RESEARCH ARTICLE

MiR-1297 Regulates the Growth, Migration and Invasion of Colorectal Cancer Cells by Targeting Cyclo-oxygenase-2

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Abstract

Cyclo-oxygenase-2(Cox-2), a key regulator of inflammation-producing prostaglandins, promotes cell proliferation and growth. Therefore, a better understanding of the regulatory mechanisms of Cox-2 could lead to novel targeted cancer therapies. MicroRNAs are strongly implicated in colorectal cancer but their specific roles and functions have yet to be fully elucidated. MiR-1297 plays an important role in lung adenocarcinoma and laryngeal squamous cell carcinoma, but its significance in colorectal cancer (CRC) has yet to be reported. In our present study, we found miR-1297 to be down regulated in both CRC-derived cell lines and clinical CRC samples, when compared with normal tissues. Furthermore, miR-1297 could inhibit human colorectal cancer LOVO and HCT116 cell proliferation, migration, and invasion *in vitro* and tumorigenesis *in vivo* by targeting Cox-2. Moreover, miR-1297 directly binds to the 3`-UTR of Cox-2, and the expression level was drastically decreased in LOVO and HCT116 cells following overexpression of miR-1297. Additionally, Cox-2 expression levels are inversely correlated with miR-1297 expression in human colorectal cancer xenograft tissues. These results imply that miR-1297 has the potential to provide a new approach to colorectal cancer therapy by directly inhibiting Cox-2 expression.

Keywords: Cox-2 - miR - miR1297 - colorectal cancer - HCT116 cells - LOVO cells

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the gastrointestinal tract. With an estimated 140 thousand new cases and over 50 thousand deaths in 2013, colorectal cancer (CRC) is the fourth most common cancer that leads to mortality in the United States (Committee et al., 2013). In addition, the incidence and mortality of colorectal cancer has continued to rise rapidly in China over the past few decades. The incidence of CRC has increased by an annual average of 5.73% from 1992 to 2005 (13.06 to 23.54/100000) in the Nangang District of Harbin, China (Hu et al., 2013). Though the exact mechanism of CRC is still unknown, it has been well established that smoking, obesity, red meat consumption, excessive alcohol consumption, and chronic intestinal inflammation are risk factors for CRC; (Wang et al., 2010; Theodoratou et al., 2012; Burn et al., 2013). Of these risk factors, chronic intestinal inflammation has been considered one of the most significant causes of colon cancer (Wang and Dubois, 2010).

Many studies have confirmed the relationship of cyclooxygenase-2 (Cox-2) and the CRC development and progression leading to chronic inflammation (Williams et al., 1999). Cox-2 is an early inflammation response gene, previously shown to be up-regulated in 40-50% of colorectal adenomas and 85% of CRC, leading to the extracellular accumulation of prostaglandins (PGs) (Eberhart et al., 1994). Cox-2-derived PGE2, the major PG produced in colorectal tumors, contributes to the onset of cancer, by stimulating cell proliferation, invasiveness, and migration, as well as enhancing angiogenesis, evading apoptosis, and modulating the antitumor immune response (Wang et al., 2005; Pereira et al., 2014). Therefore, the regulation of Cox-2/PGE2 signaling may serve as a novel target for CRC treatment.

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules (19~22 nucleotides) that regulate post-transcriptional gene expression. By binding with partially complementary sequences of messenger RNA (mRNA), miRNAs target mRNA molecules for degradation and/or inhibit translation, thereby decreasing the expression of proteins (Kim et al., 2009; Zhang et al., 2014). Several studies have been focused on the impact of miRNAs in tumor development, and have implicated that these molecules play a role in cancer development. MiRNAs are known to act as both regulators and targets of oncogenes, and can also act as tumor suppressor genes, depending on its modulation of gene expression in specific tissues, which leads to a change in protein levels of tumor suppressors or oncogenes (Kong et al., 2012; Malumbres, 2012).

In previous research, our lab has used miRNA microarrays to detect abnormal expression of miRNAs in CRC specimens, one of which is miR-1297 (Microarray

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data is published in an additional article). The expression of miR-1297 in CRC tumors was significantly lower than those in corresponding normal tissues. So we focus on the function of miR-1297 in CRC. In this study, we confirmed the expression of miR-1297 in CRC specimens and CRC cell lines by qRT-PCR. Next, we assessed the clinical significance of miR-1297 in colorectal cancer, as well as investigated the effects of miR-1297 on CRC cells growth, migration, and invasion. Lastly, we will further discuss the mechanism of action of miR-1297 by identifying its target gene Cox-2.

Materials and Methods

Patient specimens

Clinical CRC samples were obtained from Zhongshan Hospital, Fu Dan University, Shanghai, China. Written informed consents were obtained from the subjects who participated in this study. The tissue samples were collected and used after obtaining the approval from the Ethics Committee of Zhongshan Hospital. The demographic and clinical features of the colorectal cancer (CRC) carcinoma tissues and the corresponding normal tissues are presented in Table 1.

Cell lines and reagents

Human colorectal cancer cells (LOVO, HCT116, SW480, SW-620, HT29 and HCT8) were purchased from the American Type Culture Collection (ATCC, VA, USA), cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, UT, USA), supplemented with 10% heated-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 10μg/mL streptomycin sulfate and then held at 37°C in a humidified 5% CO_2 incubator. Live/Dead Double Staining Kit (QIA76) was purchased from Merck (NJ, USA). SYBR Premix Ex Taq kit and Trizol were ordered from Takara (Dalian, China). Other laboratory reagents were obtained from Sigma (Shanghai, China).

MiRNA mimic or inhibitor transfection

All cells were seeded into 6-well, 12-well, 24-well, or 96-well plates at 90% confluence and incubated at 37°C and 5% CO_2 overnight. The miR-1297 mimic, miR-1297 negative control mimic (NC mimic), and miR-1297 inhibitor were purchased from Gene Pharma (Shanghai, China). Varying amounts of miR-1297 mimic, NC mimic, and miR-1297 inhibitor were transfected into HCT116 and LOVO cells using Lipofectamine 2000 (Invitrogen, CA,USA). The transfected cells were incubated at 37°C in a 5% CO_2 incubator for 24 or 48 h. Total cellular RNA and protein were harvested separately and stored at -80°C until use. The mimic for mir-1297 has the following sequence: 5'-UUCAAGUAAUUCAGGUG-3` and the inhibitor for mir-1297 sequence is as follows: 5'-UUCAAGUAAUUCAGGUG-3`.

Table 1. Demographic and Clinical Features of the Colorectal Cancer (CRC) Carcinoma Tissues and the Corresponding Normal Tissues

Characteristics	CRC cases	n=20
Age (year, Mean±SD)		61.45±2.43
Gender	Male	12
	Female	8
TNM stage	I	2
	II	6
	III	9
	IV	3
Location	Colon	7
	Rectum	13
Histological grading	Well, moderate	9
	Poor, mucinous	11
Lymph node metastasis	Negative	6
	Positive	14

well: well-differentiated, moderate: moderately differentiated, poor: poorly differentiated, mucinous: mucinous carcinoma

SYBR green quantitative PCR analysis

1 mg of total RNA containing small RNA extracted from tissue samples was first polyadenylated by poly (A) polymerase and then reverse transcribed to cDNA using a mixture of oligo (dT) adaptor provided in the kit. Mature miR-1297 expression in cells and tissues was detected using the Hairpin-it TM miRNAs qPCR kit (GeneCopoeia, MD, USA). The primers for β-actin (forward primer: 5·-TGGAGTCCTGTGGCATCCACGAAA-3·, reverse primer: 5·-TGTAACGCAACTAAGTCATAGTCCG-3·), Cox-2 (forward primer: 5`- TTACAATGCTGACTATGG CTAC -3`, reverse primer: 5`- CTGATGCGTGAAGTG CTG-3`) and miR-1297 (forward primer: 5`- GCGGCGGTTCAAGTAATTCAGG-3`, reverse primer: 5`- ATCCAGTGCAGGGTCCGAGG-3`) were purchased from BioSune (Shanghai, China). Expression of U6 was used as an endogenous control. SYBR PCR was performed in an MX3005p Fast Real-time PCR system (Stratagene, CA, USA). Each sample was analyzed in triplicates.

Cell viability assay

The transfected HCT116 and LOVO cells ($2*10^4$ cells/well) were seeded in 96-well plates and the cell proliferation assay was performed on days 1, 2, 3and 4. Cell viability was determined by MTT (Sigma, Shanghai,China) with a Bio-Radmicroplate reader.

Wound healing assay

The transfected HCT116 and LOVO cells were seeded into full confluence in 6-well plates and then were incubated with 10μg/mL mitomycin C for 2 hours to inactivate cell proliferation. Cells were washed with PBS and wounds were made by sterile pipette tips. DMEM supplemented with 0.5% FBS was added into wells. Cell images were taken after 10 hours of incubation with a Live/Dead Double Staining Kit (Merck, NJ, USA).

Transwell migration assay

The transwell (Corning Incorporated, NY, USA) were coated with 50μl 1:8 diluted Matrigel (Growth factor reduced, BD Biosciences, MD, USA) for 30 min in the cell incubator. The bottom chambers of transwell were filled with DMEM with 0.5% FBS and the top chambers were seeded with mitomycin C, then inactivated, with $4×10^4$ cells/well in 100μL DMEM (0.5% FBS). After 12h, the cells on the top surface of the membrane (non-migrated cells) were scraped with a cotton swab and the cells that migrated onto the bottom sides of the membrane (invasive

cells) were fixed with cold 4% paraformaldehyde and stained with crystal Violet. Images were taken using an OLYPUS inverted microscope.

Xenograft mouse model

Xenograft mouse model was used to evaluate the effect of miR-1297 regulated tumor growth as described by Yi et al (Yi et al., 2008). Specific pathogen-free female athymic BALB/c nude mice, aged 4-6weeks old (20-30 g), were obtained from SLAC Laboratory Animal (Shanghai, China) . Mice were divided into three groups with 6 animals for each group: NC group, miR-1297 mimic group and miR-1297 inhibitor group. The transfected LOVO or HCT116 cells, 5×10^6 cells per mouse, were s.c. injected into the mice. The mouse body weight and tumor sizes were recorded every two days and the tumor sizes were determined by Vernier caliper measurements and calculated as 1/2×length×width×height. After 21 days, the mice were sacrificed. Animals used in the present study were approved by the Ethics Committee of Fudan University where the work was undertaken and that it conforms to the provisions of the Declaration of Helsinki in 1995.

Protein isolation and western blotting

For the protein expression analyses, standard western blotting was carried out. Cultured or transfected cells were washed twice with cold phosphate-buffered saline (PBS) and were lysed on ice in RIPA buffer with proteinase inhibitors (Roche, Shanghai, China). Protein lysates were resolved on 10% SDS polyacrylamide gel, transferred to nitrocellulose membranes and blocked in 0.1% Tween 20 and 5% BSA in Tris Buffer Saline. Proteins were probed with rabbit anti-Cox-2 monoclonal antibody (1:1000, Cell Signaling Technology, MA, USA) and rabbit anti-beta-actin antibody (1:10000, Sigma, Shanghai, China) overnight at 4°C. The membrane was washed and visualized with horseradish peroxidase (HRP) - conjugated secondary antibodies for 1h. Signals were detected by enhanced chemiluminescence (Odyssey, MI, USA).

Immunochemistry

The tumors were removed and fixed with formalin and embedded with paraffin. Cox-2 were performed on the 5μm sections with according antibody (1:200, Cell Signaling Technology, MA, USA). Images were taken with Olympus IX71 microscope.

Statistical analysis

The data are presented as mean ± SE, and statistical comparisons between groups were performed using T-test. The *P* value of ≤0.05 was considered statistically significant.

Results

MiR-1297 is frequently down-regulated in tissues and CRC cell lines

The expression level of miR-1297 was examined in CRC specimens and the corresponding normal tissues. The average expression level of miR-1297 was significantly lower in CRC specimens compared with adjacent normal tissues ($P<0.01$) (Figure 1a). We also determined the expression level of miR-1297 in different human CRC cell lines by qRT-PCR. Compared with the normal intestinal epithelial cell line, the expression level of miR-1297 was lower in the six examined CRC cell lines (Figure 1b).

MiR-1297 inhibited CRC cells proliferation

To test the function of miR-1297 in CRC cells, stable cell lines LOVO and HCT116 expressing miR-1297 (miR-1297 mimic) and negative control (NC) were established by Lipo2000 transduction. We examined the effect of miR-1297 on the proliferation of LOVO and HCT116 cells from 0h to 96h ($P<0.05$). We observed that over-expression of miR-1297 dramatically suppressed the proliferation of LOVO and HCT116 cells. On the contrary, using a miR-1297 inhibitor promoted the proliferation of LOVO and HCT116 cell ($P<0.05$) (Figure 1c).

MiR-1297 inhibited CRC cell migration and invasion

To test the function of miR-1297 in CRC cells, the cell migration and invasion were determined. Overexpression of miR-1297 in LOVO cells significantly suppressed cell migration ($P<0.01$) and cell invasion ($P<0.01$), whereas loss of its expression promoted LOVO cellular migration ($P<0.01$) (Figure 2a) and cellular invasion ($P<0.01$) (Figure 2b). These observations suggest that miR-1297 plays an important role in inhibiting migration and invasion of CRC cells.

MiR-1297 inhibits tumor growth in vivo

In order to further explore the role of miR-1297 in tumor growth in vivo, we established the human colorectal cancer xenograft model. LOVO (miR-1297 mimic) cells, LOVO (NC) cells, HCT116 (miR-1297 mimic) cells and HCT116 (NC) cells were injected subcutaneously to the blank of nude mice, respectively, into nude mice. The tumor growth rate of LOVO (miR-1297 mimic) and HCT116 (miR-1297 mimic) were slower than that of LOVO (NC) cells and HCT116 (NC) cells. As shown in Figure 3, 3 weeks after injection, tumors in LOVO (NC)

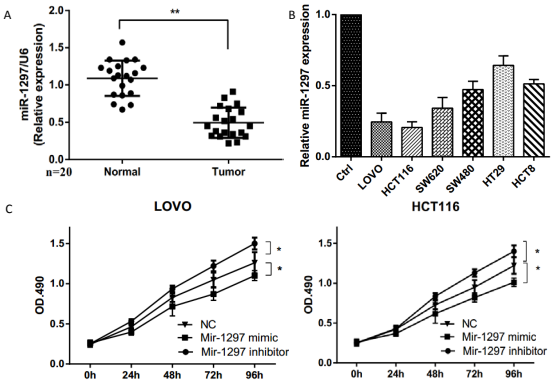

Figure 1. MiR-1297 is Frequently Down-Regulated in Tissues and CRC Cell Lines and it Inhibited CRC Cell Proliferation. (a) The expression level of miR-1297 was examined in CRC specimens and the normal tissues. (b) The expression level of miR-1297 in different human CRC cell lines. (c) MiR-1297 inhibited CRC cell proliferation. *($P<0.05$, **$P<0.01$, versus control)

and HCT116 (NC) grew rapidly (from 193.66±29.87 to 1527±194.65mm3 and form 177.29±25.44 to 1173.19±122.47 mm3), whereas tumors in LOVO (miR-1297 mimic) and HCT116 (miR-1297 mimic) groups grew much slower (from 155.25±19.91 to 578.23±72.41 mm3 and from 168.51±27.13 to 654.12±95.18 mm3) (Figure 3a). Accordingly, the average tumor weight in miR-1297 mimic groups mice was significantly lower compared with that in the NC group (Figure 3b), suggesting that miR-1297 strongly inhibited tumor growth.

Cox-2 is a target for miR-1297 in CRC cells

In order to identify downstream targets of miR-1297, we used three miRNA target prediction programs, i.e., miRBase, Targetscan, and DAVID, to identify potential targets. Cox-2 is one of the predicated targets of miR-1297. A putative miR-1297 binding site that perfectly matched nucleotides was defined at the 3`-UTR of Cox-2 (Figure 4a). Overexpression of miR-1297 significantly inhibited Cox-2 mRNA and protein levels in LOVO and HCT116 cells (Figure 4b, 4c). Furthermore, Cox-2 level was inversely correlated with miR-1297 expression in the human colorectal cancer xenograft tissues (Figure 4d).

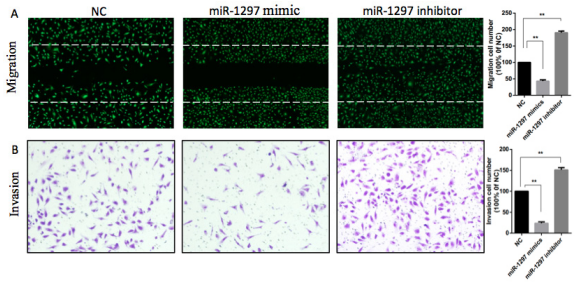

Figure 2. MiR-1297 Inhibited CRC cellular migration and invasion. (a) Overexpression of miR-1297 in LOVO cells significantly suppressed cellular migration (P<0.01), but loss of its expression promoted LOVO cellular migration (P<0.01). (b) Overexpression of miR-1297 in LOVO cells significantly suppressed cellular invasion (P<0.01), but loss of its expression promoted LOVO cellular invasion (P<0.01). **(P< 0.01, versus control)

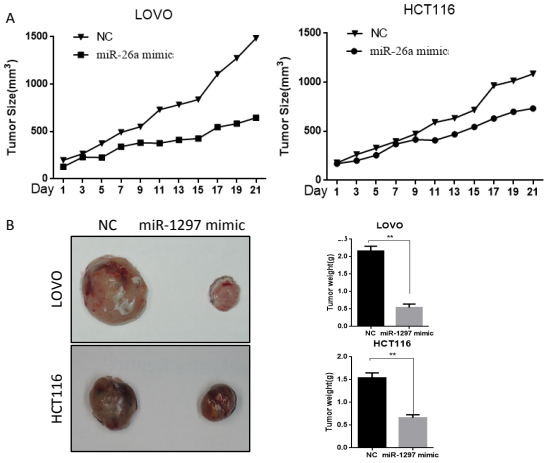

Figure 3. MiR-1297 Inhibits Tumor Growth *In Vivo*. (a) 3 weeks after injection, tumors in LOVO (NC) and HCT116 (NC) groups grew rapidly (from 193.66±29.87 to 1527±194.65 and form 177.29±25.44 to 1173-.19±122.47), whereas tumors in LOVO (miR-1297 mimic) cells and HCT116 (miR-1297 mimic) cells groups grew much slower (from 155.25±19.91 to 578.23±72.41 and from 168.51±27.13 to 654.12±95.18). (b) The average tumor weight in miR-1297 mimic group mice was significantly lower compared with that in the NC group

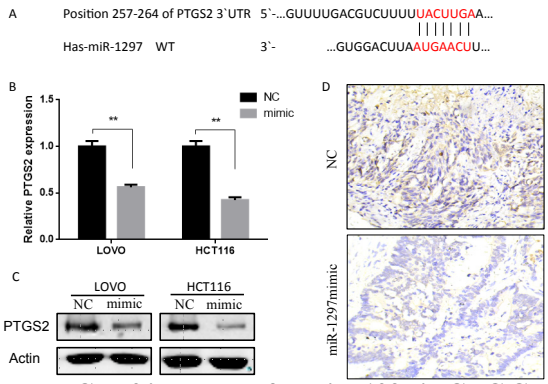

Figure 4. Cox-2 is a Target for miR-1297 in CRC Cells. (a) Cox-2/PTGS2 is one of the predicated targets of miR-1297. (b,c) Overexpression of miR-1297 significantly inhibited Cox-2 mRNA and protein levels in LOVO and HCT116 cells. (d) Cox-2 levels are inversely correlated with miR-1297 expression in the human colorectal cancer xenograft tissues

Discussion

Research in animal models gave us direct molecular evidence that Cox-2 plays a key role in colorectal cancer (Wang and Dubois, 2010). Genetic studies demonstrate that deletion of the Cox-2 gene results in decreased tumor formation in both the small intestine and colon of $Apc^{min/+}$ mice (Oshima et al., 1996; Chulada et al., 2000). Several studies also reported that the Cox-2 expression was elevated in colorectal tumors, in comparison to normal colorectal tissue (Sano et al., 1995). Since elevated Cox-2 expression was found in most colorectal cancer tissue and is associated with decreased likelihood of survival among CRC patients, investigators have sought to evaluate selective Cox-2 inhibitors (such as Celecoxib) on CRC prevention and treatment (Lin et al., 2006; Chan et al., 2011; Xie et al., 2012). Our results illustrated that miR-1297 could directly bind to the 3`-UTR of Cox-2 and the expression level of Cox-2 drastically decreased in LOVO and HCT116 cells following the overexpression of miR-1297. Furthermore, Cox-2 levels were inversely correlated with miR-1297 expression in human colorectal cancer xenograft tissues.

Many studies have revealed miRNAs could influence colorectal tumor growth, migration, invasion, angiogenesis, metastasis, diagnosis and prognosis (Dang et al., 2014; Yin et al., 2014; Zhao et al., 2014). Li et al showed that miR-451 is downregulated in colorectal carcinoma and has a repressive role in CRC cells by inhibiting cell growth through down-regulating the P13K/AKT pathway (Li et al., 2013). Wang et al reports that miR-150-5p may suppress CRC metastasis through directly targeting MUC4, highlighting its potential as a novel agent for the treatment of CRC metastasis (Wang et al., 2014). The role of miR-1297 in lung adenocarcinoma and laryngeal squamous cell carcinoma were reported in 2012, but their role in CRC was not. Chao Zhang et al reported that miR-1297 act as a tumor suppressor, which could suppress A549 cell proliferation *in vitro* and in vivo by suppressing

TRIB2 and further increasing C/EBPα expression (Zhang et al., 2012). In laryngeal squamous cell carcinoma, miR-1297 could inhibit cancer cell proliferation, migration, and tumorigenesis by targeting PTEN (Li et al., 2012). In this study, we demonstrated that miR-1297 was down regulated in both CRC-derived cell lines and clinical CRC samples compared with normal tissues and miR-1297 could inhibit human colorectal cancer LOVO and HCT116 cellular proliferation, migration, and invasion *in vitro*, as well as tumorigenesis in vivo by targeting Cox-2.

In principle, it is currently believed that miRNAs which are overexpressed in tumor cells might function as inhibitors of different tumor suppressor genes. Mechanisms involve translocation, pleomorphism or mutation in miRNAs transcribing genes and nuclear over production of pri-miRNAs. By contrast, those miRNAs which are silenced in tumors might play a role in down-regulating oncogenes in normal tissues. In this case mechanisms involve mutation, deletion, promoter methylation, or any abnormalities in the biogenesis of miRNAs, which leads to the over expression of oncogenic mRNAs (Esquela-Kerscher et al., 2006). In our research, the expression levels of miR-1297 in normal corresponding tissues were higher than those in CRC tissues. It might be because miR-1297 down-regulates the expression of Cox-2, leading to control the normal cell growth.

MiRNAs are present in plasma and feces at detectable levels and are stable, since miRNAs are not degraded by endogenous ribonucleases. Thus, miRNAs are being validated as biomarkers for screening and diagnosis, with potential therapeutic reflections (Bonfrate et al., 2013). In 2014, miR-205 was reported that its potential environmental activators might be a promising therapeutic option to prevent malignant progression toward metastasis (Orang et al., 2014). We believe that miR-1297 might be as a diagnostic biomarker to assess the stage of CRC, or as a prognosis biomarker to determine the outcome of CRC. It may also serve as a therapy tool to neutralize Cox-2 overexpression, thereby terminating the malignant tumor proliferation.

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