MicroRNA-200c is involved in proliferation of gastric cancer by directly repressing p27Kip1

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A R T I C L E   I N F O

Article history:
Received 14 December 2015
Received in revised form
10 August 2016
Accepted 6 September 2016
Available online 19 September 2016

Keywords:
MiR-200c
P27Kip1
Proliferation
Gastric cancer

A B S T R A C T

P27Kip1, 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, P27Kip1 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 P27Kip1 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 P27Kip1. It was also verified in gastric epithelial-derived cell lines that overexpression of miR-200c significantly inhibited the expression levels of P27Kip1, whereas knockdown of miR-200c promoted P27Kip1 expression in AGS and BGC-823 cells. Furthermore, we identified the direct binding of miR-200c on the P27Kip1 3′-UTR sequence by luciferase assay. MiR-200c could enhance the colony formation of cells by repressing P27Kip1 expression. In addition, the negative correlation between P27Kip1 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 P27Kip1 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 checkpoint genes can always be found in tumorigenesis, including TP53, P27Kip1, P21Cip1, P16Ink4a and C-Myc [4–8]. P27Kip1, 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 P27Kip1 [13,14]. These observations support that P27Kip1 is strongly negatively correlated with gastric carcinogenesis. It is important to better understand the regulation of P27Kip1 expression in tumorgenesis for anti-cancer therapy.

Currently, microRNAs (miRNAs) has been thought one of the most important regulators in tumorgenesis [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 P27Kip1 expression in tumorgenesis, including miR-211/222 [23,24]. We still need more evidence on specific miRNA regulation on P27Kip1 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...
P27\textsuperscript{kip1} in vivo and in vitro. To our knowledge, this is the first time to explore the relationship between miR-200c and P27\textsuperscript{kip1} in gastric carcinoma. We propose that antagomiRNAs of miR-200c might be the potential for an anti-cancer therapy by reactivation of P27\textsuperscript{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\textsubscript{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\textsuperscript{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 (Promega, USA). Real-time PCR was involved by the 2-\textsuperscript{ΔΔCt} 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-\textsuperscript{ΔΔCt} method. The primer sequences for p27\textsuperscript{kip1}, 5'-ATGCTAACAGCTGAGTCTCTAAA-3', and antisense, 5'-TTACGTTT-GAGCTCTTACTGAG-3', and β-actin, sense, 5'-AGTTGCGTTA-ACCCCTTTCGTAACGTCA-3', and antisense, 5'-CACCTTCACCGTTCAATGTTT-3'.

2.3. Western blotting

The protein level of P27\textsuperscript{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 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\textsuperscript{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\textsuperscript{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-REPORT (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\textsuperscript{kip1}. For the transfection of the plasmids, cells were seeded into 6-well plates (3 x 10\textsuperscript{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\textsuperscript{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 Shandong University 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\textsuperscript{kip1} in human species was determined by Immunohistochemistry. Resected tissue pairs were embedded with paraffin and sliced into 5-μm 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\textsubscript{2}O\textsubscript{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\textsuperscript{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

| Category | No. of patients | miR-200c high | miR-200c low | P | P27 high | P27 low | P |
|----------|----------------|--------------|--------------|---|----------|--------|---|
| Age      |                |              |              |   |          |        |   |
| < 60 years | 6             | 4            | 2            | 5 | > 0.05   |        |   |
| ≥ 60 years | 9             | 6            | 3            | 5 | > 0.05   |        |   |
| Sex      |                |              |              |   |          |        |   |
| Male     | 9              | 6            | 3            | 6 | > 0.05   |        |   |
| Female   | 6              | 4            | 2            | 4 | > 0.05   |        |   |
| Differentiation |        |              |              |   |          |        |   |
| Poor     | 10             | 6            | 4            | 7 | > 0.05   |        |   |
| Tumor size |              |              |              |   |          |        |   |
| ≤ 5 cm   | 8              | 5            | 3            | 5 | > 0.05   |        |   |
| > 5 cm   | 7              | 5            | 2            | 5 | > 0.05   |        |   |
Fig. 1. MiR-200c downregulated the expression of P27Kip1 in gastric cancer cell lines. QRT-PCR analyses of (A) miR-200c and (B) P27Kip1 mRNA level in control and miR-200c mimics-transfected AGS, BGC-823 cell lines after 48 h. *P < 0.05 and **P < 0.01. (C) miR-200c and (D) P27Kip1 mRNA level in control and miR-200c inhibitors-transfected AGS, BGC-823 cell lines after 48 h. *P < 0.05 and **P < 0.01. (E) Western blot analyses of P27Kip1 protein levels in gastric cancer cells treated with control and miR-200c mimics or inhibitors. (F,G) Western blot analyses of P27Kip1 protein levels. *P < 0.05 and **P < 0.01. Data are mean ± SEM of 3 independent experiments.
2.8. Statistical analysis

Quantitative data are expressed as mean ± SEM. Statistical analysis was in the use of SPSS 15.0 (SPSS, Inc., Chicago, IL) by two-tailed Student’s t-test. Statistical significance was set at $P < 0.05$.

3. Results

MiR-200c downregulated the expression of $P27^{kip1}$ at the protein level in gastric cancer cell lines. The bioinformatics analysis with miRanda (http://www.microrna.org/microrna), TargetScan (http://www.targetscan.org), and miRBase (http://www.mirbase.org) predicted $P27^{kip1}$ as a downstream target of miR-200c which could directly bind to the 3′-UTR of $P27^{kip1}$. To determine the correlation between miR-200c and $P27^{kip1}$ mRNA expression level, we transfected the miR-200c mimics into AGS and BGC-823 cells for 48 h. The high expression of miR-200c was detected by QRT-PCR (Fig. 1A), which repressed the mRNA level of $P27^{kip1}$ without significant difference (Fig. 1B). Similarly, the transfection of miR-200c inhibitor (Fig. 1C) also did not obviously increase the mRNA expression of $P27^{kip1}$ (Fig. 1D). Then Western blotting was checked to determine the effect of miR-200c on the expression of $P27^{kip1}$ protein. The results showed that with miR-200c overexpression, the $P27^{kip1}$ protein level decreased. While with the inhibition of miR-200c, the $P27^{kip1}$ protein level recovered to a higher level (Fig. 1E, F and G). Therefore, miR-200c inhibited $P27^{kip1}$ expression in the gastric cancer cells by degrading the protein level of $P27^{kip1}$.

3.1. $P27^{kip1}$ is the direct target of miR-200c

The complementary sequence of miR-200c was found on the 3′-UTR site of $P27^{kip1}$ (Fig. 2A). Then the direct regulation of miR-200c on $P27^{kip1}$ expression by the binding of the special 3′-UTR site was determined by luciferase report assay. AGS cells were cotransfected with miR-200c mimics and $P27^{kip1}$ wild-type or mutant-type 3′-UTR plasmids. Cotransfection of miR-200c and wild-type 3′-UTR plasmid reduced the luciferase activity by approximately 60% relative to the control, whereas mutant 3′-UTR cotransfection almost restored the luciferase activity (Fig. 2B). Thus, miR-200c directly targeted the binding site located at $P27^{kip1}$ 3′-UTR and $P27^{kip1}$ is one of direct targets of miR-200c.

3.2. MiR-200c was involved in the proliferation of gastric cancer cells

Colonies formation assay in BGC-823 cells revealed that the expression level of miR-200c truly affected the proliferation of gastric cancer cells (Fig. 3A). The enforced expression of miR-200c enhanced the clone formation of cells, while the knockdown of miR-200c significantly inhibited the number of colonies (Fig. 3B). Thus, miR-200c could work as an oncogene in human gastric cancer cells to activate cell proliferation.

3.3. MiR-200c expression was enhanced in human primary gastric cancer

We determined the expression of miR-200c in human primary gastric cancer specimens and matched normal tissues from 15 patients. HE staining showed the pathology of the samples (Fig. 4A). QRT-PCR and IHC results showed that $P27^{kip1}$ protein expression was inhibited in human gastric cancer specimens which was the same as the research before. We also found that $P27^{kip1}$ might be negative covalent in vivo (Fig. 4D). We found no association of miR-200c or $P27^{kip1}$ expression and patient age, gender or tumor size (Table 1).

4. Discussion

In this study, we showed that miR-200c played an oncogenetic role in human gastric carcinogenesis by directly downregulated the expression of $P27^{kip1}$ and promoted the gastric cancer cell proliferation.

Gastric cancer (GC) is one of the most common human malignant diseases and the second leading cause of tumor-related deaths worldwide [1,2]. The incidence and mortality rate are particularly high in eastern Asia [3]. Despite recent advances in surgical techniques and adjuvant therapy after surgery, the 5-year survival rate is still low [25]. Carcinogenesis is a multi-factor, multi-phase and long-term interaction process on malignant transformation of normal cells. In this process, the abnormal regulation of cell cycle is involved in all the stages [26]. In previous studies, we have identified $P27^{kip1}$ is an important target which could be regulated by different factors in the proliferation of gastric cancer cells, including H. pylori infection, the escape of cancer cells senescence, epigenetics, et al. [13,14,27]. We also find that $P27^{kip1}$ can express in different cancer cell lines, which means it might be a valuable target for the diagnosis and treatment of gastric cancer.

In most cancers, reduced levels of $P27^{kip1}$ are correlated with increased tumor size, increased tumor grade, and a higher propensity for metastasis [28]. There might be different mechanisms by which levels of $P27^{kip1}$ are regulated in different types of cancers. The expression level of $P27^{kip1}$ can be regulated from the different steps of gene expression, such as transcription, translation and proteolysis [29]. $P27^{kip1}$ can also be regulated by changing its subcellular location [30]. Both mechanisms act to reduce the inhibition effect of $P27^{kip1}$ on the cell cycle, allowing for the activation of Cdk1 and Cdk2, which can begin the process of the cell cycle. We have identified that FoxM1 can inhibit the promoter...
activity of P27Kip1 and thus is involved in gastric carcinoma. Since P27Kip1 levels can also be moderated at the post transcriptional level, it has been proposed that P27Kip1 may be regulated by miRNAs.

MiRNAs have recently been discovered as one of the crucial players in gastric carcinogenesis through posttranscriptional regulation of tumor suppressor and oncogenes [31–33]. A substantial number of deregulated miRNAs have been revealed in gastric cancer and the biological significance of those miRNAs has been confirmed in multiple functional experiments. Only a few researches focus on the cell cycle inhibitors. In this study, we first predicted miR-200c as the up-stream regulator of P27Kip1 by bioinformatics. Then the negative regulative effect of miR-200c was determined in gastric cancer cell lines only at the translational level, which means that miR-200c does not affect the P27Kip1 mRNA cleavage, but only repressed P27Kip1 translation. Luciferase assay further suggested that P27Kip1 was the direct target of miR-200c. The oncogenetic role of miR-200c was identified by the increase of clone number with the overexpression of miR-200c in gastric cancer cells. The higher level of miR-200c and reduced
expression of P27Kip1 in human primary gastric cancer samples proved their negative correlation in vivo. P27Kip1 has been showed to have prognostic value in ovarian cancer in which P27Kip1 negative tumors progressed in 23 months compared to 85 months in P27Kip1 positive tumors [19]. Similar studies have correlated low levels of p27 with a worse prognosis in breast cancer [20]. The correlations were also observed in patients with non-small cell lung cancer, colorectal cancer, and prostate cancer [21–23]. So P27Kip1 can also be correlated with treatment response and work as a therapeutic target in cancer. In this study, we also identified the inhibition of P27Kip1 expression in gastric carcinoma with the negative control of miR-200c. The antagomiRs of miR-200c that can block the activity of the miR-200c and allow for P27Kip1 cell growth inhibition to take place might be a new way for gastric cancer treatment.

In summary, our results showed that miR-200c, which could negatively regulate the expression of P27Kip1, was an important oncogene involved in the cell proliferation of gastric cancer. These findings may provide further information miR-200c to be useful as
an anti-cancer therapeutic agent in gastric cancer.

Acknowledgments

The study was supported by the National Natural Science Foundation of China (Nos. 81272564, 81172354, 81171536 and 81371781) and the National Basic Research Program of China (973 Program, 2012CB911202).

References

[1] L. Yang, Incidence and mortality of gastric cancer in China, World J. Gastroenterol. 12 (1) (2006) 17.
[2] D. Kanagavel, M. Fedyanin, A. Tryakin, et al., Second-line treatment of metastatic gastric cancer: Current options and future directions, World J. Gastroenterol. 21 (41) (2015) 11621.
[3] L.P. Carcas, Gastric cancer review, J. Carcinog. 13 (1) (2014) 14.
[4] V.J.N. Bykov, K.G. Wiman, Mutant p53 reactivation by small molecules makes an anti-cancer therapeutic agent in gastric cancer. 
[5] J. Kota, R.R. Chivukula, K.A. O
[6] M.J. Langlois, S.A. Roy, E. Roy, et al., Su1867 Role of PTEN and p27 tumor suppressors in intestinal morphogenesis and neoplasia, Gastroenterology 142 (4) (2012), S-522.
[7] J. Zeng, L. Wang, Q. Li, et al., FoxM1 is up-regulated in gastric cancer and its inhibition leads to cellular senescence, partially dependent on p27kip1, J. Pathol. 218 (4) (2009) 419–427. 
[8] A.M. Petrich, C. Nabhan, S.M. Smith, MYC-associated and double-hit lymphomas: a review of pathobiology, prognosis, and therapeutic approaches, Cancer Res. Commun. 443 (3) (2014) 1105–1109.
[9] S. Tane, A. Ikenishi, H. Okayama, et al., p16Ink4a epimutation causes tumorigenesis and reduces survival in mice, J. Clin. Investig. 124 (9) (2014) 3708.
[10] A.M. Petrich, C. Nahban, S.M. Smith, MYC-associated and double-hit lymphomas: a review of pathobiology, prognosis, and therapeutic approaches, Cancer Res. 70 (14) (2010) 5923–5930.
[11] D. Wang, C. Qiu, H. Zhang, et al., Human microRNA oncogenes and tumor suppressors show significantly different biological patterns: from functions to targets, PLoS One 5 (9) (2010) e13067.
[12] L.A. Lorimer, Regulation of p27kip1 by miRNA 221/222 in glioblastoma, Cell Cycle 8 (17) (2009) 2685.
[13] M. Frenquelli, M. Muzio, C. Scezlo, et al., MicroRNA and proliferation control in chronic lymphocytic leukemia: functional relationship between miR-221/222 cluster and p27, Blood 115 (19) (2010) 3949–3959.
[14] M. Malumbres, M. Barbacid, Cell cycle, CDKs and cancer: a changing paradigm, Nat. Rev. Cancer 9 (3) (2009) 153–166.
[15] J. Zeng, Z. Ge, L. Wang, et al., The histone demethylase RBP2 Is overexpressed in gastric cancer and its inhibition triggers senescence of cancer cells, Gastroenterology 138 (3) (2010) 981–992.
[16] A. Borriello, D. Bencivenga, M. Criscuolo, et al., Targeting p27Kip1 protein: its relevance in the therapy of human cancer, Expert Opin. Ther. Targets 15 (6) (2011) 677–693.
[17] S.A. Wunder, D. Zhao, J.M. Slingerland, p27: a barometer of signaling deregulation and potential predictor of response to targeted therapies, Clin. Cancer Res. 17 (1) (2011) 12–18.
[18] H.K. Lin, G. Wang, Z. Chen, et al., Phosphorylation-dependent regulation of cytotoxic localization and oncogenic function of Skp2 by Akt/PKB, Nat. Cell Biol. 11 (4) (2009) 420–432.
[19] T. Ueda, S. Volinia, H. Okumura, et al., Relation between microRNA expression and progression of gastric cancer: a microRNA expression analysis, Lancet Oncol. 11 (2) (2010) 136–146.
[20] W.K.K. Wu, C.W. Lee, C.H. Cho, et al., MicroRNA dysregulation in gastric cancer: a new player enters the game, Oncogene 29 (43) (2010) 5761–5771.
[21] M.M.B. Baldoni, C.M.C. Camillo, R.D. Puga, et al., MicroRNA expression associated with response grade in gastric cancer patients’ tumor, Cancer Res. 75 (2015), 3988–3988.