Characterization of microRNA-29 family expression and investigation of their mechanistic roles in gastric cancer

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Increasing evidence shows that abnormal microRNAs (miRNAs) expression is involved in tumorigenesis. They might be the novel biomarkers or therapeutic targets in disease treatment. miR-29 family was previously reported to act as tumor suppressors or oncogenes in diverse cancers. However, their accurate expression, function and mechanism in gastric cancer (GC) are not well known. Here, we found that the expression of miR-29 family members was significantly reduced in GC compared with adjacent controls. Among them, miR-29c had the most reduced percentage in GC and was associated with aggressive and progressive phenotypes of GC. We further demonstrated that miR-29 family acted as tumor suppressors through targeting CCND2 and matrix metalloproteinase-2 genes in GC. Moreover, the inverse relationship between miR-29 family and their targets was verified in patients and xenograft mice. Finally, reintroduction of miR-29 family significantly inhibited tumor formation of GC cells in the xenograft mice. Take together, our finding characterized the expression properties of miR-29 family, contributed to the function and molecular mechanism of miR-29 family in GC and implied that miR-29 family might be employed as novel prognostic markers and therapeutic targets of GC.

Introduction

Gastric cancer (GC) is one of the most common cancers and the leading cause of cancer-related death globally. Adenocarcinomas represent the vast majority of GC, whereas the remaining cases comprise non-Hodgkin lymphomas, sarcomas and carcinoid tumors. The incidence of GC varies depending on the geographic region, diet, and genetic background (1). According to the National Cancer Institute, the incidence of GC is highest in Japan, South America and Eastern Europe and part of Middle East (2). In China, the incidence of GC is still high despite advances in treatment and subsequent improvement in prognosis. However, in the West, where the incidence has continuously decreased, the overall and stage-specific survival is worse than that in Eastern Asia (3). GC is twice more frequent in men than in women in most of these regions. Although the tremendous improvements in diagnosis and treatment technologies, the prognosis of advanced GC remains poor and the survival of affected patients is <40% even after a curative surgical resection and adjuvant therapy (4). Hence, powerful biomarkers that can identify the early stages or patients at risk for GC, or predict the therapeutic responses of GC are urgently needed.

MicroRNA (miRNA) is a novel class of gene regulators that suppress protein expression through base pairing with the 3′-untranslated region (3′-UTR) of target messenger RNA. Accumulating evidence suggests that miRNAs play important roles in diverse biological processes and the dysfunction of miRNAs is involved in many diseases including cancer (5). Although dysregulation of miRNAs has been observed in various types of cancers, the molecular mechanisms by which miRNAs modulate the process of carcinogenesis and the behavior of cancer cells are still largely unknown. Currently, altered miRNA expression patterns collected from different study cohorts have been observed in GC. Furthermore, several deregulated miRNAs (e.g. miR-21, miR-375, miR-124, miR-125b) have been shown to contribute to gastroesophageal carcinogenesis by altering expression of oncogenes and tumor suppressors to affect cell proliferation, apoptosis, and motility and invasion (6). Meanwhile, a number of miRNAs, including circulating miRNAs, have been associated with tumor types or stages, or patient survival, and might be developed as diagnostic or prognostic markers (7). Therefore, greater understanding of the roles of miRNAs in gastric carcinogenesis could provide insights into the mechanisms of tumor development and identify diagnostic biomarkers and therapeutic targets.

It has been reported that the members of miR-29 family are downregulated in a few tumor types and critical miRNAs with tumor suppressor functions (8). They regulate the expression of many oncogenes, such as MCL-1, TCL-1, CDC42, p85α, DNMT3a and DNMT3b (9,10). Although a study described the roles of miR-29a in GC cell lines (11), the accurate expression, mechanistic function and therapeutic roles of them in GC are largely unknown. In this study, we used Taqman probe stem-loop real-time PCR to accurately measure the levels of miR-29a, b and c in gastric carcinomas. Interestingly, we found that although the whole miR-29 family was downregulated in the GC, repressed miR-29c expression was the most significant and associated with aggressive phenotypes of GC. We also explored the molecular mechanisms underlying the tumor suppression role of these miRNAs and probably functional significance of this observation through in vitro and in vivo studies.

Materials and methods

Clinical samples

One hundred and fifteen cases of newly diagnosed patients were collected with informed consent between 2009 and 2012 from Cancer Institute and Hospital of Chinese Academy of Medical Sciences, The First Hospital of Shanxi Medical University, The General Hospital of the People’s Liberation Army, Shaxi Cancer Hospital. The study was approved by the ethical board of each hospital and the ethical board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. All clinical samples were obtained
from patients undergoing GC and collected at surgery, immediately snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. GC pathological diagnosis was performed by at least two pathologists. Tumor and non-cancerous tissues were confirmed histologically by hematoxylin and eosin staining. The retrospective study was performed in these samples to analyze the expression level of miR-29a and the correlation of miR-29 family expression with clinicopathological variables of patients.

**Cell culture**

GC cell lines, including HGC-27, BGC-823, SGC-7901, MKN-26 and MGC-803, were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium RPMI-1640 with 10% fetal bovine serum (Gibco) at 37°C in 5% CO2, cell culture incubator. 293T cells were obtained from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted from the cells and tissues harvested using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. The RNA was quantified by absorbance at 260 nm. To measure the level of miR-29a, b and c, quantitative real-time PCR (q-PCR) was performed by using Taqman probes (Invitrogen) in the Bio-Rad IQ5 real-time PCR system according to the manufacturer’s instruction. The data were normalized using the endogenous U6 snRNA. The 2^(-ΔΔCT) method was used in the analysis of PCR data.

**Constructs, reagents and assays**

The 3'-UTR of the human CCND2 and matrix metalloproteinase-2 (MMP2) messenger RNA was cloned in between the NotI and XbaI sites of pGL-3 (Promega). Mutations of their 3'-UTR sequence were created by using a QuickChange Site-Directed Mutagenesis kit (Stratagene). Three luciferase reporters (pGL-3-29a/b/c PER) containing the miR-29 family consensus target sequence were used as the positive controls. miRNA mimics/inhibitors specifying miR-29 family and scramble control were obtained from Dharmacon (Chicago, IL). 293T cells were seeded onto 24-well plates (1 x 10^5 cells per well) the day before transfections. Cells (>70% confluence) were transfected with pGL-3 firefly luciferase reporter (50 ng per well), pRL-TK Renilla luciferase reporter (10 ng per well) and miR-29 family mimics (50 nM). The pRL-TK vectors serve as the internal control. For HGC-27 and MGC-803 cells, pGL-3 firefly luciferase and pRL-TK luciferase reporter were cotransfected into GC cells preseeded onto 24-well plates. All transfections were carried out in triplicate with Lipofectamine 2000 (Invitrogen). Cell lysates were prepared with Passive Lysis Buffer (Promega) 48 h after transfection, and luciferase activities were measured by using the Dual Luciferase Reporter Assay (Promega).

**Oligonucleotides and transfection**

miR-29a, b and c mimics/inhibitors and negative control molecules (scramble control mimic and inhibitor) were obtained from Dharmacon (Austin, TX) and transfected with DharmaFECT1 (Dharmacon) in HGC-27 or MGC-803 cells at a final concentration of 50 nM. Small interfering RNAs (siRNAs) (specifically for CCND2 or MMP2) and control siRNA were synthesized by GenePharma and transfected into HGC-27 or MGC-803 cells (100 nM) using Lipofectamine 2000 (Invitrogen). Medium was changed after 6 h, cells were cultured for 48 h and harvested for western blot analyses.

**Northern blot**

Northern blot analysis of miRNAs was performed as described previously (12). The oligonucleotide probes’ sequences were listed in Supplementary Table 2, available at Carcinogenesis Online.

**Western blot**

Whole cell lysate or nuclear extract was subjected to western blot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were purchased from Santa Cruz Biotechnology. MMP2, CCND2, Rb and phosphorylated-Rb (Ser780) were purchased from Cell Signal Technology (CST) Company. Immunoblots were quantified by Image J software.

**Cell proliferation and apoptosis assay**

To make the effect of miRNA mimics on cellular proliferation rates, cells were incubated in 10% CCK-8 (DOJINDO) diluted in normal culture media at 37°C until visual color conversion appears. Proliferation rates were determined at 24, 48, 72, 96 h posttransfection, and quantification was done on a microtiter plate reader (Spectra Rainbow; Tecan) according to the manufacturer’s protocol. Apoptosis assay was performed on HGC-27 and MGC-803 cell lines at 72h after transfection with either miR-29a, b, c mimics or control mimics with the Annexin V-FITC Apoptosis Detection kit I (BD Biosciences). Data was collected and processed by using FACScalibur Flow Cytometer (BECTON DICKINSON).

**Cell invasion assay**

HGC-27 and MGC-803 cells were grown to 50–70% confluence and transfected with miR-29 family or control mimics. Twenty-four hours posttransfection, cells were seeded onto a Matrigel-coated membrane matrix (BD Biosciences) present in the insert of a 24-well culture plate. Fetal bovine serum was added to the lower chamber as a chemoattractant. After 24 h, the non-invasive cells were gently removed with a cotton swab. Invasive cells located on the lower surface of chamber were stained with the 0.1% crystal violet (Sigma) and counted.

**Immunohistochemistry**

Human and mouse tumor tissues were made into paraffin sections and pretreated at 65°C for 2 h, followed by deparaffinization. Antigen retrieval was carried out before application of the primary antibodies (CCND2, 1:200, CST Company; MMP2, 1:200, CST Company; Ki-67, 1:100, DAKO) overnight at 4°C. As a negative control, sections were incubated with normal immunoglobulin G. Thereafter, slides were incubated for 2 h at room temperature with the secondary antibody conjugated to horseradish peroxidase (1:100; DAKO). Horseradish peroxidase activity was detected using the Liquid DAB+ Substrate Chromogen System (DAKO). Finally, sections were counterstained with hematoxylin and photographed.

**Immunofluorescence staining**

HGC-27 cultures grown on glass coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min. After washing in phosphate-buffered saline, cells were blocked and permeabilized in phosphate-buffered saline containing 2.5% goat serum and 0.1% Triton X-100 at room temperature for 90 min. Cells were incubated with human CCND2 or MMP2 antibodies (1:25; CST company) overnight at 4°C. Detection was with Alexa Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen) at room temperature for 1 h. As a negative control, antibodies against rabbit immunoglobulin G were used. Cells were viewed and photographed with a Nikon TE2000 microscope.

**Cell cycle assay**

Cell cycle analysis was performed on HGC-27 and MGC-803 cells 72 h after transfection with either miR-29a, b, c mimics or control mimics as described previously (14). Data was collected and processed by using the Modfit Cell Cycle Analysis Software.

**In vivo studies**

Animal xenograft model studies were performed according to institutional guidelines: 3 x 10^6 HGC-27 viable cells were injected subcutaneously into the anterior flanks of 6-week-old female nude mice, five mice per group. When tumors reached 50 mm^3, miRNA mimics (miR-29a, b, c or scrambled control) diluted in Lipofectamine 2000 (Invitrogen) solution (100 nmol mimics in 100 μl total volume) were injected directly into the tumors, respectively. The tumors were injected every 3 or 4 days for a total of six times. Tumor diameters were measured after 10 days from injection and then every 3 days. At 28 days after injection, mice were killed and tumors were weighed after necropsy. Tumor volume was calculated as follows: length x width^2 x 1/2.

**Statistics**

The comparison of miR-29a/b/c expression between GC tissue and adjacent non-cancer tissue was evaluated by independent sample t-test (two tailed). Correlation of miR-29 family expression with patients’ clinicopathological variables was evaluated by independent sample t-test (two tailed) or one-way analysis of variance, followed by Tukey post hoc test. All the analyses were completed by the software SPSS17.0 and P ≤ 0.05 was considered statistically significant.

**Results**

**Members of miR-29 family were differently downregulated in GC**

To accurately analyze the expression of miR-29 family in GC, q-PCR by using Taqman probes was conducted to measure the levels of miR-29a, b and c in 115 pairs of GC and adjacent non-neoplastic tissues. Here, the non-tumor samples from the macroscopic tumor margin (>5 cm from tumor tissues), which was further confirmed by pathologist for their normal origin that they do not have tumor cells, were isolated at the same time and used as the matched adjacent non-neoplastic tissues. The results showed that 39/115 (33.9%) of cases had reduced levels of miR-29a, 52/115 (45.2%) had reduced levels of miR-29b and 72/115 (62.6%) had reduced levels of miR-29c in GC.

**patients with GC**

The comparison of miR-29a/b/c expression between GC tissue and adjacent non-cancer tissue was evaluated by independent sample t-test (two tailed). Correlation of miR-29 family expression with patients’ clinicopathological variables was evaluated by independent sample t-test (two tailed) or one-way analysis of variance, followed by Tukey post hoc test. All the analyses were completed by the software SPSS17.0 and P ≤ 0.05 was considered statistically significant.
tissues (C) compared with non-neoplastic gastric tissues (N) when the cutoff was set up as 1.5 (Figure 1A). Interestingly, miR-29c had the most reduced percentage in GC compared with non-tumorous tissues. Collectively, the data suggested that average expression of each member of miR-29 family was significantly attenuated in tumor tissues compared with adjacent non-neoplastic tissues (miR-29a, \( P < 0.05 \); miR-29b, \( P < 0.001 \); miR-29c, \( P < 0.001 \)) (Figure 1B).

**Low-level expression of miR-29c was associated with aggressive and progressive phenotypes of GC**

To further investigate the clinicopathological and prognostic significance of miR-29 levels in patients with GC, the levels of miR-29 family in 77 pairs of GC tissues (including fully clinical information) were statistically analyzed. Correlation analysis showed that low-level expression of miR-29c in GC was significantly associated with a more extensive venous invasion (\( P = 0.05 \)) (Figure 1C, Supplementary Table 1, available at Carcinogenesis Online) and a more aggressive tumor phenotype (\( P < 0.05 \), stage I/II versus III/IV) (Figure 1D, Supplementary Table 1, available at Carcinogenesis Online). However, no significant differences were observed in age, gender, position or Borrmann typing. Meanwhile, the analysis showed that there were not significant associations between low level of miR-29a or 29b and almost all of tumor phenotypes of GC (Supplementary Table 1, available at Carcinogenesis Online).

**Fig. 1.** miR-29a, 29b and 29c are differentially downregulated in GC. (A) q-PCR analysis of the expression of miR-29 family in 115 pairs of GC tissues (C) and the matched adjacent normal regions (N). (B) Relative miR-29a/b/c expression levels in primary GC tissues and adjacent normal regions. Horizontal line represents the average value for each group. (C) Correlation of miR-29c expression with venous invasion of GC tissues used for miR-29 expression analysis. (D) Correlation of miR-29c expression with clinicopathological stage of GC tissues used for miR-29 expression analysis.
Exogenous overexpression of miR-29 family suppressed GC cell growth and cell cycle progression and promoted cell apoptosis in vitro

Interestingly, when analyzing the levels of miR-29 family in the same patient, we found that all of three members of miR-29 family were commonly downregulated in 35/115 (30.4%) of cases, miR-29b and 29c were commonly reduced in 48/115 (41.7%) of cases (Supplementary Figure 2, available at Carcinogenesis Online). The data suggested that miR-29 family might collaborate to exert tumor suppressor roles in GC. To validate the hypothesis, we then explored the potentially biological function of miR-29 family in GC tumorigenesis and/or progression. Northern blot analysis was conducted to measure the level of miR-29 family in five GC cell lines. We found that HGC-27 and MGC-803 had lower miR-29 expression and were suitable for function analysis of miRNAs (Figure 2A). The ability of cell growth was first evaluated in

HGC-27 and MGC-803 cells that were transfected with miR-29a, b, c or scramble mimics. Increased expression of miR-29 family upon transfection in these GC cell lines was confirmed by q-PCR (Supplementary Figure 1, available at Carcinogenesis Online). We discovered that miR-29-transfected cells had much fewer growth percentage than that in the scramble mimics-transfected cells (Figure 2B, Supplementary Figure 3A, available at Carcinogenesis Online), indicating a growth-inhibitory role of miR-29 family in GC cells. We subsequently used the propidium iodide staining assays to examine the effect of miR-29a/b/c overexpression on cell cycle of GC cells. As expected, the percentage of S-phase cells was markedly reduced by miR-29 overexpression in HGC-27 and MGC-803 cells (Figure 2C, Supplementary Figure 3B, available at Carcinogenesis Online). Meanwhile, fluorescence-activated cell sorting analysis was performed to evaluate the apoptosis percentage in miR-29 overexpressed GC cells and showed increases

Fig. 2. miR-29 family inhibits the growth of GC cell in vitro. (A) Northern blot analysis of the expression of miR-29a/b/c in five GC cell lines. U6 snRNA works as the loading control. (B) Cellular viability assay showing that miR-29a/b/c overexpression significantly decreases the viability of HGC-27 cells, respectively. (C) Cell cycle assay is done by flow cytometry on HGC-27 cells after scramble control, or miR-29 mimics treatment for 48h. (D) Apoptosis assay in HGC-27 cells showing induction of apoptosis by miR-29a, b and c overexpression. The biparametric histogram shows cells in early (bottom right quadrant) and late apoptotic states (upper right quadrant). Viable cells are double negative (bottom left quadrant). (E and F) Representative images (E) and bar graphs (F) depicting the invasion ability of HGC-27 or MGC-803 after scramble control, or miR-29 mimics 48h transfection (**P < 0.01; ***P < 0.001).
in apoptotic cell fractions of HGC-27 and MGC-803 cells treated by miR-29a/b/c mimics for 48 h (Figure 2D, Supplementary Figure 3C, available at Carcinogenesis Online). In addition, invasion assay was then performed to evaluate the effect of miR-29 family on the cell invasiveness in miR-29a/b/c overexpressing HGC-27 and MGC-803 cells and showed attenuated invasiveness of GC cells (Figure 2E and F, Supplementary Figure 3D, available at Carcinogenesis Online). Together, these results indicated that miR-29 family could efficiently inhibit GC cell proliferation, promote cell apoptosis and repress cell invasiveness of GC cells in vitro, respectively.

miR-29 family targeted CCND2 and MMP2 genes in GC cells

Since miRNAs usually exert their function by negatively regulating the expression of their target genes and our results above indicate the tumor suppressive role of miR-29 family in GC, putative oncotargets of miR-29 family were predicted using target prediction programs, TargetScan, PicTar and miRanda. Our analysis revealed that CCND2 and MMP2 were potential targets of miR-29 family. The 3′-UTR of CCND2 or MMP2 messenger RNA contains a complementary site for the seed region of miR-29 family (Figure 3A). Although CCND2 was shown previously to be a target for miR-29 in rhabdomyosarcoma (15), and MMP2 was demonstrated to be targeted by miR-29b in hepatocellular carcinoma (16), the interaction between miR-29 and CCND2/MMP2 has not been experimentally validated in GC.

To determine whether CCND2 and MMP2 are direct targets of miR-29 family, wild-type and mutant 3′-UTR lacking miR-29 binding sites were cloned into the downstream of firefly luciferase coding region in pGL-3 luciferase reporter vector. The constructs were then cotransfected with pRL-TK and miR-29a, b, c or scramble mimics into 293T cells, respectively. The relative luciferase activity was reduced by 60 and 50% in pGL-3 vectors with wild-type CCND2 and MMP2 3′-UTR, respectively, but not in those with respective mutant 3′-UTRs (Figure 3B). More ever, the endogenous miR-29s indeed inhibited the luciferase activity of the pGL-3 vectors with wide-type CCND2 and MMP2 3′-UTR but did not affect the luciferase activity of the pGL-3 vectors with mutant 3′-UTR (Figure 3C) in HGC-27 and MGC-803 cells.

**Fig. 3.** CCND2 and MMP2 are direct targets of miR-29 family in GC cells. (A) Schematic representation of CCND2 and MMP2 3′-UTRs showing putative miR-29 target site. (B) Relative luciferase activity of the indicated CCND2 or MMP2 reporter constructs in 293T cells. Error bars presented standard deviation obtained from three independent experiments. (C) Luciferase activity assay with wild-type CCND2 or MMP2 3′-UTRs constructs and mutated luciferase constructs in HGC-27 and MGC-803 cells. Firefly luciferase values were normalized to Renilla luciferase activity and plotted as relative luciferase activity (*P < 0.05 and **P < 0.01 compared with mutated constructs). (D) Western blot analysis of CCND2 and MMP2 expression in HGC-27 and MGC-803 cells transfected with scramble oligonucleotide or miRNA mimics/miRNA inhibitors. (E) Immunofluorescence microscopy of HGC-27 cells stained with anti-CCND2/DAPI and anti-MMP2/DAPI showing a marked decrease in staining after treatment with miR-29a, b and c mimic for 48 h, respectively. DAPI, 4′,6-diamidino-2-phenylindole.
MGC-803, suggesting that miR-29 family cognate sites are essential for negative regulation of luciferase expression driven by CCND2-3'-UTR and MMP2-3'-UTR.

To confirm that miR-29 can indeed suppress expression of endogenous CCND2 and MMP2, HGC-27 and MGC-803 cells were transfected with miR-29a, b, c or scramble mimics, followed by detection of their protein levels. The protein levels of CCND2 and MMP2 were substantially decreased after ectopic overexpression of miR-29 family in HGC-27 and MGC-803 cell lines as evidenced by western blot assays (Figure 3D). On the other hand, knocking down of miR-29 family by anti-miR-29a, b or c in HGC-27 and MGC-803 cells increased protein levels of CCND2 and MMP2 (Figure 3D). Inhibition expression of miR-29 family upon transfection in these GC cell lines was confirmed by q-PCR (Supplementary Figure 4 available at Carcinogenesis Online). Similarly, CCND2 and MMP2 immunoreactivities were diminished in HGC-27 cells transfected with the miR-29a/b/c mimics when compared with cells treated with the scramble mimic (Figure 3E). Taken together, these findings indicate that CCND2 and MMP2 can be negatively regulated by miR-29 family in GC cells.

miR-29 family regulated cell cycle through inhibiting CCND2 and p-Rb

CCND2 is one member of the cyclins participating in promoting the transition from G1 phase of the cycle to S phase (17). To verify whether miR-29 family inhibited tumorous phenotypes of GC cells through targeting CCND2, we first knocked down endogenous CCND2 through its specific siRNAs in HGC-27 and MGC-803 cells and analyzed the effect on cell proliferation (Figure 4A). We observed that downregulating CCND2 inhibited the cell growth and G/S transition in these cells (Figure 4B and C). Previous studies indicated that CCND2 functions through regulating the phosphorylation level of Rb to regulate the cell cycle (18,19). To analyze whether miR-29 family affects cell cycle progression through inhibiting CCND2/p-Rb, we transfected miR-29 mimic into HGC-27 and MGC-803 cells and measured the levels of p-Rb and total Rb. A decreased p-Rb level was detected in GC cells transfected with miR-29 mimics as compared with the cells transfected with the scramble mimics, whereas the level of total Rb remained consistent (Figure 4D). Meanwhile, we found that CCND2 siRNAs also promoted cell apoptosis of HGC-27 and MGC-803 cells (Figure 4E). These data indicated that miR-29 family could inhibit cell proliferation and cell cycle progression through downregulating p-Rb activity.

Inhibition of miR-29 targets decreased GC cell invasiveness

Our data suggested that miR-29 family might regulate tumor cell metastasis in vitro by seeding on 3'-UTR sites of their targets. To investigate whether miR-29 family affects cell invasiveness by targeting CCND2 and MMP2 in GC cells, we used specific siRNAs to knock down endogenous CCND2 and MMP2 (Figure 4F) and examine cell invasiveness of HGC-27 and MGC-803 cells. We observed that siRNAs targeting CCND2 and MMP2 inhibited the invasiveness of HGC-27 and MGC-803 cells, respectively (Figure 4G). These results suggest that miR-29 family regulates CCND2 and MMP2, which affect GC cell invasiveness.

miR-29 family targeting CCND2 and MMP2 was clinically validated in GC patients

To address the functional relevance of miR-29 family and their target levels in human GC patients, we analyzed the expression of miR-29a/b/c, CCND2 and MMP2 expression in four pairs of GC and their corresponding adjacent non-cancer tissues. Northern blot was first performed to evaluate the expression of miR-29 family in these tissues and showed markedly reduced miR-29a, b and c in GC compared with the corresponding controls (Figure 5A). Immunoblot analysis was simultaneously conducted in the same tissues and indicated that the levels of these proteins were evidently upregulated in GC as compared with the matching non-neoplastic tissues (Figure 5B).

Meanwhile, immunohistochemistry was used to assess the expression of CCND2 and MMP2 in the same GC tissues. Consistent with the immunoblot findings, CCND2 and MMP2 staining was also increased in GC tissues as compared with the matching normal gastric tissues (Figure 5C). To further validate our findings, the levels of miR-29 family, CCND2 and MMP2 were measured in additional 16 of human primary GC and pair-matched peritumoral gastric tissues (Supplementary Figure 5A and B, available at Carcinogenesis Online). Comparison of miR-29a/b/c levels and protein levels corresponding to CCND2 and MMP2 in GC exhibited significantly inverse correlation between CCND2 and miR-29c (r² = 0.2248, P = 0.0347), and inverse correlation between MMP2 and miR-29a (r² = 0.1995, P = 0.0483), or miR-29c (r² = 0.3593, P = 0.0052) (Figure 5D). Collectively, these findings provide strong evidence that CCND2 and MMP2 are direct targets of miR-29 family in GC.

miR-29 family inhibited the growth of HGC-27-engrafted tumors

Our above findings indicated that miR-29 family was potential therapeutic targets in GC. To investigate the therapeutic effect of miR-29s on gastric tumorigenicity in vivo, ~3 × 10⁶ HGC-27 cells were inoculated subcutaneously in posterior flanks of immunocompromised 'nude' mice. When tumors reached 50 mm³, synthetic miR-29a/b/c or scrambled oligonucleotides were injected directly into the tumors. The tumors were injected every 3 days for a total of six times (Figure 6A). After 4 weeks, we found that injection with miR-29s inhibited the growth of HGC-27-engrafted tumors with respect to scrambled oligonucleotides-treated tumors (Figure 6B). In agreement with the tumor growth curve, the volumes and weights of tumors treated by miR-29 mimics were significantly lower than scrambled mimics-injected tumors (Figure 6C and D). In view of these observations, we reasoned that increased levels of miR-29s in mouse tumors after injection with mimics might affect tumor cell proliferation. To address this point, immunohistochemical analysis was used to measure the protein levels of Ki-67 in the tumor tissues, showing decreases of Ki-67 in miR-29a/b/c-injected tumors (Figure 6E). These data indicated that introduction of miR-29a, b and c remarkably inhibited the tumorigenicity of HGC-27 cells in the nude mouse xenograft model. Moreover, we also performed immunohistochemistry to detect the expression of CCND2 and MMP2 in randomly selected xenograft mouse tumors and found that miR-29 mimics-injecting tumors expressed lower levels of CCND2 and MMP2 than scramble controls (Figure 6F). Thus, the possibility that introduction of miR-29 mimics may contribute to GC control provides a novel method for GC therapy.

Discussion

Our recent study reported high frequency of miR-29a downregulation in human acute myeloid leukemia and demonstrated its potential tumor suppressor function in acute myeloid leukemia (20,21). This article showed that all members of miR-29 family were downregulated in GC and miR-29c was more significant than miR-29a or 29b as a signature miRNA of GC. Furthermore, our results indicated that miR-29 family directly targeted CCND2 and inhibited Rb phosphorylation activity, leading to repressed cell proliferation, arrested G1/S transition and enhanced cell apoptosis in GC cells. Our results also indicated that miR-29 family directly targeted CCND2 and MMP2 to inhibit cell invasiveness in GC cells. Moreover, our investigation for the expression of CCND2, MMP2 and miR-29 family in 20 GC patients indicated that there was an inverse correlation between miR-29 family and CCND2/MMP2 levels. Importantly, overexpression of miR-29 family ameliorated progression of GC in an established experimental xenograft model, respectively.

An emerging body of evidence suggests that miRNAs serve as importantly therapeutic targets in a wide range of complex human diseases by targeting multiple transcripts (22,23). We argued that to identify key regulatory miRNAs in GC, it would be necessary to examine accurate miRNA expression patterns in an unbiased manner. Downregulation of the miR-29 family has been reported in acute
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myeloid leukemia (24), chronic lymphocytic leukemia (25), lung cancer (26,27) and nasopharyngeal cancer (28), and a possible role of miR-29 as a tumor suppressor has been hypothesized. However, the expression and function of miR-29 family was not well known in GC. Accordingly, we elaborately examined expression properties of miR-29 family through q-PCR in samples obtained from GC patients. Our experimental design identified downregulation of miR-29a, b and c as marked signatures in GC. Although a few studies indicated that miR-29 family might act as oncogenes in metastatic breast cancer and indolent lymphocytic leukemia (27,29), downregulated miR-29a or 29b was demonstrated to be significant in most of cancers, including our findings. Interestingly, we found that the level of the miR-29c was able to monitor tumor progression, implying that lower level of the miRNA would potentially predict more aggressive and progressive phenotypes of the GC patients. Actually, miR-29c was identified as a tumor suppressor gene rather than miR-29a or

Fig. 4. miR-29 family regulates cell proliferation, cell cycle and cell invasiveness through targeting CCND2 and MMP2 in GC cells. (A) Immunoblot analysis of CCND2 in HGC-27 or MGC-803 cells transfected with three different siRNAs targeting CCND2 for 48 h. GAPDH was used as a loading control. (B) Cellular viability analysis in HGC-27 or MGC-803 cells treated with CCND2 siRNAs #1 or control siRNAs for 24, 48, 72 and 96 h. (C) Cell cycle assay in HGC-27 or MGC-803 cells by flow cytometry after siRNA control or CCND2 siRNAs #1 treatment for 48 h. (D) Immunoblot analysis of p-Rb and Rb in HGC-27 or MGC-803 cells treated with scramble control, miR-29a, b or c mimics for 48 h. GAPDH is used as a loading control. (E) Apoptosis assay in HGC-27 or MGC-803 cells after transfection with si_CCND2 #1 and the corresponding negative controls. (F) Immunoblot analysis of MMP2 in HGC-27 or MGC-803 cells transfected with three different siRNAs targeting MMP2 for 48 h. GAPDH is used as a loading control. (G) Representative images (top) and bar graphs (bottom) depicting the invasion ability of HGC-27 and MGC-803 cells after si_CCND2 #1 or si_MMP2 #3 transfection compared with corresponding negative controls (**P < 0.01; ***P < 0.001).
miR-29 family is inversely correlated to CCND2 and MMP2 in human primary GC. (A) Northern blot analysis of miR-29a/b/c expression in four pairs of GC samples. U6 snRNA is used as the loading control. (B) Immunoblot analysis of CCND2 and MMP2 in four pairs of GC samples as described in A. GAPDH is used as the loading control. (C) Representative images of the immunohistochemistry analysis of CCND2 and MMP2 in the same samples as described in A. (D) Inverse correlation between CCND2, MMP2 protein level and miR-29a/b/c expression in 20 GC samples.
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Targeting MMP2 in GC. Meanwhile, we also demonstrated that miR-29 family inhibited cell invasiveness through targeting CCND2 in GC cells.

Upregulation of CCND2 and MMP2 has been observed in different types of malignancy, including GC (35). We have also analyzed the correlation between miR-29 family expression and protein expression of their targets in 20 primary GC samples. Consistent with the cell line data, CCND2 and MMP2 were enriched in the primary GC tissues that inversely correlated to miR-29a, 29b or 29c levels. This result was also observed at the immunostaining in a few primary GC samples. However, further studies using large numbers of primary GC samples will be needed to confirm this interaction.

Although a few studies have tested whether members of miR-29 family could reduce tumorigenicity in a xenograft model, we also tested the tumor-suppressive role of miR-29 family in vivo through direct miRNA mimics injection and found that miR-29a/b/c significantly inhibited the growth of GC cells, respectively. Further immunohistochemistry analysis in xenograft carcinoma tissues demonstrated the negative regulation of miR-29 to CCND2 and MMP2.

Fig. 6. miR-29 family inhibits GC growth in vivo. (A) Diagram illustrating the experimental design of the nude mice xenograft experiment. (B) Photographs of four mice injected with scramble (left flank) or miR-29a, b, c (right flank). (C) Graph representing tumor volumes at the indicated days during the experiment for the four groups: scrambled mimics, miR-29a mimics, miR-29b mimics and miR-29c mimics. Five mice in each group. (D) Tumor weight averages between scrambled and miR-29a/b/c mimics-treated mice groups at the end of the experiment (day 28), *P < 0.05; **P < 0.01. (E) Immunohistochemistry analysis of Ki-67 expression in tumors from xenograft mice. (F) Immunohistochemistry analysis of CCND2 and MMP2 in tumors from xenograft mice.
suggesting that miR-29a, b, and c might be better therapeutic choices in GC. In conclusion, a better knowledge of miR-29a/b/c functions, their interrelationships with these cellular processes and the already available treatments against unwanted proliferation can generate new approaches, such as combined therapies, in which the manipulation of miRNA expression can play a pivotal role.

Taken together, our work demonstrated that miR-29 family was differentially expressed in GC and acted as tumor suppressor genes by regulating critical oncogenic targets through in vitro and in vivo experiments. Reintroducing miR-29a/b/c expression downregulated CCND2 and MMP2, which induced cell apoptosis, suppressed cell invasion and dampened cell growth in GC cells, thereby suggesting miRNA-based therapeutic pattern in GC.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–5 (reference 36 is cited in Supplementary Figure 2) can be found at http://carcin.oxfordjournals.org/

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