Abstract. Colorectal cancer (CRC) is the third-most prevalent cancer and the fourth-most common cause of cancer-associated fatality worldwide. The expression and biological roles of microRNAs (miRNAs/miRs) in tumourigenesis, and their regulatory function in a number of biological processes correlated with cancer have been investigated. miR-383 has been reported to be deregulated in several human cancer types. However, the involvement and effects of miR-383 on CRC progression and its underlying mechanism remain unknown. Therefore, the present study aimed to examine miR-383 expression, investigate the biological functions of miR-383 and identify its mechanism of action in CRC cells. In the present study, miR-383 was significantly downregulated in CRC tissues and cell lines. Low miR-383 expression was negatively associated with tumour size, lymph node metastasis and TNM stage. Function experiments demonstrated that miR-383 upregulation inhibited the proliferation and invasion of CRC cells. Paired box 6 (PAX6) was confirmed as a direct target of miR-383. PAX6 was upregulated in CRC tissues and was negatively correlated with miR-383 expression. Induced PAX6 overexpression effectively rescued the tumour-suppressing roles of miR-383 on CRC cell proliferation and invasion. These findings suggested that miR-383 may act as a tumour suppressor in CRC by directly targeting PAX6 and may serve as a promising therapeutic target for CRC treatment.

Introduction

Colorectal cancer (CRC) is the third-most prevalent cancer and the fourth-most common cause of cancer-related deaths worldwide (1). Approximately 1.36 million new CRC cases and 694,000 deaths attributed to CRC are documented worldwide (2). In China, CRC ranks the fifth-most common cancer type (3). Several CRC risk factors, including older age, hereditary components, obesity, excess alcohol and red meat, smoking and a lack of physical exercise, have been validated (4-7). The diagnosis and treatment of patients with CRC have been considerably enhanced; however, their prognosis remains satisfactory (8). Local recurrence and distant metastasis are the main causes of the unfavourable prognosis of patients with CRC (9). Therefore, the molecular mechanisms associated with CRC occurrence and development should be understood to develop novel effective therapeutic strategies for patients with this disease.

MicroRNAs (miRNAs/miRs) are a class of single-stranded, endogenous, non-coding and short RNAs, which are typically composed of 21-25 nucleotides (10). miRNAs have been identified as novel gene expression regulators through their direct interaction with binding sites in the 3'-untranslated regions (3'-UTRs) of their corresponding target mRNAs. This process results in translational repression or target mRNA degradation (11). More than 1,000 miRNAs possibly exist in the human genome and can regulate as much as 60% of protein-encoding genes in humans (12). Dysregulated miRNA expression has been reported in numerous human diseases, such as neoplasm, metabolic disease, autoimmune disease, cardiovascular disease and nervous system disease (13-16). miRNAs are essential for various biological processes associated with tumourigenesis and tumour development, such as cell proliferation, cycle, apoptosis, migration, invasion, metastasis and angiogenesis (17-19). In human cancer, miRNAs may function as either oncogenes or tumour suppressors depending on the type of tumour (20). Hence, miRNAs can be investigated as diagnostic markers or possible therapeutic targets in various types of cancers.

miR-383 has been reported to be deregulated in several human cancers, including testicular embryonal carcinoma (21), ovarian cancer (22) and gastric cancer (23). However, the involvement and effects of miR-383 on CRC progression and its underlying mechanism remain unknown. Therefore, this study aimed to examine miR-383 expression, investigate the biological functions of miR-383 and identify its mechanism of action in CRC cells.

Materials and methods

Clinical tissue samples and cell lines. Forty-five clinical CRC tissues and the adjacent normal tissues were obtained from
patients who received surgical resection at The Eighth People's Hospital of Shanghai between July 2014 to October 2016. Patients who underwent radiotherapy or chemotherapy prior to surgery were excluded form this research. All clinical tissues were immediately frozen in liquid nitrogen at the time of surgery and stored at -80˚C until further use. This study was approved by the Ethics Committee of The Eighth People's Hospital of Shanghai. Written informed consent was also obtained from all participants.

The normal human colon epithelium cell line FHC was obtained from the American Type Culture Collection (Manassas, VA, USA). Six human CRC cell lines, SW480, SW620, HT29, HCT116, CaCo-2 and LoVo, were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and cultured at 37˚C in a humidified incubator containing 5% CO₂.

**Oligonucleotide and plasmid transfection.** miRNA mimics negative control (miR-NC) and miR-383 mimics were acquired from Shanghai GenePharma, Co., Ltd. (Shanghai, China). Paired box 6 (PAX6) overexpression plasmid (pcDNA3.1-PAX6) and empty plasmid (pcDNA3.1) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were seeded in 6-well plates at a density of 5x10⁵ cells per well. At 24 h later, cell transfection was carried out using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), in accordance with the protocol provided by the manufacturer. Cell culture medium was replaced with fresh DMEM medium containing 10% FBS at 8 h posttransfection.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A Nanodrop spectrophotometer (Nanodrop Technologies; Shanghai Sangon Co., Ltd.) was used to determine the quantity and the quality of total RNA. To quantify miR-383 expression, total RNA was reverse-transcribed into cDNA using a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform quantitative PCR with an ABI7300 thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). For PAX6 mRNA expression, cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corporation, Madison, WI, USA), followed by qPCR with SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The miR-383 and PAX6 mRNA expression levels were normalized to those of U6 snRNA and GAPDH, respectively. Relative expression was calculated using the 2^{-ΔΔCt} method (24).

**Cell Counting Kit-8 (CCK8) assay.** CCK8 assay was performed to evaluate CRC cell proliferation. At 24 h after transfection, cells were collected and seeded into 96-well plates at a density of 3x10⁴ cells/well. Cells were allowed to grow at 37˚C with 5% CO₂ for 0, 24, 48 and 72 h. At each time point, 10 μl of CCK8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into each well and incubated at 37˚C for another 2 h. The optical density (OD) was detected at a wavelength of 450 nm using a microplate reader (Bio-Rad, Laboratories, Inc., Hercules, CA, USA).

**Transwell invasion assay.** *In vitro* Transwell invasion assay was performed in 24-well Transwell chambers (8 µm pores; Costar; Corning Incorporated, Cambridge, MA, USA) precoated with Matrigel (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. At 48 h after transfection, cells were harvested and suspended into single cell solution. 5x10⁵ cells were seeded into the upper chamber. DMEM medium with 10% FBS as the chemotactic factor was added into the lower chambers. Following 24 h incubation at 37˚C with 5% CO₂, cells remaining on the upper membrane of the Transwell chambers were carefully removed with cotton swab, while invasive cells were fixed with 90% alcohol at room temperature for 15 min and stained with 0.05% crystal violet for 15 min at room temperature. Five random fields per chamber were photographed and counted under an IX71 inverted microscope (Olympus Corporation, Tokyo, Japan).

**Bioinformatic analysis and luciferase reporter assay.** Bioinformatic analysis was conducted to predict the potential targets of miR-383 using online prediction programs: microRNA.org (www.microrna.org) and TargetScan (www.targetscan.org/vert_60/). PAX6 was predicted as a candidate of miR-383. The 3'-UTR of PAX6 containing the miR-383 putative binding site and mutant site were designed and produced by Shanghai GenePharma, Co., Ltd, and subcloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) to construct pGL3-PAX6-3'-UTR wild type (Wt) and pGL3-PAX6-3'-UTR mutant (Mut). Cells were seeded into 24-well plates at a density of 1x10⁵ cells each well. After incubation overnight, miR-383 mimics or miR-NC was transfected into cells, together with pGL3-PAX6-3'-UTR Wt or pGL3-PAX6-3'-UTR Mut, using Lipofectamine 2000, according to the manufacturer's protocol. The luciferase activities were detected at 24 h posttransfection using the Dual-Luciferase assay system (Promega Corporation). Renilla luciferase activity was used to normalize the luciferase activity.

**Western blot analysis.** Total protein was extracted using radioimmunoprecipitation assay (RIPA) cell lysis buffer (Beyotime, Shanghai, China). The concentration of total protein was determined by Bicinchoninic Acid Protein Assay Kit (Beyotime). Equal amounts of protein were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Then the membranes were blocked with 5% skimmed milk in TBS containing 0.05% Tween-20 (TBST) for 2 h, and incubated overnight at 4°C with primary antibodies: Mouse anti-human monoclonal PAX6 antibody (1:1,000 dilution; sc-53106; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human monoclonal GAPDH (1:1,000 dilution; sc-32233; Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were washed with TBST and probed with
goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000 dilution; sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Finally, protein bands were visualized using electrochemiluminescent substrates (EMD Millipore), and analyzed with Sigma Photo Pro 6.0 software (SPSS, Inc., Chicago, IL, USA).

Statistical analysis. Data are presented as the mean ± standard deviation, and analyzed with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Student's t-test was used for comparisons between two groups, whereas more than two groups were compared by one-way ANOVA followed by SNK test. Spearman's correlation analysis was adopted to examine the association between miR-383 and PAX6 mRNA expression in CRC tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-383 is downregulated in CRC tissues and cell lines. To investigate the expression pattern of miR-383 in CRC, we measured its expression in 45 pairs of CRC tissues and adjacent normal tissues through RT-qPCR. In comparison with adjacent normal tissues, miR-383 expression was significantly downregulated in CRC tissues (Fig. 1A, P<0.05). Additionally, miR-383 expression levels in a normal human colon epithelium cell line FHC and six human CRC cell lines, consisting of SW480, SW620, HT29, HCT116, CaCo-2 and LoVo cell lines, were analysed. RT-qPCR data showed that miR-383 expression levels were significantly lower in CRC cell lines than that in FHC (Fig. 1B, P<0.05). These results suggested that miR-383 may play important roles in CRC progression.

Underexpression of miR-383 correlates with adverse clinical parameters of patients with CRC. To explore the clinical significance of miR-383 in CRC, 45 patients with CRC were divided into miR-383 low expression group (n=23) and miR-383 high expression group (n=22) using the medium value of miR-383 expression level as cutoff. As shown in Table I, miR-383 expression was associated with tumour size (P=0.025), lymph node metastasis (P=0.011) and TNM stage (P=0.001). miR-383 overexpression decreased cell invasion abilities compared with that of miR-NC group in SW620 and HCT116 cells (Fig. 2C, P<0.05). Collectively, these data suggested that miR-383 may play a tumour-suppressing role in CRC growth and metastasis.

PAX6 is a direct target of miR-383 in CRC. miRNAs exert functional roles mainly by base-pairing with the complementary sequence of their targets. Hence, we explored the direct targets of miR-383 in CRC. Bioinformatic analysis was performed to predict the potential target genes of miR-383. PAX6 (Fig. 3A), which has been previously aberrantly expressed in CRC and associated with CRC occurrence and development (25,26), was predicted as a major target of miR-383 and was selected for further confirmation. To confirm this hypothesis, luciferase reporter assays were conducted in SW620 and HCT116 cells co-transfected with miR-383 mimics or miR-NC, and pGL3-PAX6-3'-UTR Wt or pGL3-PAX6-3'-UTR Mut. Results revealed that miR-383 overexpression decreased the luciferase activities of pGL3-PAX6-3'-UTR Wt (Fig. 3B and C, P<0.05) but did not affect the luciferase activities of pGL3-PAX6-3'-UTR Mut in SW620 and HCT116 cells. To further investigate whether miR-383 can affect the endogenous expression of PAX6 in CRC, we transfected miR-383 mimics or miR-NC in SW620 and HCT116 cells and detected the mRNA and protein levels of PAX6. As shown in Fig. 3D and E, induced miR-383

<table>
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<td>26</td>
<td>12</td>
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<td>6</td>
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P<0.05; miR-383, microRNA-383; CRC, colorectal cancer.
Figure 1. miR-383 is downregulated in human CRC tissues and cell lines. (A) Relative miR-383 expression levels in 45 pairs of CRC tissues and adjacent normal tissues were detected through RT-qPCR. *P<0.05 compared with normal tissues. (B) miR-383 expression levels were measured in a normal human colon epithelium cell line FHC and six human CRC cell lines through RT-qPCR. *P<0.05 compared with FHC.

Figure 2. miR-383 overexpression suppresses CRC cell proliferation and invasion in vitro. (A) SW620 and HCT116 cells were transfected with miR-383 mimics or miR-NC. At 48 h later, RT-qPCR was performed to evaluate the transfection efficiency. *P<0.05 compared with miR-NC. (B) CCK8 assays were performed to assess the proliferation of SW620 and HCT116 cells transfected with miR-383 mimics or miR-NC. *P<0.05 compared with miR-NC. (C) Cell invasion capacities were evaluated by Transwell invasion assay in SW620 and HCT116 cells after transfection with miR-383 mimics or miR-NC. *P<0.05 compared with miR-NC. NC, negative control.
expression significantly reduced the mRNA (P<0.05) and protein (P<0.05) levels of PAX6 in SW620 and HCT116 cells. These data suggested that PAX6 is a novel target of miR-383 in CRC.

miR-383 level negatively correlates with the PAX6 level in CRC tissues. In order to further examine the association between miR-383 and PAX6 in CRC, the mRNA and protein expression levels of PAX6 in CRC tissues and the adjacent normal tissues were determined using RT-qPCR and western blot analysis. The results showed that PAX6 was obviously upregulated in CRC tissues at mRNA (Fig. 4A, P<0.05) and protein (Fig. 4B) levels compared with that in adjacent normal tissues. Spearman's correlation analysis further demonstrated that miR-383 level was negatively correlated with the mRNA expression level of PAX6 in CRC tissues (Fig. 4C; r=-0.6163, P<0.0001).

Restored PAX6 expression rescued the tumour-suppressive effects induced by miR-383 overexpression in CRC. To evaluate whether PAX6 is responsible for the functional roles of miR-383 in CRC cells, we performed rescue experiments. PAX6-overexpressing plasmid pcDNA3.1-PAX6 was transfected in SW620 and HCT116 cells overexpressing miR-383. Western blot analysis indicated that the ectopic expression of PAX6 restored the expression of the PAX6 protein that had been suppressed by the overexpression of miR-383 (Fig. 5A, P<0.05). Then, CCK8 and Transwell invasion assays were conducted to determine the cell proliferation and invasion of the cells. The results showed that restoration of PAX6 expression markedly rescued the suppression of cell proliferation (Fig. 5B, P<0.05) and invasion (Fig. 5C, P<0.05) induced by miR-383 overexpression in SW620 and HCT116 cells. These results make it clear that miR-383 exerted its suppressive role in CRC cells, at least in part, by regulation of PAX6.

Discussion

The expression and biological roles of miRNAs in tumourigenesis and their regulatory function in a number
of biological processes correlated with cancer have been investigated (27-29). Thus, further investigations on miRNAs involved in the tumourigenesis and tumour development of CRC may help improve diagnostics and more effective therapeutic methods for patients with this malignancy. In this study, the miRNA-383 expression in CRC tissues and cell lines was lower than that in adjacent normal tissues and the normal human colon epithelium cell line FHC. Low miR-383 expression was associated with tumour size, lymph node metastasis and TNM stage. Its upregulation reduced the proliferation and invasion of CRC cells. PAX6 was identified as a direct target of miR-383 in CRC, and its mRNA level was inversely correlated with the expression levels of miR-383 in the CRC tissues. PAX6 upregulation rescued the tumour-suppressing roles of miR-383 in CRC cells. Our results demonstrated that miR-383 may be a novel therapeutic target for patients with CRC.

miR-383 is aberrantly expressed in several cancer types. For example, the miR-383 expression level is lower in thyroid cancer tissues and cell lines, and its expression is significantly associated with the clinical stage and lymph node metastasis of thyroid cancer (30). In hepatocellular carcinoma, miR-383 expression is downregulated and correlated with tumour size and TNM stage. Kaplan-Meier analysis showed that low miR-383 expression is related to the poor overall survival of patients with hepatocellular carcinoma. Cox regression analysis identified miR-383 as an independent prognostic factor for patients with hepatocellular carcinoma (30). In glioma, miR-383 expression is reduced and is negatively

Figure 4. miR-383 level negatively correlates with the mRNA level of PAX6 in CRC tissues. (A) The mRNA and (B) protein expression levels of PAX6 in CRC tissues and adjacent normal tissues were determined by RT-qPCR and western blot analysis, respectively. *P<0.05 compared with normal tissues. (C) Spearman's correlation analysis showed an inverse relationship between miR-383 and PAX6 mRNA expression levels in CRC tissues. r=-0.6163, P<0.0001.
associated with pathological grading (31). In lung cancer, the miR-383 expression level is decreased in tumour tissues cell lines (32,33). miR-383 expression is associated with advanced TNM stages, positive lymph node metastasis and shorter overall survival for patients with non-small cell lung cancer (32). miR-383 is also downregulated in testicular embryonal carcinoma (21), ovarian cancer (22) and gastric cancer (23). These findings suggested that miR-383 may act as a diagnostic and prognostic biomarker in these cancer types.

The involvement of miR-383 has been demonstrated in the initiation and progression of certain cancer types. For instance, miR-383 upregulation inhibits thyroid cancer cell growth and metastasis in vitro and reduced tumourigenesis in a nude mouse xenograft model system (30). Fang et al (34) reported that miR-383 overexpression attenuates cell proliferation, invasion, glycolysis and promotes cell cycle arrest and cell apoptosis in hepatocellular carcinoma (30,34). Multiple studies found that the resumption miR-383 expression suppresses glioma cell proliferation, motility and angiogenesis and induces apoptosis in vitro (31,35-38). Shang et al (32) showed that ectopic miR-383 expression reduces lung cancer cell proliferation, metastasis in vitro and tumour growth in vivo (32,33). Huang et al indicated that miR-383 reexpression increased cell cycle arrest in testicular embryonal carcinoma (21). Han et al revealed that the restored miR-383 expression represses the proliferation, invasion and aerobic glycolysis of ovarian cancer cells (22). These findings suggested that miR-383 may be a candidate in the treatment of these cancers.

Several direct miR-383 targets, including AKT3 (30) in thyroid cancer; APRIL (30) and LDHA (34) in hepatocellular carcinoma; VEGF (35), CCND1 (36), IGF1R (37) and PRDX3 (38) in glioma; EPAS1 (33) in lung cancer; and PNUTS in testicular embryonal carcinoma (21), have been...
validated. In our current study, PAX6 was demonstrated to be a novel target of miR-383 in CRC. PAX6, a highly conserved transcription factor, contributes to the development of tissues, including those of the eyes, in the central nervous system and in endocrine glands of vertebrates and invertebrates (39,40). PAX6 is upregulated in various types of human cancers, such as gastric cancer (41), lung cancer (42), breast cancer (43) and retinoblastoma (44).

Functional assays indicated that PAX6 is involved in the regulation of multiple biological processes, including proliferation, cell cycle, apoptosis, invasion and angiogenesis (44-47). PAX6 is also highly expressed in CRC. PAX6 upregulation promotes cell proliferation, cell cycle progression, colony formation and invasion in CRC (25). Considering the possible correlation of PAX6 with aggressive CRC progression, we may explore PAX6 as a therapeutic target for patients with this disease.

In conclusion, miR-383 was downregulated in CRC and was correlated with tumour size, lymph node metastasis and TNM stage. miR-383 might serve as a tumour suppressor in CRC. According to our results, miR-383 upregulation might be a novel therapeutic strategy for patients with CRC.

References