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Original Article

miR-218 suppresses gastric cancer cell cycle progression through the CDK6/Cyclin D1/E2F1 axis in a feedback loop

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ABSTRACT

Studies in several cancers have suggested that miR-218 has anti-tumor activities, but its function is yet to be elucidated. In this study, we investigated the regulation and function of miR-218 (miR-218-5p) in the cell cycle progression of gastric cancer (GC). We found that miR-218 could suppress proliferation of gastric cancer cells, induce cell cycle arrest at the G1 phase and inhibit tumor growth and metastasis in vivo. We also demonstrated that miR-218 specifically targeted the 3′-UTR regions of CDK6 and cyclin D1 and inhibited the expression of these molecules, which in turn repressed the pRb/E2F1 signaling pathway. Overexpression of CDK6 and Cyclin D1 reversed miR-218-mediated inhibition of pRB/E2F1 signaling and attenuated the miR-218-induced cell cycle arrest. More importantly, miR-218 expression was significantly reduced and inversely correlated with the levels of CDK6 and Cyclin D1 in gastric cancer tissues. Decreased miR-218 expression was also correlated with advanced clinical stage, lymph node metastasis, and poor prognosis in gastric cancer patients. Furthermore, we showed that miR-218 expression was directly activated by E2F1 through the transactivation of miR-218 host genes, SLIT2 and SLIT3, revealing a negative feedback regulation of miR-218 expression. Taken together, our results describe a regulatory loop miR-218-CDK6/CyclinD1-E2F1 whose disruption may contribute to cell cycle progression in gastric cancer and indicate the potential application of miR-218 in cancer therapy.

Introduction

Gastric cancer is one of the most common malignancies and remains the third leading cause of cancer-related mortality worldwide [1]. Despite recent progress in the detection and treatment of early gastric cancer, the long-term survival rate for advanced gastric cancer is low. A better understanding of the molecular pathogenesis of this disease and effective targeted therapies are needed to improve patient outcomes.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression by binding to partially complementary sequences of target mRNAs [2]. Emerging evidence has revealed that miRNAs play key roles in various biological processes, including cell differentiation, proliferation, apoptosis, stress resistance, lipid metabolism, tumorigenesis, and metastasis [3,4]. Alterations in microRNA (miRNA) expression have been observed in gastric cancer, suggesting that a dysfunction of miRNA may be associated with tumorigenesis and progression of this disease [5–9].

miR-218 (has-miR-218-5p, previous ID hsa-miR-218) is a vertebrate-specific intronic miRNA encoded by two genes, miR-218-1 and miR-218-2, which are located in the introns of tumor suppressor gene SLIT2 and SLIT3 [10]. Accumulating evidence from studies in colorectal cancer [11], nasopharyngeal cancer [12], breast cancer [13], clear cell renal cell carcinoma [14], suggested that miR-218 is frequently down-regulated in various cancers and acts as a tumor suppressor. Recently, the results of two large-scale microarray assays demonstrated that miR-218 expression is significantly reduced in gastric cancer [15,16]. Reduced miR-218 expression is

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correlated with advanced clinical stage, lymph node metastasis and poor prognosis in patients, and re-expression of miR-218 in metastatic cells inhibits migration, invasion, and metastasis both in vitro and in vivo [10]. Another studies also showed that miR-218 inhibits cell proliferation and induces cell apoptosis via targeting EGFC/NF-κB signaling [17]. These studies suggest an important role of miR-218 in gastric tumorigenesis and tumor progression. However, the molecular mechanisms by which miR-218 exerts its effects and its significance in GC remain largely unknown.

Here, we demonstrated that miR-218 suppressed gastric cancer cell proliferation and induced cell cycle arrest at G1 phase, as well as inhibited tumor growth and metastasis in vivo. We further discovered an intriguing negative feedback loop involving miR-218, CDK6/CyclinD1, and E2F1, in which miR-218 specifically targeted CDK6 and cyclin D1, which in turn repressed the Rb/E2F1 signaling, whereas E2F1 directly activated miR-218 expression through transactivation of miR-218 host genes, SLIT2/SLIT3. Our findings provide an experimental basis for investigating miR-218 as a potential therapeutic target for gastric cancer.

Materials and methods

Cell culture and cell transfection

The human gastric cancer cell lines AGS, SGC7901, MGC803, MKN-28, MKN-45, and BGC823 were obtained from the Chinese Academy of Medical Science (Beijing, China). The gastric epithelial cell line GES-1 was purchased from the Beijing Institute for Cancer Research (Beijing, China). Cells were cultured in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA), 100 U/ml penicillin sodium, and 100 mg/mL streptomycin sulfate at 37 °C in a humidified incubator with 5% CO_2. For selection of gastric cancer stable cell lines, miR-218-expressing retroviruses were transduced into gastric cancer AGS cells in the presence of polybrene (6 μg/mL, Sigma). Cells were selected with 2 μg/mL puromycin for 14 days. miRIDIAN miRNA mimics and hairpin antagomirs were obtained from Dharmacon and were transfected using lipofectamine RNAimax at a final concentration of 10–100 nM.

Clinical sample

All tissue samples used in this study were collected from the Affiliated Tumor Hospital of Guangzhou Medical University. Written informed consent was obtained from all study participants. This study was approved by the Ethics Committee of Guangzhou Medical University Authority. The collection and use of tissues followed the procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration.

Cell proliferation assay

Cells were seeded into 6-well plates and the cell numbers were counted after 0 h, 24, 48, 72 and 96 h of incubation using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA) in triplicate.

Cell viability assay

Cell viability was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), following the manufacturer's indications.

Colony formation assay

Six-well plates were covered with a layer of 0.6% agar in medium supplemented with 20% fetal bovine serum. A total of 1000 cells were prepared in 0.3% agar and cultured for 2 weeks at 37 °C. The numbers of colonies per well was counted.

Cell invasion assay

Cells were seeded onto basement membrane matrix on inserts of a 24-well culture plate (EC matrix, Chemicon, Temecula, CA) and fetal bovine serum was added to the lower chamber as a chemoattractant. After 48 h, the non-invading cells and EC matrix were gently removed with a cotton swab. The invasive cells located on the lower side of the chamber were stained with Crystal Violet, counted and imaged.

BrdU cell proliferation assay

Cells were treated with 0.03 mg/mL BrdU for 6–12 h, at 37 °C, fixed with 4% paraformaldehyde, washed in 0.1 M PBS (phosphate-buffered saline, pH 7.4) with 1% Triton X-100, and incubated with 1 M Hcl (hydrochloric acid) and 2 M Hcl. A borate buffer (0.1 M) was added and cells were blocked with 5% normal goat serum in 0.1 M PBS in the presence of 1% Triton X-100, 1.0 M glycine. The cells were sequentially incubated with anti-BrdU and secondary antibodies.

Cell cycle analysis

Cells were collected into flow cytometry tubes and centrifuged at 2000 rpm for 5 min to obtain cell pellets. The cells were washed with PBS, and fixed with 70% ethanol (−20 °C ice-cold) for 1 h at 4 °C. The fixed cells were washed with PBS and incubated with RNAase A (0.1 mg/mL) for 30 min followed by incubation with propidium iodide (50 μg/mL) for 30 min at room temperature. Cell cycle analysis was performed with a Coulter Epics XL Flow Cytometry System (Beckman-Coulter, Miami, USA). In each analysis, 10,000 events were recorded. The percentage of cells at sub-G1, G0/G1, S, and G2/M phases was analyzed using FlowJo software (TreeStar, Inc., Ashland, OR).

Western blot and coimmunoprecipitation

Cells were lysed in cell lysis buffer containing 1% NP-40, 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/mL aprotinin, and 5 mg/mL leupeptin. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA, USA). After blocking, the blots were probed with the indicated primary antibodies. After washing and incubating with secondary antibodies, the blots were visualized with ECL reagent (Millipore). Co-immunoprecipitations were performed using the Dynabeads Protein G Immunoprecipitation Kit (Life Technologies) following the manufacturers' instructions. Negative controls were performed on all runs using an equivalent concentration of a subclass-matched immunoglobulin.

Plasmids and infection

The 3′-untranslated regions (UTR) of CDK6 and Cyclin D1, containing the predicted binding sites of miR-218, were amplified by PCR from genomic DNA and inserted into the luciferase reporter vector pmirGLO (Promega, Madison, WI, USA). By using these vectors as templates, we also generated mutant vectors with point mutations in the miR-218-binding sites using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA).

Recombinant lentiviruses containing miR-218 precursor or scrambled sequences were purchased from GeneCopeia (Guang zhou, China). CDK6 ORF cDNA and CCND1 ORF cDNA were purchased from GeneCopoeia (Rockville, MD, USA) and sub-cloned into the eukaryotic expression vector pcDNA3.1 (+) (Invitrogen), and the constructs were called pCDK6 and pCCND1, respectively. To construct the inducible E2F1-expressing vector, E2F1 cDNA was obtained via PCR amplification and then subcloned into the tetracycline-inducible lentiviral vector pLenti-Tet-MCS-Puro (GeneChem, Shanghai, China) and called pTet-E2F1. Recombinant lentiviruses were generated by co-transfecting the plasmid pTet-E2F1 and packaging plasmids into HEK293T packaging cells. The SLIT2 promoter plasmids SLIT2-L, SLIT2-1, SLIT2-2, SLIT2-3, SLIT2-4, SLIT2-5, and SLIT3 promoter plasmids SLIT3-L, SLIT3-1, SLIT3-2 and SLIT3-3 were constructed by ligating the respective PCR products into a promoter less vector pGL3 basic (Promega) upstream of the firefly luciferase gene at the Bgl I and Xho I restriction sites.

RNA extraction and real-time RT-PCR

Total cellular RNA was extracted using TRIzol (Invitrogen, California, USA). For mRNA detection, CDK6, Cyclin D1 and GAPDH mRNA expression was analyzed by the SYBR Green qRT-PCR according to the manufacturer's instructions (Applied Biosystems). The following primers were used: GAPDH forward, 5′-GACTCATGACCACAGTCCATGC-3′, and reverse, 5′-AGAGGCAGGGATGATGTTCTG-3’; CDK6 forward, 5′-ACGTGGTCAGGTTGTTT-3′, and reverse, 5′-TTTATGGTTTCAGTGGG-3’; CCND1 forward, 5′-GCGAGGAACAGAAGTGC-3′, and reverse, 5′-GAGTTGTCGGTGTAGATGC-3’.

For miRNA detection, the reverse transcribed cDNA was synthesized with the All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA). miR-218 expression was determined with the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) and U6 snRNA was used as the endogenous control.

In situ hybridization and immunohistochemistry analyses

Tissue microarrays (TMAs) consisting of 126 GC and 41 adjacent normal stomach tissues [18], were used for in situ hybridization and immunohistochemistry analyses. In situ hybridization for the detection of miR-218 was carried out as described previously [18]. Briefly, tissue microarray slides were deparaffinized, treated with proteinase K and subsequently blocked endogenous peroxidase activity with 3% H_2O_2. Hybridization was performed at 52 °C overnight after the addition of 50 nM of DIG-labelled LNA probe specific for miR-218 (Exiqon, Vedbaek, Denmark), followed by a stringency wash in SSC buffer. The probe-target complex was visualized with an anti-DIG-POD antibody and DAB complex.

The immunohistochemical analysis of human GC specimens was conducted using anti-CDK6 (Cell Signaling Technology) and anti-Cyclin D1 antibodies (Cell Signaling Technology). We quantitatively scored the tissue sections according to the percentage of positive cells and staining intensity, as previously described [19].