miR-205-5p/PTK7 axis is involved in the proliferation, migration and invasion of colorectal cancer cells

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Abstract. MicroRNAs (miRNAs) are small non-coding RNAs, which are critical in a diverse range of biological processes, including development, differentiation, homeostasis, and in the formation of diseases by accelerating and/or inhibiting the translation of mRNAs. The present study aimed to examine the potential role of miRNA (miR)-205-5p in the developmental process of colorectal cancer (CRC) through protein-tyrosine kinase 7 (PTK7). Initially, TargetScan was used to predict the miRNA target sites in the sequence of the PTK7 3’-untranslated region. It was then found that the mRNA expression level of miR-205-5p was lower in CRC cells, determined using reverse transcription-quantitative polymerase chain reaction analysis, and there was a negative correlation between miR-205-5p and PTK7 in CRC tissues. It was also found that miR-205-5p regulated the gene transcription of PTK7, determined using a luciferase reporter assay. The results of RT-qPCR and western blot analyses in human colorectal cancer revealed that miR-205-5p suppressed the expression of PTK7. Finally, it was revealed that miR-205-5p restricted the proliferation ability of CRC cells through inhibiting PTK7. Therefore, miR-205-5p is involved in the proliferation, migration and invasion of CRC through inhibiting PTK7.

Introduction

Colorectal cancer (CRC) is a frequently occurring type of cancer and is a primary contributor to morbidity and mortality rates worldwide (1). The incidence rates of CRC have gradually increased in developing countries, which previously showed a decrease in cases of CRC (2). Although methods have become available for the diagnosis and treatment of CRC during the last few decades, the overall five-year survival rate remains at 40-45% due to the limited ability to detect cancer at an early stage and provide prognostic predictions (3). At present, the molecular and functional mechanisms of CRC remain to be fully elucidated. Therefore, investigations aimed at determining biomarkers and effective molecular targets for CRC are urgently required.

MicroRNAs (miRNAs), which are non-coding RNAs of ~20 nucleotides, are involved in post-transcriptional regulation by targeting the 3’untranslated region (UTR) of target genes (4-6). There have been an increasing number of studies showing that miRNAs are vital in the occurrence and development of various diseases, including cancer in humans (4,7-9). Increasing evidence indicates that miRNAs are involved in the regulation of various biological processes, including proliferation, differentiation, apoptosis, migration and invasion (10). Several studies have indicated that certain miRNAs are involved in the developmental process of CRC, including miR-135b (11), miR-34a (12), miR-25 (13) and miR-21 (14). However, the biological functions and molecular mechanisms of miR-205-5p in CRC remain to be fully elucidated.

Protein-tyrosine kinase 7 (PTK7) belongs to the defective receptor protein-tyrosine kinases, and includes an extracellular domain, a transmembrane domain and a tyrosine kinase domain (15,16). PTK7 is involved in the development of planar cell polarity, functioning as a regulator. Studies have indicated that PTK7 is involved in the Wnt pathway and PCP signaling pathway (17-19). Studies have also demonstrated that PTK7 is expressed at high levels in various types of cancer, including colon cancer (20), lung cancer (21), gastric cancer (22) and acute myeloid leukemia (23). Studies have also shown that PTK7 can affect the proliferation and invasion abilities of liposarcoma cells (24), and the migration and invasion abilities mediated by vascular endothelial growth factor (25).
However, the interaction and association between miR-205-5p and PTK7, and the effects of miR-205-5p on the proliferation, migration and invasion abilities of CRC through PTK7 remain to be elucidated.

The present study investigated the potential role of miR-205-5p in the development of CRC through PTK7. The miRNA target sites in the sequence of the PTK7 3'UTR were predicted, which revealed that the expression of miR-205-5p was low in CRC. The correlation between miR-205-5p and PTK7 was examined in CRC tissues and the regulatory association between miR-205-5p and PTK7 was examined in CRC cells. The study also aimed to measure the effects of miR-205-5p on the proliferation, apoptosis, migration and invasion abilities of CRC cells through PTK7.

Materials and methods

Cell lines and transfection. HT29 and SW480 human CRC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The HT29 and SW480 cell cultures were collected at 37°C in an appropriate incubator containing 5% CO₂ in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml). For treatment, 2x10⁵ HT29 and SW480 cells were seeded into 6-well plates and then transfected with 200 µl mature hsa-miR-NC (negative control) and the predicted miRNAs [predicted using TargetScan (http://www.targetscan.org/); miRBase (http://www.mirbase.org/), and MiSiM (http://cmbi.bjmu.edu.cn/misim)], including hsa-miR-409-5p, hsa-miR-205-5p, hsa-miR-495-3p, hsa-miR-5688, and hsa-miR-503-5p (GenePharma Co., Ltd., Shanghai, China), respectively, for 72 h. The MiR-205-5p mimic sequence was 5’-UCCUUCUAUUCCACCGAGUCUG-3’, the miR-control sequence was 5’-ACUACAGUGAGUAGAGA A-3’, the inhibitor NC sequence was 5’-CAGUACUUUUGU GUAGUACAA-3’, and the miR-205-5p inhibitor sequence was 5’-CAGACUCGGUGAGUAAAGGA-3’. The HT29 and SW480 cells were then transfected with miR-205-5p (50 nM), miR-control (50 nM), inhibitor NC (50 nM), and miR-205-5p inhibitor (50 nM), respectively. Finally, HT29 and SW480 cells were transfected with miR-control, miR-205-5p, miR-205-5p and vector, and miR-205-5p and PTK7, respectively, for 72 h. All transfections were performed with Lipofectamine™ 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Clinical specimens. In the present study, tissue samples were collected from 46 patients (age 50.34±5.87 years, range 29-91; man/woman=25/21) with CRC in The People's Hospital of Tianjin (Tianjin, China) between January 2015 and November 2016. All patients were treated with liquid nitrogen, and then ground into powder; the cell suspensions were centrifuged (1,000 x g, 5 min, 4°C). Trizol (1 ml) was added and ground for 5 min on ice, and then 0.2 ml chloroform was added and centrifuged (12,000 x g, 15 min, 4°C). The supernatant solution was obtained, precipitated by alcohol, and then centrifuged (7,500 x g, 5 min, 4°C). RNA purity can be detected using the NanoDrop (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The optical density 260/280 ratio is used as an indicator for RNA purity. A ratio >1.8 is regarded as suitable for gene expression measurements. Total RNA (1 µg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.).

Western blot analysis and antibodies. The treated HT29 and SW480 cells were lysed in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The concentrations of proteins were measured using a bicinchoninic acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Equivalent quantities of protein were separated by 10% SDS-PAGE on gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The PVDF membranes were blocked in 5% skim milk (BD Biosciences, Franklin Lakes, NJ, USA) for 2 h at room temperature. The PVDF membranes were then incubated with anti-PTK7 antibody (1:1,000; cat. no. MAB4499; R&D Systems, Inc., Minneapolis, MN, USA); anti-GAPDH antibody (1:4,000; cat. no. 12255; Cell Signaling Technology, Inc., Beverly, MA, USA) at 4°C overnight, and were then incubated with HRP-conjugated secondary antibodies (goat anti-mouse; 1:5,000; cat. no. SC-2005, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; goat anti-rabbit; 1:5,000; cat. no. SC-2004, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Finally, the proteins were detected using an enhanced chemiluminescence detection kit (EMD Millipore, Billerica, MA, USA). The signals were detected.
using a chemiluminescence detection system with Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., cat. no. 34080). Anti-GAPDH antibody was used as an internal control.

Luciferase reporter assays. The sequences of the wild-type (WT) and mutant type (Mut) PTK7-3’UTR were amplified by PCR using human genomic DNA of the HT29 cell line and cloned into the pGL3-promoter vector (Promega Corporation, Madison, WI, USA; cat. no. E1751). The HT29 and SW480 cells (5x10^4 cells/well) were cultured in 24-well plates and co-transfected with 50 nM miR-205-5p, miR-control, inhibitor NC, or 100 nM miR-205-5p inhibitor, respectively, with 15 ng of WT pGL3-promoter-PTK7-3’UTR or 15 ng Mut type pGL3-promoter-PTK7-3’UTR, and the Renilla plasmid (RL-SV40) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. According to the manufacturer’s protocol, the luciferase activity of PTK7 was detected using a Dual-Luciferase reporter assay system (Promega Corporation). The duration was 10 h between activity measurement and transfection, and the results were normalized to pRL-CMV Renilla.

Colony formation assay. The HT29 and SW480 cells were transfected with miR-control, miR-205-5p, and miR-205-5p and vector, respectively, for 72 h. The treated HT29 and SW480 cells were incubated in complete medium for 14 days. The colonies were fixed with methanol for 15 min at room temperature, and dyed with giemsa dye solution for 10 min at room temperature. The colonies were then identified and counted under a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The treated HT29 and SW480 cells (2,000 cells/well) were seeded in 96-well plates with complete medium for 0, 24 and 48 h, respectively. Each group consisted of five wells and each well was treated with MTT (20 µl/well) solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) at 0, 12, 24 and 48 h. After 4 h, 100 µl dimethyl sulfoxide (Sigma-Aldrich, Merck KGaA) was added to dissolve the crystal. The absorbance (optical density) was detected using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm.

Flow cytometric analysis of cell apoptosis. According to the manufacturer’s protocol, the treated HT29 and SW480 cells were stained with Annexin V-fluorescein isothio-cyanate (FITC)/propidium iodide (PI) kit (cat. no. 4830-01-K; R&D systems, Inc.). Samples were analyzed for apoptosis using a FACSCalibur flow cytometer (BD Biosciences). FlowJo software 7.6.5 (Tree Star Inc., Ashland, OR, USA) was used to analyze the results of the flow cytometry.

Migration and invasion assays. For the migration assay, the treated HT29 and SW480 cells (1x10^5 cells/well) were seeded in the top of each well containing serum-free medium, and 600 µl complete medium was added to the lower chamber. After 24 h, the migrated cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.1 % crystal violet solution (Sigma-Aldrich; Merck KGaA) for 20 mins at room temperature. The migrated cells were identified and counted using a light microscope (BX51; Olympus Corporation). For the invasion assay, the diluted Matrigel (BD Biosciences, ) was added to the Transwell chamber for 1 h at 37˚C, and the remaining steps were similar to those of the migration assay.

Statistical analysis. The data were analyzed using SPSS 18.0 version (SPSS, Inc. Chicago, IL, USA). The results were compared using one-way analysis of variance followed by Dunnett’s posttest for multiple comparisons. All results are expressed as the mean ± standard deviation from three replicates. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of PTK7-integrated miRNAs. To identify miRNAs, which were potential target sites in the sequence of the PTK7 3’UTR. TargetScan (http://www.targetscan.org/) was used. It was found that there were five potential miRNAs, including hsa-miR-409-5p, hsa-miR-205-5p, hsa-miR-495-3p, hsa-miR-5688 and hsa-miR-503-5p (Fig. 1A). The HT29 and SW480 cells were then transfected with hsa-miR-NC (negative control) and the predicted miRNAs (miR-409-5p, miR-205-5p, miR-495-3p, miR-5688, and miR-503-5p, respectively). The results revealed that the mRNA expression level of PTK7 was decreased in HT29 cells transfected with miR-205-5p, compared with that in the NC cells (P<0.05; Fig. 1B). Similarly, the mRNA expression level of PTK7 was decreased in SW480 cells transfected with miR-205-5p, compared with that in the NC cells (P<0.05; Fig. 1C). To investigate whether miR-205-5p was physically associated with PTK7 in CRC tissues (n=46), the correlation between the expression of miR-205-5p and PTK7 in CRC tissues was detected using RT-qPCR analysis. The result showed that there was a negative correlation between the gene expression of PTK7 and miR-205-5p and in the CRC tissues (Fig. 1D).

miR-205-5p regulates the gene transcription of PTK7 and suppresses the expression of PTK7 in human CRC. The present study further demonstrated that miR-205-5p may directly regulate the transcriptional level of PTK7. According to the binding site of the miR-205-5p targeting PTK7-3’UTR, the promoter region of PTK7-3’UTR was designed and cloned into pMIR-report, including a WT (PTK7-3’UTR-WT) vector and Mut type (PTK7-3’UTR-Mut) vector (Fig. 2A). The PTK7-3’UTR-WT or PTK7-3’UTR-Mut luciferase reporter was co-transfected into HT29 and SW480 cells with the miR-control, miR-205-5p, inhibitor NC, and miR-205-5p inhibitor, respectively. The results of luciferase reporter gene assays indicated that miR-205-5p decreased the promoter activity of PTK7 in HT29 cells (P<0.05; Fig. 2B); and miR-205-5p decreased the promoter activity of PTK7 in SW480 cells (P<0.05; Fig. 2C). The results of the RT-qPCR analysis also showed that miR-205-5p decreased the mRNA expression level of PTK7 in the HT29 and SW480 cells; the inhibition of miR-205-5p by the inhibitor increased the mRNA expression level of PTK7 in the HT29 and SW480 cells (Fig. 2D). The results of the western blot
analysis indicated that miR-205-5p also decreased the protein expression levels of PTK7 in the HT29 and SW480 cells; the inhibition of miR-205-5p by the inhibitor increased the protein expression level of PTK7 in HT29 and SW480 cells (Fig. 2E).
miR-205-5p accelerates cell proliferation and inhibits apoptosis through PTK7 in CRC cells. The effects of miR-205-5p on the proliferation and apoptosis capacities of the CRC cells were investigated. The HT29 and SW480 cells were transfected with miR-control, miR-205-5p, miR-205-5p and vector, miR-205-5p and PTK7, respectively. The mRNA expression level of PTK7 was detected using RT-qPCR analysis. The results demonstrated that miR-205-5p decreased the mRNA expression level of PTK7 in the HT29 and SW480 cells; PTK7-transfection increased the mRNA expression level of PTK7 mediated by miR-205-5p (P<0.05; Fig. 3A). Simultaneously, the results of the western blot analysis showed that miR-205-5p inhibited the protein expression level of PTK7 in the HT29 and SW480 cells; PTK7-transfection increased the protein expression level of PTK7 mediated by miR-205-5p (Fig. 3B). Subsequently, the MTT assays indicated that miR-205-5p inhibited the proliferation ability of HT29 cells; the overexpression of PTK7 promoted the proliferation ability of HT29 cells mediated by miR-205-5p (P<0.05; Fig. 3C). It was also indicated that miR-205-5p inhibited the proliferation ability of SW480 cells; the overexpression of PTK7 promoted the proliferation ability of SW480 cells mediated by miR-205-5p (P<0.05; Fig. 3D). The apoptosis of cells was detected using Annexin V-FITC/PI staining. It was found that apoptosis was increased in HT29 and SW480 cells transfected with miR-205-5p and vector, compared with those transfected with miR-205-5p and PTK7 respectively. miR, microRNA; PTK7, protein-tyrosine kinase 7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide.
miR-205-5p promotes the migration and invasion abilities of CRC cells through PTK7. The effects of the expression of miR-205-5p on the migration and invasion abilities of CRC cells were detected in HT29 and SW480 cells transfected with miR-control, miR-205-5p, miR-205-5p and vector, and miR-205-5p and PTK7, respectively. The results showed that the invasion capacities of the HT29 and SW480 cells transfected with miR-205-5p were significantly decreased compared with those transfected with miR-control (P<0.05); the invasion capacities of the HT29 and SW480 cells transfected with miR-205-5p and PTK7 were significantly increased, compared with those transfected with miR-205-5p and vector (P<0.05; Fig. 3E).

miR-205-5p/PTK7 axis in CRC. The results of the present study confirmed the functional mechanism of PTK7-integrated miR-205-5p in CRC. The overexpression of miR-205-5p inhibited the proliferation, migration and invasion abilities of CRC through inhibition of the expression of PTK7. The decreased expression of miR-205-5p accelerated the proliferation, migration and invasion abilities of CRC through activation of the expression of PTK7 (Fig. 4E).

Discussion

Several studies have demonstrated that PTK7 is expressed at high levels in various types of cancer, including CRC,
liposarcoma, esophageal cancer, and gastric cancer (24,29-31). High expression levels of PTK7 have been associated with poor prognosis in esophageal cancer. PTK7 is involved in the proliferation, survival, invasion and migration of tumors, including CRC (32), esophageal cancer (30), and lung cancer (33). Furthermore, is have been shown that PTK7 may be a potential oncogene (30,31,34). However, the regulatory association between PTK7 and miRNA remains to be fully elucidated.

miRNAs, a class of small non-coding RNAs of 19-24 nucleotides, are differentially expressed in various types of cancer suggesting the important function of miRNAs in tumorigenesis (35-37). An increasing number of studies have indicated that miRNAs can act as oncogenes or tumor suppressors (36,37), and can affect the proliferation, metastasis, angiogenesis and inflammation of tumors by targeting miRNAs (38,39). There is evidence that various miRNAs are associated with the occurrence and development of CRC. For example, miR-21 has been found to stimulate invasion, intravasation and metastasis in CRC by downregulating Pdcd4 (40); miR-135b, as a downstream effector of oncogenic pathways, also promotes the progression of CRC (11) and, as a potential tumor suppressor, miR-25 has been found to be involved in CRC by targeting small mothers against decapentaplegic 7 (13).

miR-218 inhibits cell cycle and promotes apoptosis of CRC cells (41); miR-498 is downregulated in CRC and affects the functions of CRC cells (42). Furthermore, the expression of miRNAs can provide biomolecular and prognostic characteristics (43,44). However, the function of miR-205-5p in CRC remains to be fully elucidated.

In the present study, five potential miRNAs, including hsa-miR-409-5p, hsa-miR-205-5p, hsa-miR-495-3p, hsa-miR-5688 and hsa-miR-503-5p, were identified as potential target sites in the sequence of PTK7 3’UTR. It was indicated that the mRNA expression levels of PTK7 were decreased in HT29 and SW480 cells transfected with miR-205-5p, compared with levels in NC cells. In addition, there was a negative correlation between the gene expression of PTK7 and miR-205-5p in CRC tissues. It was confirmed that miR-205-5p directly regulated the transcriptional level of PTK7, with miR-205-5p simultaneously decreasing the mRNA and protein expression levels of PTK7 in HT29 and SW480 cells. It was also revealed that miR-205-5p accelerated the proliferation, migration and invasion abilities of CRC cells, and inhibited apoptosis through PTK7.

In conclusion, the results of the present study confirmed the functional mechanism of PTK7-integrated miR-205-5p in CRC. It was found that there was a negative correlation between miR-205-5p and PTK7 in CRC tissues. Furthermore, miR-205-5p was indicated to be involved in the processes of proliferation, apoptosis, migration and invasion in CRC through the regulation of PTK7.

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