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MicroRNA-200c is involved in proliferation of gastric cancer by directly repressing p27^{Kip1}

Yangyang Wang [a,1], Jiping Zeng [b,1], Jianyong Pan [c], Xue Geng [a], Yansong Liu [d], Jing Wu [a], Ping Song [b], Ying Wang [b], Jihui Jia [e], Lixiang Wang [a,*]

[a] Department of Pharmocology, Shandong University School of Medicine, Jinan 250012, PR China
[b] Department of Biochemistry and Molecular Biology, Shandong University School of Medicine, Jinan 250012, PR China
[c] Department of Hepatobiliary Surgery, Qilu Hospital of Shandong University, Jinan 250012, PR China
[d] Shandong Tumor's Hospital and Institute, Jinan 250117, PR China
[e] Department of Microbiology/Key Laboratory for Experimental Teratology of Chinese Ministry of Education, Shandong University School of Medicine, Jinan 250012, PR China

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ABSTRACT

P27^{Kip1}, also known as Cyclin-dependent kinase inhibitor 1B, is an important check-point protein in the cell cycle. It has been identified that although as a tumor suppressor, P27^{Kip1} is expressed in different cancer cell types, which shows the therapeutic potential in tumor genesis. In this study, we examined the upstream regulatory mechanism of P27^{Kip1} at the microRNA (miRNA) level in gastric carcinogenesis. We used bioinformatics to predict that microRNA-200c (miR-200c) might be a direct upstream regulator of P27^{Kip1}. It was also verified in gastric epithelial-derived cell lines that overexpression of miR-200c significantly inhibited the expression levels of P27^{Kip1}, whereas knockdown of miR-200c promoted P27^{Kip1} expression in AGS and BGC-823 cells. Furthermore, we identified the direct binding of miR-200c on the P27^{Kip1} 3′ -UTR sequence by luciferase assay. MiR-200c could enhance the colony formation of cells by repressing P27^{Kip1} expression. In addition, the negative correlation between P27^{Kip1} and miR-200c in human gastric cancer tissues and matched normal tissues further supported the tumor-promoting action of miR-200c *in vivo*. Our finding suggested that miR-200c directly regulates the expression of P27^{Kip1} and promotes cell growth in gastric cancer as an oncogene, which may provide new clues to treatment.

1. Introduction

Gastric cancer is one of the most common and aggressive forms in China [1]. Patients with gastric cancer are often given a poor prognosis due to the difficulty of early diagnosis with highly growth and high rate of recurrence [2,3].

It has been suggested that the abnormal expression of check point genes can always be found in tumorgenesis, including TP53, P27^{Kip1}, P21^{Cip2}, P16^{Ink1} and C-Myc [4–8]. P27^{Kip1}, also known as Cyclin-dependent kinase inhibitor 1B, binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1 stage [9]. It is always thought the inhibitor of cell cycle with a tumor suppressor [10,11]. In cancers it is often inactivated *via* impaired synthesis, accelerated degradation, or mislocalization [12]. We previously reported that FoxM1, the main positive regulator of cell cycle, can regulate gastric cancer cells proliferation and senescence through inhibition of P27^{Kip1} [13,14]. These observations support that P27^{Kip1} is strongly negatively correlated with gastric carcinogenesis. It is important to better understand the regulation of P27^{Kip1} expression in tumorgenesis for anti-cancer therapy.

Currently, microRNAs (miRNAs) has been thought one of the most important regulators in tumorgeneis [15–18]. MiRNAs are the small non- translated RNA molecules with approximately 18–24 nucleotides in length. Every miRNA can directly bind to the complementary sequence on the 3′-untranslated region (3′-UTR) of many possible target mRNAs, which thus regulates the genes expression by post-transcriptional gene silencing, producing sequence specific mRNA cleavage, or translational repression [19,20]. MiRNAs can be the tumor suppressors or oncogenes which are determined by the functions of their target genes [21,22]. There are only a few research on the miRNAs regulation on P27^{Kip1} expression in tumorgenesis, including miR-221/222 [23,24]. We still need more evidence on specific miRNA regulation on P27^{Kip1} and its function in gastric carcinoma.

In this study, we demonstrated that miR-200c is an oncogene in gastric carcinoma which can directly inhibit the expression of

* Corresponding author.
E-mail address: wanglx@sdu.edu.cn (L. Wang).
[1] The first two authors contributed equally to this article.

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$P27^{Kip1}$ *in vivo* and *in vitro*. To our knowledge, this is the first time to explore the relationship between miR-200c and $P27^{Kip1}$ in gastric carcinoma. We propose that antagomiRNAs of miR-200c might be the potential for an anti-cancer therapy by reactivation of $P27^{Kip1}$.

2. Materials and methods

2.1. Cell lines and culture

The gastric epithelial-derived cancer cell lines AGS and BGC-823 were obtained from the cell repository for Academia Sinica (Shanghai). AGS cells were grown in Ham's F12 (Gibco, USA). BGC-823 cells were grown in RPMI1640 medium (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (Gibco, USA). Both the cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. RNA extraction and QRT-PCR

Total RNA was extracted by use of Trizol reagent (Invitrogen, USA). For qRT-PCR of $P27^{Kip1}$, total RNA was reverse transcribed by use of the RevertAid First Strand cDNA synthesis Kit (Fermentas, Canada). Real-time PCR was involved by the SUBY Green mixture (Takara, Japan) with the Biorad Sequence Detection System. Gene expression was normalized to that of β-actin. Results were calculated by the $2^{-\Delta\Delta Ct}$ method. The level of mature miR-200c expression was analyzed by TaqMan miRNA Assay (Applied Biosystems). cDNA was synthesized from total RNA samples by use of the TaqMan miRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers. Real-time PCR involved TaqMan miRNA Assay primers with the TaqMan Universal PCR Master Mix. The reactions were run in the Biorad Sequence Detect System. The relative level of miRNA expression was normalized to that of U6 small noncoding RNA and the fold change for miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences for $p27^{Kip1}$, 5′-ATGTCAAACGTGCGAGTGTCTAA-3′, and antisense, 5′-TTACGTTTGACGTCTTCTGAGG-3′, and β-actin, sense, 5‘-AGTTGCGTTACACCCTTTCTTG-3′, and antisense, 5‘- CACCTTCACCGTTCCAGTTTT-3′.

2.3. Western blotting

The protein level of $P27^{Kip1}$ was determined by Western blotting which was normalized by β-actin. In brief, the cells were lysed in RIPA buffer. Then the lysate was spun down and the supernatant was harvested. The concentration of the proteins was detected by BCA assay. Equal amounts of proteins were separated by SDS polyacrylamide gels and transferred onto membranes (Millipore), which were blocked with 5% non-fat milk protein for 1 h, then incubated with primary antibodies overnight at 4 °C. The antibodies used were for $P27^{Kip1}$ (1:300, Santa Cruz Biotechnology) and β-actin (1:5,000, Sigma). The secondary antibodies were horseradish peroxidase-conjugated goat-anti-rabbit IgG (1:5,000, Santa Cruz Biotechnology). Immune complexes were detected by use of the Chemiluminescent HRP Substrate Kit (Millipore).

2.4. Luciferase assay

The mimics and the inhibitor of miR-220c were purchased from Ruibo (Guangzhou, China). The special fragment of the $P27^{Kip1}$ 3′-UTR containing the miR-200c predicted target sites was synthesized by Invitrogen (USA). Then the fragment was cloned into the multiple cloning sites of the luciferase reporter pMIR-REPOTR (Applied Biosystems, USA), designated as pMIR-REPORT-P27-3′-UTR, which was also used in PCR to generate pMIR-REPORT-P27-3′-UTRmut plasmid with mutation of the binding sites on the 3′-UTR of $P27^{Kip1}$. For the transfection of the palsmids, cells were seeded into 6-well plates (3×10^5 cells/well) for 18–24 h. Then the plasmids were transfected by the use of Lipofectamine 3000 (Invitrogen, USA). To examine the direct conjugation of miR-200c to the 3′-UTR of $P27^{Kip1}$, pMIR-REPORT-P27-3′-UTR and pMIR-REPORT-P27-3′-UTRmut were co-transfected into AGS cells with miR-200c mimics. pMIR-REPORT β-gal plasmid was used as a negative control. Luciferase activity in the cell lysates was determined by a single luciferase reporter assay (Promega, USA) 48 h after transfection, and target promoter-driven firefly luciferase activity was normalized to that of β-gal.

2.5. Clone formation assay

BGC-823 cells were incubated in 6-well plates for 18–24 h, which were transfected with the corresponding mimics/inhibitor for 48 h. Single cells were seeded on 6-well plates (300 cells/well). After 10 days of incubation, plates were stained with Giemsa for 20 min. The number of colonies with more than 50 cells was counted.

2.6. Patients and tissue specimens

Resected pairs of cancer tissue and distal normal gastric tissue (> 5 cm from the margin of the tumor) from 15 patients with gastric cancer were harvested during surgery at Qilu Hospital of Shandong University 2014–2015. None of the patients had received adjuvant chemotherapy before surgery. The diagnosis of gastric cancer was histopathologically confirmed. The general information for patients is in Table 1. The study was approved by the ethics committee of Shandong University School of Medicine.

2.7. Immunohistochemistry

The expression of $P27^{Kip1}$ in human species was determined by Immunohistochemistry. Resected tissue pairs were embedded with paraffin and sliced into 5- mm pieces, which were deparaffinized and dehydrated with xylene and a graded series of alcohol. Antigen retrieval was treated in 0.1 M citrate buffer at pH 6.0 with heating. Then 3% H_2O_2 was used to block the endogenous peroxidase activity. The slides were incubated with goat serum for 30 min, then with rabbit anti-human $P27^{Kip1}$ (Santa Cruz, USA) overnight at 4 °C. The results were detected with Diaminobenzidine (DAB) staining (Vector Laboratories, USA) which were calculated with the microscope images (Olympus BX60, Tokyo, Japan).

Table 1
Association of miR-200c and p27 expression in human gastric cancer tissues.

Category	No. of patients	miR-200c			P27		
		high	low	*P*	high	low	*P*
Age				> 0.05			> 0.05
< 60 years	6	4	2		1	5	
≥ 60 years	9	6	3		4	5	
Sex				> 0.05			> 0.05
Male	9	6	3		3	6	
Female	6	4	2		2	4	
Differentiation				> 0.05			> 0.05
Well	5	4	1		2	3	
Poor	10	6	4		3	7	
Tumor size				> 0.05			> 0.05
≤ 5 cm	8	5	3		3	5	
> 5 cm	7	5	2		2	5	