MicroRNA-330-5p negatively regulates ITGA5 expression in human colorectal cancer

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Received March 2, 2016; Accepted March 30, 2016

DOI: 10.3892/or.2016.5092

Abstract. Colorectal cancer (CRC), one of the most prevalent malignant cancers, has high rates of incidence and is the fourth leading cause of cancer-related death for both men and women worldwide. MicroRNAs (miRNAs) play critical roles in the development of various types of cancers. miRNA-330-5p has been implicated in the progression of prostate, neuronal and pancreatic cancers by regulating proliferation, migration, invasion and epithelial-mesenchymal transition of cells. The purpose of the present study was to investigate the expression of miR-330-5p in CRC and identify its target gene(s) that may act in CRC tumorigenesis. We found that miR-330-5p expression was significantly lower in CRC tissues than that in adjacent non-tumorous tissues. Furthermore, we identified integrin α5 (ITGA5) as a new target of miR-330-5p and found that it inhibits ITGA5 expression by directly binding to the 3’ untranslated region of ITGA5 mRNA. These results suggest that downregulation of miR-330-5p expression may affect CRC development via modulation of ITGA5 expression.

Introduction

Colorectal cancer (CRC), one of the most prevalent malignant cancers worldwide, is the third most common cancer in men and the second in women, accounting for 10.0 and 9.2% of cancer cases, respectively (1.2). The main cause of the CRC patient death is metastasis of CRC cells to other organs. The 5-year survival rate of early stage CRC patients (stage I) is higher than 90%, but that of advanced stage CRC patients with metastases (stage IV) decreases to less than 5% (3-5). Although the CRC incidence rate is gradually decreasing in developed countries, it is still increasing in numerous developing countries. Numerous studies are ongoing to elucidate the molecular mechanisms related to CRC development in order to develop effective treatments and prognostic markers.

MicroRNAs (miRNAs), endogenous small RNAs of 19-25 nucleotides, regulate translation through mRNA degradation or translational inhibition by binding to the 3’ untranslated regions (UTRs) of target mRNAs (6). More than 50% of miRNAs identified in human cells are encoded in cancer-associated genomic regions and are deeply involved in human cancer development (7,8). Among these, miR-330-5p was first discovered by Weber in 2005 (9), and several studies have identified miR-330-5p as a tumor-suppressor in prostate and pancreatic cancers and in CRC cell lines as well (10-13). In contrast, a study suggested that miR-330-5p is an oncomiR in glioblastoma cells (14,15). In any case, these lines of evidence implicate miR-330-5p in human cancers. Although several target genes are known for miR-330-5p, the list of its molecular targets is likely to be incomplete and the mechanisms of its involvement in cancer are not well understood.

Integrins are a family of transmembrane proteins that mediate communications between different cells or between cells and extracellular matrix. They play important roles in tumor development through regulation of cell proliferation, survival, migration and invasion (16). Functional integrins are heterodimeric proteins composed of one α and one β chain; in humans, 18α and 8β integrin subunits can be present in any combination (17). Among the α subunits, α5 (ITGA5) forms a dimer predominantly with β1 (ITGB1). Integrin α5β1 recognizes the arginine-glycine-aspartic acid sequence in its ligand fibronectin, which is one of the proteins organizing the extracellular matrix. Recently, it was revealed that integrin α5β1 promotes metastasis of CRC cells in the hepatic microenvironment (18). Furthermore, ITGA5 was shown to regulate peritoneal dissemination of ovarian cancer cells and adhesion and invasion of CRC cells, thus affecting metastasis (19-22).

In the present study, we showed that the level of miR-330-5p expression was decreased and was inversely related to that of ITGA5 in the CRC tissue. Our data identified ITGA5 as a new miR-330-5p target and demonstrated negative regulation of ITGA5 expression by direct binding of miR-330-5p to its 3’UTR. Together with previous reports, our results suggest that miR-330-5p acts as an antimitastatic miRNA in CRC by regulating ITGA5 expression.

Materials and methods

Human tissue samples. Human CRC and non-tumor colorectal tissue samples were obtained from the Seoul St. Mary's
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Biobank (2013-05). Collection and use of all samples were approved by the Institutional Review Board (IRB) of the College of Medicine at the Catholic University of Korea.

**Cell culture and transfection.** The human CRC cell lines, DLD-1, SNU-C5 and HCT116, were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). Cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin with 5% CO₂ in a 37°C incubator. miRNAs used in the present study were purchased from Dharmacon (Lafayette, CO, USA). CRC cells were seeded onto 60 mm dishes at 70% confluency. After 24 h, miR-330-5p mimic transfection was performed using DharmaFECT I (Dharmacon) following the manufacturer's instructions. Transfected cells were harvested 72 h post-transfection.

**Quantitative RT-PCR (qRT-PCR).** Total RNA was extracted from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complimentary DNA (cDNA) was synthesized using the PrimeScript II First Strand cDNA Synthesis kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. The cDNA synthesis for miRNA was carried out using the Mir-X miRNA First-Strand Synthesis kit (Clontech, Mountain View, CA, USA). The qRT-PCR for ITGA5 and miR-330-5p was performed using the SYBR Premix Ex Taq II (Takara) following the manufacturer's instructions. ITGA5 expression levels were normalized against glyceraldehyde-3-phosphated dehydrogenase (GAPDH) gene expression. Expression of miR-330-5p was determined by qRT-PCR using the miR-330-5p primer and miRQ 3' Primer (both from Clontech) and normalized against U6 expression. The sequences of the gene-specific primers are shown in Table I.

**Western blot analysis.** Cell lysates were prepared using RIPA buffer [150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8.0)] according to the standard method. Protein concentrations were determined by the Bradford assay. Proteins were resolved on 8% sodium dodecyl sulfate polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with a rabbit polyclonal antibody against ITGA5 (1:1,000; Applied Biological Materials, Vancouver, Canada) and a mouse polyclonal antibody against β-actin (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin level was used for normalization.

**Plasmid construction.** The full length 3'UTR cDNA of ITGA5 was amplified from total RNAs of DLD-1 cells by RT-PCR following the standard protocol. The amplified PCR product was cloned into the pGEM-T Easy vector and the integrity of its sequence was confirmed by sequencing. The inserted DNA fragment was subcloned into the psiCHECK-2 vector (Promega, Madison, WI, USA) using the NotI site (Takara) to generate psiCHECK-2/h_ITGA5 3'UTR. The deletion constructs, psiCHECK-2/h_ITGA5 3'UTR_deletion I, psiCHECK-2/h_ITGA5 3'UTR_deletion II and psiCHECK-2/h_ITGA5 3'UTR_deletion III, were generated by site-directed mutagenesis using the QuikChange Site-Directed
Mutagenesis kit (Stratagene, La Jolla, CA, USA). Primer sequences for plasmid construction are listed in Table II.

Luciferase reporter assay. Cells were plated onto 60 mm dishes at 70% confluency. After 24 h, the cells were co-transfected with miR-330-5p mimic and 1 µg of the luciferase reporter construct containing the ITGA5 3'UTR using the Lipofectamine 2000 reagent (Invitrogen). At 72 h post-transfection, the lysates of the transfected cells were prepared and the luciferase activity was measured using the Dual-Luciferase Reporter Assay reagent (Promega) following the protocols recommended by the manufacturer.

Bioinformatic analysis. Target genes of miR-330-5p were searched using the TargetScan version 5.2 (http://www.targetscan.org), miRBase (http://www.mirbase.org/) and TargetMiner (http://www.isical.ac.in/~bioinfo_miu/targetminer20.htm) databases. To determine the relationship between miR-330-5p and ITGA5, data sets of miRNAs and mRNAs from the NCI60 cell line were obtained from CellMiner (http://discover.nci.nih.gov/cellminer/). Correlation was measured between the expression levels of hsa-miR-330-5p (MI0000803) and ITGA5 probe set (201389_at) in CRC cell lines.

Statistical analysis. Statistical significance was determined by Student's t-tests and p-values <0.05 were regarded as statistically significant.

Results

miR-330-5p is downregulated in CRC patients. Although miR-330-5p expression has been shown to be reduced in various tumors in vivo, it has not been studied in CRC. To investigate the expression of miR-330-5p in CRC, we compared its expression in human CRC tumors and paired adjacent non-tumorous tissues (control) using qRT-PCR. Among the 17 CRC tumors tested, 12 showed reduced miR-330-5p expression compared to the controls (Fig. 1A).

Since miRNAs regulate mRNA expression through recognition of seed-match sequences of the target mRNA, we searched for putative target genes of miR-330-5p using online software for prediction of miRNA targets including miRDB, TargetScan and TargetMiner. Each of these software predicted numerous candidate target genes and 16 genes were predicted by all three (Fig. 1B). Among them, we focused on ITGA5 since it plays important roles in the development of...
various types of cancers (17,19-23). Our analysis of the data in the Gene Expression Omnibus (GEO) database (accession nos. GSE37364 and GSE4107) showed that ITGA5 expression was significantly increased in the CRC tumors (Fig. 1C and D).

Expression of miR-330-5p is inversely correlated with ITGA5 expression in CRC cell lines. The relationship between the expression of miR-330-5p and ITGA5 was also investigated using the NCI-60 database of the US National Cancer Institute, which contains mRNA and miRNA expression data for 60 human cancer cell lines. The analysis revealed that the expression of miR-330-5p and ITGA5 was inversely correlated in the 7 CRC cell lines (R=-0.367) (Fig. 2A).

The relationship between miR-330-5p and ITGA5 protein levels was studied in 5 CRC cell lines. The levels of ITGA5 were determined by western blot analysis and those of miR-330-5p by qRT-PCR. As shown in Fig. 2B-D, the inverse relationship was found between the levels of miR-330-5p and ITGA5 in all 5 cell lines.

miR-330-5p downregulates ITGA5 expression. To investigate whether miR-330-5p regulates ITGA5 expression, we transfected the CRC cell lines DLD-1, HCT116 and SNU-C5 with a miR-330-5p mimic and measured the levels of the ITGA5 mRNA. ITGA5 expression was decreased by >50% in the cell lines transfected with the miR-330-5p mimic compared to that in cells transfected with the control RNA (Fig. 3A). Western blot analysis revealed that the levels of the ITGA5 protein were also reduced by miR-330-5p: by 51.9% in DLD-1, 52.54% in HCT116 and 59.68% in SNU-C5 cells (Fig. 3B and C). These results indicate that miR-330-5p downregulates ITGA5 expression.

miR-330-5p directly targets the 3'UTR of the ITGA5 mRNA. To determine whether miR-330-5p directly binds to the 3'UTR of the ITGA5 mRNA, we used a heterologous reporter system. First, we cloned the full-length 3'UTR of ITGA5 into a reporter plasmid in which luciferase gene expression is affected by the cloned 3'UTR. The cloned reporter construct was transfected into the cells along with the miR-330-5p mimic, and luciferase activity was determined. Luciferase activity was significantly inhibited by miR-330-5p in both cell lines tested (DLD-1 and SNU-C5; Fig. 4A). This result indicated that miR-330-5p directly regulated ITGA5 expression by binding to the 3'UTR of its mRNA. We further investigated which region of the ITGA5 3'UTR is recognized by miR-330-5p. Each of the 3 regions predicted as seed matches (Fig. 4B) was deleted to generate constructs lacking nucleotides 124-131, 409-415 or 843-850. Each construct was co-transfected with the miR-330-5p mimic, and luciferase activity was measured. The construct with the deletion of nucleotides 124-131 was the only one that did not show a reduction of luciferase activity by miR-330-5p, indicating that this region is the target of miR-330-5p (Fig. 4C). This result strongly suggests that miR-330-5p regulates ITGA5 expression by directly binding to nucleotides 124-131 of the ITGA5 3'UTR.

Figure 2. Expression levels of miR-330-5p and ITGA5 are inversely correlated in colorectal cancer (CRC) cell lines. (A) NCI 60 databases indicated that expression of miR-330-5p and ITGA5 was inversely correlated in 7 CRC cell lines. (B) The levels of miR-330-5p expression and ITGA5 protein were inversely correlated in 5 different CRC cell lines as shown by western blot analysis. (C and D) The ITGA5 protein level was quantified using ImageJ, which revealed an inverse correlation with miR-330-5p expression in 5 CRC cell lines. ITGA5 protein level was normalized against β-actin expression and U6 expression level was used to normalize miR-330-5p expression.
Figure 3. miR-330-5p regulates expression of ITGA5 in colorectal cancer cell lines. (A) qRT-PCR showed that expression of ITGA5 mRNA was significantly downregulated by overexpression of miR-330-5p in the DLD-1, HCT-116 and SNU-C5 cell lines. The data were normalized using GAPDH mRNA expression. These results are the average of 3 independent experiments performed in duplicate; *P<0.05, ***P<0.001. (B) Western blotting indicated that the protein level of ITGA5 was downregulated by overexpression of miR-330-5p in the DLD-1, HCT116 and SNU-C5 cell lines. The results were normalized against the protein level of β-actin. (C) Ratio of ITGA5/β-actin was quantified using ImageJ analysis. The results showed that ITGA5 expression was decreased in the miR-330-5p-transfected cells; ***P<0.001.

Figure 4. ITGA5 is a direct target of miR-330-5p in CRC cell lines. (A) Luciferase activity was significantly inhibited in miR-330-5p-overexpressing DLD-1 and SNU-C5 cells. (B and C) miR-330-5p regulated ITGA5 expression by directly binding to the 124-131 nt region of ITGA5 3’UTR. The results are the average of 3 independent experiments carried out in duplicate; ***P<0.001. NS, not significant.
Accumulating lines of evidence have led to the view that microRNAs (miRNAs) play important roles in the progression of human cancers through modulation of proliferation, survival, metastasis and invasion of the cancer cells. Therefore, the functions of miRNAs in cancer have been intensively investigated; this research facilitates the development of miRNA-based diagnosis, prognosis and therapy (24). Several studies have identified the function of miR-330-5p in cancer progression. miR-330-5p was shown to play a role as an oncomiR by targeting the SH3-domain GRB2-like 2 (SH3GL2) gene in glioblastoma stem cells (14). In contrast, other studies demonstrated that miR-330-5p inhibits cell motility by targeting the Sp1 transcription factor (SP1) and induces apoptosis through E2F transcription factor 1 (E2F1)-mediated suppression of Akt phosphorylation in prostate cancer (10,11). Thus, the role of miR-330-5p in tumorigenesis is controversial. A recent study showed that miR-330-5p inhibited proliferation of CRC cell lines by downregulating cell division cycle 42 (CDC42) expression and revealed that miR-330-5p overexpression induced apoptosis and reduced tumor weight in vivo (13). In the present study, we showed that miR-330-5p expression was downregulated in tumor compared to adjacent normal tissues in ~70% of the investigated CRC patients, raising a possibility of miR-330-5p being a tumor-suppressor in CRC.

We found an inverse relationship between the expression of miR-330-5p and ITGA5 in CRC. A similar relationship (R=0.54) was also observed in leukemia cell lines (NCI-60 database; data not shown). In contrast, a positive correlation was found in several other cancer cell lines including neuronal, non-small cell lung and renal cancer (NCI-60; data not shown). These results suggest that the relationship between miR-330-5p and ITGA5 is cancer type-specific.

ITGA5, the newly found target of miR-330-5p, was found to promote the development of various types of cancers. Downregulation of ITGA5 inhibited peritoneal dissemination of ovarian cancer cells; upregulation of ITGA5 promoted adhesion, invasion and epithelial-mesenchymal transition of CRC cells (19-22). Furthermore, ITGA5 in a complex with ITGB1...
integrin α5β1) promoted invasive migration and metastasis of cervical cancer cells (25) and regulated ionizing radiation-induced adhesion of breast cancer cells (26). Thus, there is enough evidence to support the notion that integrin α5β1 plays important roles in the development of various human cancers.

Since ITGB1 forms a heterodimer with ITGAV5, we investigated the relationship between the expression of ITGB1 and miR-330-5p in CRC using public datasets, but found no correlation in the 7 CRC cell lines (NCI-60 data; Fig. 5A), and the software for the miRNA target did not predict ITGB1 as a miR-330-5p target. Based on these data, ITGB1 seems not to be regulated by miR-330-5p in CRC.

We further investigated ITGB1 expression using the same GEO data (GSE37364 and GSE4107) as for the analysis of ITGAV5 expression in CRC, and found no consistency between these two datasets. Whereas ITGB1 mRNA level was slightly higher in the CRC tumors compared to that of the normal tissue in GSE37364, there was no difference in the GSE4170 dataset (Fig. 5B and C). At this point, it is not clear whether ITGB1 expression is related to the progression of CRC. Whether ITGB1 is in excess, the level of ITGAV5 may be limiting for α5β1 formation, which may explain the apparent irrelevance of the ITGB1 level. Thus, we compared expression of ITGAV5 and ITGB1 in the same datasets and found 6.3- (ranging from 1.0- to 10.2-fold in GSE37364) and 3.4- (ranging from 1.3- to 8.0-fold in GSE4107) fold higher expression of ITGB1 on average (Fig. 5D and E). A similar expression pattern was noted in lung cancer (27). Obviously further study is required to understand its relevance to CRC development.

In conclusion, we found that miR-330-5p was significantly downregulated in human CRC tumors, which led to the upregulation of ITGB1, a direct target of miR-330-5p. Our findings suggest that downregulation of miR-330-5p may stimulate the metastasis and invasion of CRC cells by directly targeting ITGB1. These results improve our understanding of CRC development and may be useful for future clinical applications.

Acknowledgements

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A5A2047939)

References