target of miR-150-5p. (A and B) Posttranscriptional regulation of MUC4 by miR-150-5p. LoVo and HCT-116 cells were treated with miR-150-5p mimic or miR-NC mimic for 48 h, Expression of MUC4 was examined at mRNA (A) and protein (B) levels by qRT-PCR and western blot analyses, respectively. GAPDH was used as internal controls. (C) (A) Putative miR-150-5p binding sequence in the MUC4 3'UTR. Mutation of the MUC4 3'UTR was generated in the complementary site for the seed region of miR-150-5p as indicated. (D) Analysis of the luciferase activity of pGL3-MUC4 3'-UTR WT and Mut plasmid in HEK293T cells by miR-150-5p. Numerical data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n \geq 3$

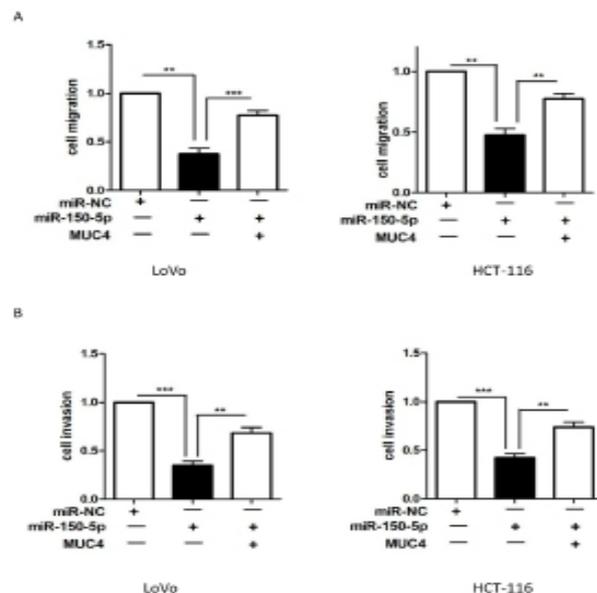


Figure 3. MiR-150-5p Suppresses CRC Cells Metastasis through Directly Targeting MUC4. (A, B) MUC4 mediates miR-150-5p induced reduced migration and invasion in CRC cells. (A) Migration assay of LoVo and HCT-116 cells transfected with miR-150-5p and/or MUC4 cDNA. (B) Invasion assay of LoVo and HCT-116 cells transfected with miR-150-5p and/or MUC4 cDNA. Numerical data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n \geq 3$

effect of miR-150-5p over-expression on cell migration was partly blocked by MUC4 re-expression (Figure 3A). Our experiment also showed that miR-150-5p reduced the invasion of LoVo and HCT-116 cells, and this effect was partially reversed by MUC4 re-expression (Figure 3B). Collectively, our data indicate that miR-150-5p exerted a metastasis-inhibiting function in human CRC via targeting MUC4.

Discussion

Although aberrant over-expression of some miRNAs occurred in CRC metastasis, such as miR-17, miR-19a and miR-21 (Zhang et al., 2012), miR-27a (Gao et al., 2013), miR-29a (Tang et al., 2014), miR-31 (Wang et al., 2010), miR-103 and miR-107 (Chen et al., 2012), miR-143 and miR-145 (Arndt et al., 2009), down-regulation of miRNAs involved in CRC metastasis was much less known, and the novel candidate for suppressing CRC metastasis with a definite molecular mechanism should be further explored. It was reported that the dysregulation of miR-150-5p performed diverse functions in different types of cancer (Wu et al., 2010; Srivastava et al., 2011; Huang et al., 2013; Cao et al., 2014). Previously, it was reported that the serum exosomal levels of miR-150-5p was significantly higher in primary CRC patients than in healthy controls, and was significantly down-regulated after surgical resection of tumors, suggesting miR-150-5p is a promising biomarkers for non-invasive diagnosis of the disease (Ogata-Kawata et al., 2014). However, it was uncovered in other researchers that CRC tumors had reduced levels of miR-150-5p expression compared with paired non-cancerous tissue, and miR-150-5p was suggested to be considered as a potential biomarker associated with the prognosis in CRC (Ma et al., 2012). In this study, the data of the CRC cell line tests indicated that miR-150-5p suppressed CRC metastasis. We also proved that MUC4 was a direct target of miR-150-5p in CRC, and miR-150-5p induced reduced CRC cells migration and invasion was partly blocked by MUC4 re-expression. Therefore, we uncovered that miR-150-5p acted as a metastasis suppressor in CRC cells and directly targeted MUC4 to suppress CRC cells metastasis.

MUC4 is frequently deregulated in a wide variety of cancers (Carraway et al., 2002), and its over-expression has been associated with a poor prognosis and survival in various carcinomas, including colon carcinomas (Shanmugam et al., 2010). Furthermore, increasing evidence also indicate MUC4 is critical for the progression of other malignancies (Ponnusamy et al., 2008; Rachagani et al., 2012; Mukhopadhyay et al., 2013). It is proposed that MUC4 may be regulated at the transcriptional level via the transforming growth factor β and epidermal growth factor pathways among others (Perrais et al., 2001; Andrianifahanana et al., 2005), and at the epigenetic level via DNA methylation and histone modifications (Vincent et al., 2008). MiRNAs were also shown to be involved in post-transcriptional regulation of MUC4 (Srivastava et al., 2011; Lahdaoui et al., 2014). However, all the above regulation manners of MUC4 were reported in other cancers but not CRC. In this study, we demonstrate that

MUC4 is a functional target of miR-150-5p, and miR-150-5p suppress MUC4 expression at both mRNA and protein levels through directly binding to the nucleotidic sites in the MUC4 3' UTR.

In summary, this study provided insight into the role of the miR-150-5p in CRC metastasis. The low expression of miR-150-5p, which suppresses CRC metastasis by regulating MUC4 expression, highlights the potential of miR-150-5p as a novel agent for the treatment of CRC metastasis. Meanwhile, there are still many questions remained to be further explored, *i)* why miR-150-5p is low expressed in CRC tumor tissue compared with paired non-cancerous tissue, *ii)* why serum exosomal levels of miR-150-5p was significantly higher in primary CRC patients and was significantly down-regulated after surgical resection of tumors, *iii)* whether there are other mechanism for miR-150-5p involving in CRC. Solving the above questions may promote our understanding of miR-150-5p in CRC metastasis.

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