miR-331-3p inhibits proliferation and promotes apoptosis by targeting HER2 through the PI3K/Akt and ERK1/2 pathways in colorectal cancer

DONGLI ZHAO¹, YANXIA SUI² and XIAOQIANG ZHENG¹

Departments of ¹Radiotherapy and ²Pathology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China

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Abstract. MicroRNAs (miRNAs) regulate cell proliferation, apoptosis and carcinogenesis by targeting related mRNAs in different types of cancer. miR-331-3p has been found to regulate the development and progression of various types of cancer cells. However, little research has been conducted on the role of miR-331-3p in colorectal cancer (CRC). The present study aimed to explore the function of miR-331-3p in CRC. We found that miR-331-3p was significantly downregulated in CRC tissues and cells compared to the level in healthy colon tissues and cells. Overexpression of miR-331-3p by transfection with pre-miR-331-3p inhibited cell proliferation, promoted apoptosis and activated caspase-3. Furthermore, the protein expression level of apoptosis-related protein Bcl-2 was downregulated and Bax was upregulated by pre-miR-331-3p. Downregulation of the expression of miR-331-3p by transfection with AS-miR-331-3p had the opposite effect. Moreover, we found that HER2 was overexpressed in the CRC cell lines, and the expression level of HER2 was negatively regulated by miR-331-3p. Additionally, knockdown of HER2 inhibited cell proliferation and phosphorylation of Akt and ERK1/2 induced by AS-miR-331-3p. Overall, we identified that miR-331-3p is underexpressed in CRC and contributes to cell growth regulation by targeting HER2 through activating the PI3K/Akt and ERK1/2 signaling pathways.

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world and the third leading cause of cancer-related death in both males and females (1). It was estimated that there were more than 1.4 million CRC cases and 693,900 deaths in 2012 worldwide (2). Smoking, red/processed meat consumption, obesity and excessive alcohol consumption are the modifiable risk factors for CRC (3). With a reduction in smoking and other modifiable risk factors, along with CRC screening and research into the molecular pathological epidemiology of CRC, decreased CRC mortality rates have been observed (4,5). However, the death rate is still high, and the molecular pathological epidemiology of CRC is not completely understood, thus further extensive investigations are required.

MicroRNAs (miRNAs) are 18-23-nucleotide small RNAs that function as negative regulators of gene expression through binding the 3' untranslated region (3'UTR) of mRNAs (6). Since evidence of the involvement of miR-15 and miR-16 in leukemia was initially reported, researchers have investigated the important roles of miRNAs in cancer (7,8). Subsequently, more and more miRNAs have been found to be involved in the development of human tumors (9). miRNAs can regulate cell proliferation, apoptosis and carcinogenesis by targeting related mRNAs in cancer (10). In CRC, eight miRNAs, including miR-25, miR-345, miR-7 and miR-331-3p, have been found to be significantly and differentially expressed when compared to the levels in healthy tissues (11).

miR-331-3p is a member of the miR-331 family, located on 12q22, and has been shown to be a tumor-suppressor miRNA in the prostate (12). Aberrant expression of miR-331-3p has been observed and found to be associated with proliferation and migration in lymphocytic leukemia (13), lung cancer (14), glioblastoma (15), gastric cancer (16) and prostate cancer (17). However, the role of miR-331-3p in the tumorigenesis of CRC remains unknown (18).

The human epidermal growth factor receptor (EGFR) family, which consists of EGFR (HER1 or ErbB1), HER2 (HER2/neu or ErbB2), HER3 (ErbB3) and HER4 (ErbB4), plays an important role in regulating cell proliferation, survival, and differentiation in cancers (19,20). HER2 is an important regulator of EGFR family signaling (21,22). Overexpression of HER2 is found in ~30% of all breast cancers and is frequently associated with poor prognosis, greater invasiveness and higher apoptotic resistance potential (23-26). It has been found that HER2 is a key factor in CRC, and cytoplasmic HER2 is
overexpressed in almost 30% of CRC patients (27). However, the prognostic value of HER-2 expression remains controversial in CRC. Thus, the present study was designed to validate the potential function of miR-331-3p in CRC.

Materials and methods

Human tissue samples. Human colon cancer tissue samples and normal adjacent colon tissues were obtained from 29 colon cancer patients before any therapeutic intervention at the First Affiliated Hospital of Xi’an Jiaotong University. All patients provided informed written consent. Samples were selected and stored in liquid nitrogen immediately after surgical resection. The study was approved by the Human Research Ethics Committee of the hospital.

Cell culture. Human colon cancer cell lines HCT-116, LoVo, HT-29, SW480, DLD-1 and Caco2 and the human normal colon epithelial cell line CRL-1831 (American Type Culture Collection, ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium containing 5% fetal bovine serum (FBS) (both from Gibco-BRL, Gaithersburg, MD, USA); 1% penicillin and streptomycin (Sigma-Aldrich, Castle Hill, NSW, Australia), and maintained in a humidified incubator at 37˚C with 5% CO₂.

Real-time quantitative polymerase chain reaction (RT-qPCR). MicroRNAs were isolated from the tissue samples and cells using the miRNeasy Mini kit (Qiagen, Valencia, CA, USA). One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology, Dalian, China) was used to synthesize cDNA. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) according to the standard protocol. RT-qPCR was performed using SYBR Green qPCR Master Mix (Thermo Fisher, Shanghai, China). U6 (Ribobio, Guangzhou, China) or β-actin was used as the normalizer for miRNA or mRNA, respectively. The data obtained were assessed using the 2⁻¹ΔΔCt method and evaluated by statistical analysis as described previously (28).

Plasmid vectors and transfection. HCT-116 cells were transfected with the miR-331-3p precursor (pre-miR-331-3p) or negative control RNA oligonucleotides (pre-miR-control) (both from Ambion Corporation, Austin, TX, USA); the miRNA inhibitor of miR-331-3p (AS-miR-331-3p) or the negative control (AS-miR-control); and HER2-siRNA (29) (sense, 5'-GGUGAAGGUGCUUGGAACUUU-3' and antisense, 5'-AGAUCAAGACCUCUACCCUU-3') or control siRNA using Lipofectamine 2000 according to the manufacturer's procedure (Invitrogen) and cultured for 48 h. The expression levels of miR-331-3p and HER2 in the HCT-116 cell line were assayed by RT-qPCR 48 h after transfection.

Caspase-3 activity assay. A caspase-3 assay kit (Abcam, Cambridge, MA, USA) was used to measure the enzymatic activity of caspase-3. HCT-116 cells transfected with pre-miR-331-3p or AS-miR-331-3p were seeded in a 24-well plate and cultured for 48 h. The cells were then harvested and the enzymatic activity of caspase-3 was detected according to the manufacturer's protocol.

Cell proliferation assay. HCT-116 cells transfected with pre-miR-331-3p or AS-miR-331-3p or HER2-siRNA or HER2-siRNA and AS-miR-331-3p were seeded in a 96-well plate. Twenty-four, 48, 72 and 96 h later, the medium was replaced by fresh medium, and 20 µl MTT (5 mg/ml) was added in each well and incubated for 4 h. Then, the medium was removed, and 200 µl DMSO was added. The OD490 was read after shaking for 10 min to fully dissolve the crystals.

Cell apoptosis assay. HCT-116 cells transfected with pre-miR-331-3p or AS-miR-331-3p or HER2-siRNA or HER2-siRNA and AS-miR-331-3p were seeded in 24-well plates and cultured for 48 h. Cells were harvested and the apoptotic cells were evaluated using the FITC-Annexin V apoptosis detection kit (BD Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Western blot analysis. HCT-116 cells transfected with pre-miR-331-3p or AS-miR-331-3p were seeded in a 6-well plate and cultured for 48 h. Cells were harvested and the total protein was extracted using mammalian protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). After measuring the concentration, the proteins were separated using 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% (v/v) dried milk and incubated with anti-HER2 (Abcam), anti-Bax, anti-Bcl-2, anti-Akt, anti-p-Akt, anti-ERK1/2 and anti-p-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4˚C overnight. The secondary HRP-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) was incubated for 1 h. β-actin (Cell Signaling Technology, Danvers, MA, USA) was used as the reference protein.

 Luciferase activity assay. The human HER2 wild-type 3'UTR containing the miR-331-3p binding site and mutated HER2-3'UTR sequence (18) were constructed into the pGL3 luciferase reporter plasmid (Promega, Madison, WI, USA). HCT-116 cells were plated in 24-well plates and co-transfected with 10 nM of either pre-miR-331-3p or the pre-miR-control, and 500 ng of pGL3-HER2 or pGL3-mutHER2 according to the manufacturers' protocols for the use of Lipofectamine (Invitrogen) and the luciferase assay kit (Promega) (16). Cells were collected and cell lysates were assayed for luciferase activity using a dual-luciferase reporter assay kit (Promega) 48 h after transfection.

Statistical analysis. Results are presented as the mean ± standard deviation. One-way analysis of variance and the Student's t-test were used to analyze differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-331-3p is downregulated in human colon cancer tissues and cells. To explore the role of miR-331-3p in human colon cancer, the expression level of miR-331-3p in human colon cancer tissues and cells was measured using RT-qPCR.
The results showed that the expression level of miR-331-3p was significantly downregulated in the colon cancer tissues compared to this level in the normal tissues (P<0.05, Fig. 1A). Compared with the normal colon epithelial cell line CRL-1831, the expression level of miR-331-3p was significantly lower in all of the colon cancer cell lines, including HCT-116, LoVo, HT-29, SW480, DLD-1 and Caco2 (P<0.05, Fig. 1B).

miR-331-3p inhibits colon cancer cell proliferation. To verify the effect of miR-331-3p on the growth of colon cancer, we examined the impact of miR-331-3p overexpression and suppression on the proliferation of colon cancer cells. HCT-116 cells were transfected with pre-miR-331-3p or AS-miR-331-3p and cultured for various time periods. The MTT assay was then used to evaluate the proliferation of the colon cancer cells. The results revealed that pre-miR-331-3p significantly upregulated and AS-miR-331-3p downregulated the expression of miR-331-3p (P<0.05, Fig. 2A). The MTT assay showed that pre-miR-331-3p inhibited and AS-miR-331-3p promoted the proliferation of the HCT-116 cells in a time-dependent manner (P<0.05, Fig. 2B).

miR-331-3p promotes the apoptosis of the colon cancer cells. To further confirm the effect of miR-331-3p on the tumorigenesis of colon cancer, the apoptotic ratio of the HCT-116 cells transfected with pre-miR-331-3p or AS-miR-331-3p was examined by FITC-Annexin V staining. The results show that overexpression of miR-331-3p significantly promoted apoptosis while suppression of miR-331-3p inhibited apoptosis (P<0.05, Fig. 3A). To further study the effect...
of miR-331-3p on colon cancer cell apoptosis, the activity of caspase-3 was also determined. As expected, pre-miR-331-3p significantly increased the activity of caspase-3 and AS-miR-331-3p had the opposite effect (P<0.05, Fig. 3B). Additionally, the protein expression levels of the apoptosis-related proteins Bcl-2 and Bax were also measured; the results demonstrated that pre-miR-331-3p decreased the protein expression level of Bcl-2 and increased the protein expression level of Bax, while AS-miR-331-3p increased the protein expression level of Bcl-2 and decreased the protein expression level of Bax (P<0.05, Fig. 3C and D).

**miR-331-3p directly downregulates the expression of HER2 in colon cancer cells.** The expression level of HER2 in the human colon cancer cells was significantly higher than that in the normal cells, which was inversely related to the expression of miR-331-3p (P<0.05, Fig. 4A and B). Next, we explored the relationship between miR-331-3p and HER2 by transfecting cells with pre-miR-331-3p and AS-miR-331-3p. The western blot analysis shows that pre-miR-331-3p significantly downregulated and AS-miR-331-3p significantly upregulated the protein expression level of HER2 in the HCT-116 cells (P<0.05, Fig. 4C), suggesting that miR-331-3p downregulates the expression of HER2 in colon cancer cells. To examine whether HER2 is a direct target of miR-331-3p in colon cancer cells, the human HER2 wild-type 3'UTR containing the miR-331-3p binding site and mutated HER2 3'UTR sequence were cloned into modified pGL-3
luciferase reporter vectors, which were co-transfected into the HCT-116 cells with pre-miR-331-3p and AS-miR-331-3p. The results showed that miR-331-3p overexpression significantly reduced the luciferase reporter activity in the pGL3-HER2-3’UTR transfected cells, compared to pGL3-mut HER-3’UTR, whereas the luciferase activity was not affected by the pre-miR-control (P<0.05, Fig. 4D).

miR-331-3p exerts its function by targeting HER2. HER2 plays an important role in cell proliferation and cell survival during the development of cancer (30). The results of RT-qPCR in our study showed that HER2-siRNA significantly downregulated the expression level of HER2 in the HCT-116 cells transfected with pre-miR-331-3p or AS-miR-331-3p. The results of the FITC-Annexin V assay showed that pre-miR-331-3p reduced apoptosis, while HER2-siRNA and the combination of HER2-siRNA and pre-miR-331-3p promoted apoptosis (P<0.05, Fig. 5C).

miR-331-3p suppresses the PI3K/Akt and ERK1/2 signaling pathways. Since HER2 plays important roles in mediating activation of the PI3K/Akt and ERK1/2 pathways (31,32), we aimed to ascertain whether miR-331-3p triggers suppression of HER2, caused by the regulation of PI3K/Akt and ERK1/2 signaling in human CRC cells. As expected, transfection of pre-miR-331-3p significantly inhibited the ratios of the levels of phosphorylated to total protein (arbitrary units) of Akt and ERK1/2, which were both increased by AS-miR-331-3p. HER2-siRNA significantly decreased the arbitrary units of p-Akt and p-ERK1/2. After treatment with HER2-siRNA, the activating effect of AS-miR-331-3p on the arbitrary units of
miR-331-3p mediates cell growth in colorectal cancers.

Figure 5. miR-331-3p inhibits cell proliferation and promotes apoptosis by targeting HER2. (A) Western blot analysis of HER2 protein levels in the HCT-116 cells transfected with control siRNA or HER2-siRNA. (B) Cell proliferation was detected by MTT assay in the HCT-116 cells transfected with AS-miR-331-3p or HER2-siRNA or AS-miR-331-3p and HER2-siRNA. (C) Apoptosis was detected by Annexin V-FITC assay in the HCT-116 cells transfected with AS-miR-331-3p or HER2-siRNA or AS-miR-331-3p and HER2-siRNA. Bars in the histograms represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control, #P<0.05 vs. AS-pre-miR-331-3p.

Figure 6. miR-331-3p suppresses the PI3K/Akt and ERK1/2 signaling pathways. (A) Image of western blot analysis of total Akt, p-Akt, total ERK1/2 and p-ERK1/2 protein levels in the HCT-116 cells transfected with pre-miR-331-3p, HER2-siRNA or AS-miR-331-3p and HER2-siRNA. (B) Quantitative arbitrary units of intensity relative to the control value of p-Akt. (C) Quantitative arbitrary units of intensity relative to the control value of ERK1/2 were normalized to β-actin. Bars in the histograms represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control, #P<0.05 vs. AS-pre-miR-331-3p.
p-Akt and p-ERK1/2 were abolished and the arbitrary units of p-Akt and p-ERK1/2 were significantly lower than these values in the control (P<0.05, Fig. 6).

Discussion

In the present study, we provide evidence that miR-331-3p is downregulated in CRC tissues and cells. Overexpression of miR-331-3p plays an important role in inhibiting proliferation and promoting apoptosis by inhibiting the expression of HER2 and deactivation of the PI3K/Akt and ERK1/2 signaling pathways in CRC cells. These results suggest an important role of miR-331-3p in regulating tumor progression of CRC.

miRNAs function as oncogenes or tumor suppressors in carcinogenesis, and dysregulation of these miRNAs is believed to be a common feature of human cancers (33). miRNAs are generally overexpressed or suppressed in tumor tissues, compared with corresponding healthy tissues (34,35). Increasing evidence has demonstrated that miRNAs are crucial regulators of the progression of cancer, with potential use in cancer diagnosis, prognosis, prediction, and therapy (9,36). miR-331-3p is regarded as a cancer-associated miRNA as studies have reported its correlation with prostate cancer, hepatocellular carcinoma, and gastric cancer (15,16,37). In a study by Chang et al. (38) on hepatocellular carcinoma, miR-331-3p was overexpressed in human hepatocellular carcinoma tissues and was found to be correlated with poor long-term survival. These authors also demonstrated that miR-331-3p can promote the proliferation and metastasis of hepatocellular carcinoma. Based on these results, they suggested that miR-331-3p may be a potential prognostic biomarker and a novel therapeutic target. Our study found that miR-331-3p was suppressed both in CRC tissues and cells compared with healthy colorectal tissues and cells, which is consistent with the research of Guo et al. (16) who found that miR-331-3p was downregulated in gastric cancer cell lines. To further understand the role of miR-331-3p in CRC, the miR-331-3p precursor or miRNA inhibitor of miR-331-3p was used to upregulate or downregulate the expression of miR-331-3p, and then cell proliferation and apoptosis were assessed. The results showed that overexpression of miR-331-3p significantly inhibited cell proliferation and promoted apoptosis, suggested a crucial role of miR-331-3p in the development of CRC. Together with these results, we confer that the role of miR-331-3p is inconsistent in different types of cancers. HER2 is overexpressed in a high percentage of CRC cell lines and has been shown to play an oncogenic role in human tumors (39). HER2 can activate signaling pathways including ERK1/2, STAT3, mTOR and Akt, which play important roles in cell proliferation and survival (31,40,41), and therefore is crucial to the development of cancer. Ross and McKenna (42) showed that overexpression of HER2 is associated with approximately one fourth of all gastrointestinal tract malignancies. Li et al. (29) found that the expression level of HER2 was elevated in seven out of eight CRC cell lines. Our study measured the expression level of HER2 in six frequently used CRC cell lines, and all showed upregulated expression of HER2, which suggests that HER2 may be an important factor in the development of CRC.

miRNAs regulate a variety of cellular pathways through targeting the expression of multiple target genes (6). Several lines of evidence indicate that E2F1 and neuropilin-2 are miR-331-3p targets (15,16). In the present study, we showed that miR-331-3p regulated cell proliferation by inhibiting the expression of HER2 in CRC, which is consistent with a study by Epis et al. (18), who also concluded that miR-331-3p has the capacity to regulate the development and progression of prostate cancer cells by targeting HER2. PI3K/Akt and ERK1/2 are two important downstream pathways of HER2, and link HER2 to its biological functions (43). While in the study by Epis et al., only the PI3K/Akt signaling pathway was discussed, our study focused on both the PI3K/Akt and ERK1/2 signaling pathways. The results showed that Akt and ERK1/2 were both deactivated by HER2-siRNA and pre-miR-331-3p, and were activated by AS-miR-331-3p. Further research found that the activity of Akt and ERK1/2 were downregulated by the combined action of HER2 and miR-331-3p. PI3K/Akt and ERK1/2 are crucial pathways in regulating cell survival in cancer. These results, taken together, showed that miR-331-3p inhibits proliferation and promotes apoptosis by suppressing HER2 and deactivating the PI3K/Akt and ERK1/2 signaling pathways.

In conclusion, our study demonstrated that miR-331-3p is suppressed in CRC and overexpression of miR-331-3p inhibits cell proliferation and induces apoptosis by targeting HER2 via activation of the PI3K/Akt and ERK1/2 signaling pathways. Our findings suggest that miR-331-3p plays an important role in the development and progression of CRC.

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