miR-338-3p Suppresses Gastric Cancer Progression through a PTEN-AKT Axis by Targeting P-REX2a

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Abstract

Results from recent studies suggest that aberrant microRNA expression is common in numerous cancers. Although miR-338-3p has been implicated in hepatocellular carcinoma, its role in gastric cancer is unknown. To this end, we report that miR-338-3p is downregulated in both gastric cancer tissue and cell lines. Forced expression of miR-338-3p inhibited cell proliferation and clonogenicity and induced a G1–S arrest as well as apoptosis in gastric cancer cells. Furthermore, P-Rex2a (PREX2) was identified as a direct target of miR-338-3p, and silencing P-Rex2a resulted in the same biologic effects of miR-338-3p expression in gastric cancer cells. Furthermore, both enforced expression of miR-338-3p or silencing of P-Rex2a resulted in activation of PTEN, leading to a decline in AKT phosphorylation. Also, miR-338-3p markedly inhibited the in vivo tumorigenicity in a nude mouse xenograft model system. These results demonstrate that miR-338-3p affects gastric cancer progression through PTEN–AKT signaling by targeting P-Rex2a in gastric cancer cells, which posits miR-338-3p as a novel strategy for gastric cancer treatment.

Implications: miR-338-3p acts as a novel tumor suppressor that blocks the growth of gastric cancer cells through PTEN–PI3K signaling by targeting P-Rex2a. Mol Cancer Res; 12(3); 1–9. ©2013 AACR.

Introduction

Gastric cancer is the fourth most common human malignant disease and the second most frequent cause of cancer-related deaths worldwide. Almost two thirds of cases occur in developing countries, of which 42% occur in China (1). Currently, treatment for gastric cancer may include surgery, chemotherapy, radiation therapy, or multimodality therapy (2). Unfortunately, gastric cancer is difficult to cure because the disease is usually diagnosed in the advanced stage. Thus, new treatment approaches such as genome therapy (3), photodynamic therapy (4, 5), and therapeutic microRNA (miRNA; ref. 6) are worth studying further.

miRNAs are small, noncoding RNAs of 19 to 24 nucleotides in length, which could bind at the 3′ untranslated region (UTR) of potentially hundreds of target genes with imperfect complementarity, resulting in degradation of target mRNAs and inhibition of translation (7). miRNAs dysfunction has been found in a variety of human diseases such as cancer. Recent evidence has strongly supported the finding that miRNAs played a part in crucial cellular processes, including development, differentiation, apoptosis, and proliferation (8). miRNA-135a contributes to breast cancer cell migration and invasion by targeting HOX10 (9), whereas miR-10b promotes cell migration and invasion in breast cancer cells by targeting HOXD10 (10). Similarly, miRNA-223 leads to the promotion of gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3 (11).

In our recent work, miR-338-3p is strongly downregulated in patients with gastric cancer, which indicates its potential role as a tumor suppressor in cancer progression. The earlier studies proved that miR-338-3p is downregulated in hepatocellular carcinoma (12) and malignant melanoma (13). In addition, miR-338-3p could suppress progression of cancer cells by targeting smoothened in both liver cancer (14) and colorectal carcinoma (15). Recently, hepatitis B virus X protein was found to be associated with the downregulated expression of miR-338-3p, which could inhibit proliferation by regulating cyclin D1 in liver cancer (16, 17). However, its role in gastric cancer is unclear until now. Meanwhile, we found that phosphatidylinositol 3,4,5-trisphosphate RAC exchanger 2a (P-Rex2a) had an overexpression in gastric cancer compared with normal tissues. By using gene target prediction databases (miRanda and TargetScan), we found that P-Rex2a might be a potential target of miR-338-3p.
target of miR-338-3p. P-Rex2a is a guanine nucleotide exchange factor (GEF) for the RAC guanosine triphosphatase (GTPase), acting as a PTEN-interacting protein, which should activate the phosphoinositide 3-kinase (PI3K) pathway by antagonizing PTEN in cancer cells (18). For the first time, we tested the hypothesis that miR-338-3p should suppress gastric cancer cell growth by targeting P-Rex2a.

Materials and Methods

Human tissue samples and cell lines

Tissues were obtained from patients who had undergone surgical gastric resection at the First Affiliated Hospital of Xi’an Jiaotong University (informed consent was obtained from each patient and was approved by the Institute Research Ethics Committee at Cancer Center, Xi’an Jiaotong University). The immortalized gastric epithelial cell lines (GES-1) and human gastric cancer cell lines (BGC-823 and AGS) were maintained in the Key Laboratory of Environment and Genes Related to Diseases at Xi’an Jiaotong University College of Medicine. These cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM; PAA) supplemented with 10% FBS (PAA) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from the cells and tissues with TRIzol reagent (Invitrogen). PrimeScript RT Reagent Kit and SYBR Premix Ex Taq II Kit were purchased from TAKARA for the detection of mature miR-338-3p expression and mRNA expression. The primer for miRNA was purchased from AuGCT Corporation, as shown in Supplementary Table S2. According to the manufacturer’s instructions, the \(2^{-\Delta\Delta Ct}\) method was used in quantitative real-time PCR (qRT-PCR) with the RNU6B (U6) gene as a control for mature miRNAs, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a control for mRNAs. All reactions were performed in triplicate using the IQ-5 Real-Time PCR System (Bio-Rad).

Expression vector construction

The miR-338-3p expression vector (pre–miR-338-3p) and control vector were constructed with synthetic oligonucleotides and cloned in between the EcoRI and HindIII sites of the pcDNA6.2-GW/EmGFP vector (Invitrogen). The 3’ UTR of human P-Rex2a mRNA was constructed by synthetic oligonucleotides and cloned in between the SacI and XhoI sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The inhibitor of miR-338-3p and small interfering RNA (siRNA) targeting P-Rex2a were purchased from GenePharma. All the vector sequence information is given in Supplementary Table S2.

Bioinformatic analysis

The information of human miR-338-3p was registered for the first time, we tested the hypothesis that miR-338-3p should suppress gastric cancer cell growth by targeting P-Rex2a.

Cell proliferation assay

The BGC-823 cells (5,000 cells/well) were seeded into 96-well plates with 100 μL of DMEM and incubated for 24 hours. Thereafter, 0.2 μg of the DNA vectors or siRNAs was transfected respectively and the cells were further cultivated for additional 1 to 3 days. Cell viability was assessed using the MTT assay FLUOstar OPTIMA (BMG). Each experiment contained 3 replicates and was repeated at least two times. The data were summarized as mean ± SD.

Cell apoptosis analysis

Cell apoptosis analysis was performed with Annexin-V FITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer’s instructions. The cells were seeded into 12-well plates at a density of 1 × 10⁶ cells per well in triplicate and transfected with DNA vectors or siRNAs for 48 hours, and then examined by using a flow cytometer (Becton), and the apoptosis populations were determined by ModFit software.

Cell cycle analysis

The BGC-823 cells at 1 × 10⁶ cells per well were cultured in 12-well plates in triplicate and transfected with DNA vectors or siRNAs for 48 hours. The cells were harvested by trypsinization and washed in PBS and fixed in ice-cold ethanol overnight at 4°C. The cells were then washed in PBS and incubated in 1 mL of staining solution (20 μg/mL propidium iodide and 10 U/mL RNaseA) for 30 minutes at room temperature. Cell cycle distributions were assayed by fluorescence-activated cell sorting based on flow cytometer.

Dual-luciferase assay

The pmirGLO-P-Rex2a-3’-UTR vector was cotransfected with miR-338-3p into HEK293 cell lines, pmirGLO vector as their control. Then, the cells were harvested and lysed for luciferase assays 24 hours after transfection. The Dual-Luciferase Reporter Assay System (Promega) was used to measure the reporter activity according to the manufacturer’s protocol.

Colony formation assay

Stably transfected cells were seeded at a density of 10,000 per 6-well plate, incubated for 2 weeks, and then stained with 0.5% crystal violet for 30 minutes. Excess dye was rinsed off two times with PBS. The number of colonies was counted by computer software.

Western blot analysis

Total protein was extracted by using a radioimmunoprecipitation assay cell lysis buffer (Wolsen) from cells harvested 48 hours after transfection, separated in 10% SDS-PAGE gels, transferred to a nitrocellulose membrane, and probed with antibodies against P-Rex2a.
SDS polyacrylamide gels, and electrophoretically transferred to polyvinylidene difluoride membrane (Millipore). After that, mouse polyclonal anti–P-Rex2a (antibody dilutions: 1 μg/mL), rabbit monoclonal antibody (mAb) anti-PTEN (antibody dilutions: 1:1,000), rabbit mAb anti-Akt (antibody dilutions: 1:1,000), rabbit mAb anti–phospho-Akt (Ser473; antibody dilutions: 1:2,000), rabbit mAb anti-CCND1/p-CCND1 (antibody dilutions: 1:1,000), rabbit mAb anti-CDK2 (antibody dilutions: 1:500), rabbit mAb anti-Bax (antibody dilutions: 1:1,000), rabbit mAb anti-Bcl-2 (antibody dilutions: 1:1,000), and mouse monoclonal anti-GAPDH antibody (antibody dilutions: 1:2,000; Cell Signaling Technology) were used for detection in enhanced chemiluminescence detection system (Pierce). The blots were scanned and the band density was measured on the Quantity One imaging software.

**Immunohistochemistry**

The human tumor tissues were made into paraffin sections and pretreated with microwave, blocked, and incubated in the primary antibodies (P-Rex2a; CST). The sections were then incubated with secondary antibody conjugated with horseradish peroxidase (ZSBIO) and developed with ABC (ZSBIO) and diaminobenzidine reagent (BOSTER). Finally, digital images were taken with a Leica Photo Microscope (Leica).

**In vivo tumor xenograft model**

Six-week-old male nude mice (BALB/c-nude) were used to examine tumorigenicity. BGC-823 stable cells overexpressing miR-338-3p or vector control cells were established by lentivirus vector and resuspension solution of 1/C2106 were injected into the nude mice. Tumor size was measured every 3 days. For endpoint experiments, the bioluminescence images in vivo were obtained by the system of photobiology (Xenogen).

**Statistical analysis**

Each experiment was repeated at least 3 times independently. The data were presented as mean ± SD and analyzed using the PASW Statistics 18 software (IBM). Differences or correlations between 2 groups were calculated with the Student t, χ², or Pearson correlation. P ≤ 0.05 was considered to be significant.

**Results**

**Aberrant miR-338-3p expression in human gastric cancer**

To validate the expression of miR-338-3p in gastric cancer, we analyzed the expression in 53 pairs of gastric cancer tissue and matched adjacent normal tissue samples by using real-time PCR. Compared with their normal tissues, significant downregulation of miR-338-3p was observed in 73.6% (39 of 53) of gastric cancer samples (Fig. 1A). Furthermore, analysis of miR-338-3p expression in 3 gastric cancer cell lines (GES-1, BGC-823, and AGS) revealed that miR-338-3p was downregulated in tumor cell lines as well (Fig. 1B). These data suggested that miR-338-3p might act as a tumor suppressor in gastric cancer.

**P-Rex2a is a direct target of miR-338-3p**

We searched 3 bioinformatic databases (TargetScan, PicTar, and miRanda) to identify a large number of potential target genes of miR-338-3p. Among these candidates, P-Rex2a was selected for the further analysis. A binding site of miR-338-3p was observed in the 3′ UTR of Figure 1. miR-338-3p is downregulated in gastric cancer tissues and cell lines. A, qRT-PCR was performed to examine miR-338-3p expression in 53 paired human gastric cancer and adjacent normal tissues. The expression of miR-338-3p was normalized to U6 with respect to specimen N1. B, qRT-PCR analysis of miR-338-3p expression in normal gastric and gastric cancer cells and normalized against an endogenous control (U6 RNA). Data were analyzed using a ΔΔCt approach. One-way ANOVA was adopted to compare miR-338-3p expression and all data are shown as mean ± SD for 3 independent experiments (*, P < 0.05, Student t test).
P-Rex2a mRNA. Moreover, the seeded sequences were exactly conserved after comparing the human sequence with different species (Fig. 2A). To validate the hypothesis that P-Rex2a might be a target of miR-338-3p, a dual-luciferase reporter system containing wild-type 3′ UTR of P-Rex2a was used. HEK293 cells were cotransfected with pre–miR-338-3p and reporter plasmid (P-Rex2a_UTR) or pmirGLO control vector. As a result, pre–miR-338-3p/P-Rex2a_UTR-transfected cells showed a significant reduction (approximately 40%) of luciferase activity (Fig. 2B), indicating that miR-338-3p may suppress gene expression through its binding sequences at the 3′ UTR of P-Rex2a. Meanwhile, the result showed that P-Rex2a protein has an overexpression in gastric cancer samples compared with their normal tissues control (Fig. 2C and D). Furthermore, a reduction of the P-Rex2a mRNA and protein expression levels was observed in BGC-823 cells transfected with pre–miR-338-3p compared with control vector–transfected cells (Fig. 2E). These results indicate that miR-338-3p directly recognizes the 3′ UTR of P-Rex2a mRNA and inhibits P-Rex2a translation.

Overexpression of miR-338-3p induces G1–S arrest and decreases gastric cancer cell growth by suppressing the Akt signaling pathway

The results of qRT-PCR showed that the expression of miR-338-3p had a more than 10-fold increase in the pre–miR-338-3p-transfected cells compared with the control vector–transfected cells (Supplementary Fig. S1). To examine the role of miR-338-3p in gastric cancer cell growth, MTT assays, cell-cycle analysis, and colony formation assay were adopted. The results showed that the transient overexpression of miR-338-3p led to the inhibition of the growth of BGC-823 at 24, 48, and 72 hours after transfection (Fig. 3A), resulted in a marked accumulation of the G1 population and a reduction of the S-phase population in the gastric cancer cell line BGC-823 (Fig. 3B), and reduced colony formation (Fig. 3C) in comparison with control vector–transfected cells. These findings suggested that miR-338-3p can arrest the cell cycle at G1–S transition phase and suppress cell proliferation in vitro.

Pre–miR-338-3p- and control vector–transfected cells were injected subcutaneously into each side of posterior flanks of the nude mouse. The mice were observed for xenograft growth for 4 weeks. The tumors injected with...
control vector cell were clearly seen from the bioluminescence images obtained by using the system of photobiology, whereas pre–miR-338-3p cells almost showed nontumors (Fig. 3D). These data indicate that miR-338-3p could remarkably inhibit the tumorigenicity of BGC-823 cells in the nude mouse xenograft model.

To further investigate the possible molecular mechanisms of miR-338-3p–induced cell proliferation repression, we detected the protein expression level of P-Rex2a and its downstream pathway regulators by Western blotting after transfection with pre–miR-338-3p and control vector. Our results show that miR-338-3p can reduce the expression of P-Rex2a protein and the phosphorylation of p-AKT at serine 473, whereas the protein expression of PTEN and total AKT remained unchanged (Fig. 3E). Moreover, we found that miR-338-3p could inhibit cell proliferation at the G1–S phase (Fig. 3B), which prompted us to investigate the underlying mechanisms of cell-cycle regulation. As a consequence, both miR-338-3p and si-P-Rex2a could reduce the expression of cyclin D and cdk2 (Fig. 3E). In addition, they also induced a clear decrease of cyclin D1 at a phosphorylation site at Thr286 (Fig. 3E). These results demonstrate that miR-338-3p affects gastric cancer cell growth, proliferation, and cell cycle by controlling P-Rex2a/PTEN/AKT pathways.

Inhibition of miR-338-3p contributes to tumorigenicity of BGC-823 cells

Loss-of-function studies were also performed by using anti–miR-338-3p oligonucleotides to silence miR-338-3p expression. Unexpectedly, the inhibitory effect was moderate because of the low expression of endogenous miR-338-3p in BGC-823 cells, but it rather showed that miR-338-3p could contribute to the tumorigenicity of BGC-823 cells. As shown in Fig. 4, transfection with the inhibitor of miR-338-3p promoted cell clone formation and induced a G1–S transition in BGC-823 cells. Furthermore, the knockdown of miR-338-3p increased the expression of P-Rex2a, CCND1/p-CCND1, and CDK2 tested by Western blot analysis. Together with the above experiments, these results suggested an essential contribution of endogenous miR-338-3p to the anticarcinogenesis of BGC-823 cells in gastric cancer progression.
miR-338-3p could induce apoptosis in BGC-823 cells

During the experiment in vitro, we found that overexpression of miR-338-3p could also induce cell apoptosis. Compared with cells transfected with a control vector, pre-miR-338-3p-transfected cells displayed higher apoptotic rates at an early phase, whereas the miR-338-3p inhibitor exhibited no significant difference when the cells were transfected with negative control (Fig. 5A and B). Data show that miR-338-3p induces apoptosis in human gastric cancer cells in vitro.
Deregulation of apoptosis is an important event in cancer as it allows the genetically unstable cells to survive and accumulate further mutations that eventually lead to tumorigenesis. We also focused on the apoptosis of gastric cancer cells and examined whether miR-338-3p modified the downstream targets of PI3K related to apoptosis. As a result, suppression of p-AKT could promote apoptosis by accelerating proapoptotic protein Bax and inactivating antiapoptotic protein Bcl-2 (Fig. 5C).

Silencing of P-Rex2a could suppress gastric cancer cell growth and induces G1–S arrest, cell apoptosis similarly to miR-338-3p

As shown earlier, the overexpression of miR-338-3p affects cell growth, proliferation, cycle, and apoptosis in gastric cancer cells BGC-823. We also validated P-Rex2a as a direct target of miR-338-3p. Therefore, we silenced P-Rex2a expression by RNA interference (RNAi) to confirm that P-Rex2a is involved in the antitumor effects of miR-338-3p. From mRNA and protein expression level, P-Rex2a can be specifically knocked down by siRNA (Fig. 6A). Moreover, silencing of P-Rex2a resulted in suppressed cell growth, proliferation, and induced G1–S arrest, cell apoptosis (Fig. 6B–E), which followed the same trend as miR-338-3p in BGC-823 cells.

Furthermore, this was confirmed by an analysis of protein expression in the AKT pathway. As shown in Fig. 6F, the expression of P-Rex2a protein and the phosphorylation of p-AKT at serine 473 were suppressed by si-P-Rex2a, whereas the protein expression of PTEN and total AKT remained unchanged. For cell-cycle regulation, si-P-Rex2a could reduce the expression of cyclin D and cdk2, and induce a clear decrease of cyclin D1 at a phosphorylation site at Thr286. Moreover, siRNA could promote apoptosis by activating proapoptotic protein Bax and inactivating antiapoptotic protein Bcl-2. On the basis of these findings, we concluded that miR-338-3p could regulate gastric cancer cell progression by directly targeting P-Rex2a through the PTEN/Akt signaling pathways.

Discussion

Gastric cancer remains as one of common cancer types and still is a leading cause of cancer-related deaths (19), and its genesis is considered to be related to predominantly genetic and environmental factors (20). Furthermore, gastric carcinogenesis is a multifactorial process associated with multiple genetic and epigenetic events. During the past years, dysregulation of miRNAs has been shown to play a role in control cell proliferation (21), metastasis (22), and cell cycle (23) in gastric cancer. The miR-338 gene, located on chromosome
17q25 within eighth intron of the apoptosis-associated tyrosine kinase (AATK) gene, produces 2 mature forms (miR-338-3p and miR-338-5p; ref. 24), and AATK activity plays an essential role in promoting neurite extension in developing neurons (25). Our studies showed that miR-338-3p was frequently downregulated in both gastric cancer tissues and cell lines, which indicates that miR-338-3p might be a novel tumor suppressor miRNA.

Furthermore, for the first time, our miR-338-3p target analysis identified P-Rex2a as a direct target of miR-338-3p. P-Rex2a is a GEF for the RAC GTPase (26), which could encode a protein-inhibiting phosphatase activity against phosphatidylinositol 3,4,5-trisphosphate (PIP3) in vitro (18). In our study, we found that P-Rex2a has an overexpression in gastric cancer compared with normal tissues, which shows an inverse correlation between P-Rex2a expression and miR-338-3p expression in gastric cancer tissues. These results implied that miR-338-3p may affect the progression of gastric cancer by targeting P-Rex2a. Further bioinformation analysis showed that there was a miR-338-3p–binding site at 4898–4917nt of the P-Rex2a 3′ UTR. The dual-luciferase reporter assays demonstrated that miR-338-3p targeted directly to P-Rex2a by recognizing the 3′ UTR of P-Rex2a mRNA and inhibited P-Rex2a translation.

The manipulation of P-Rex2 expression could affect AKT phosphorylation in a PTEN-dependent manner by binding to PTEN in cells (18). The PTEN (phosphatase and tensin homolog on chromosome; ref. 10) gene is frequently lost in cancers, and germline PTEN mutations are linked to inherited cancer predisposition syndromes (27). Biochemically, PTEN is a phosphatase that dephosphorylates PIP3, the lipid product of the class I PI3K (28). The PI3K pathway is one of the most potent prosurvival pathways in cancer (29). Inactivation of PTEN leads to accumulation of PIP3 and, as a consequence, increases activity of the kinase AKT, which contributes to oncogenesis in numerous other cancers, including glioblastoma (30), prostate (31), and liver cancers (32). From the existing literature, we found that AKT phosphorylation is essential for progression from G0 phase to G1 phase (33). In our research, P-Rex2a has been inhibited by the overexpression of miR-338-3p and siRNA, which resulted in activation of PTEN and tend to decline in phosphorylation of AKT. Then, we studied the effect on AKT downstream target genes cyclin D1 and CDK2 (34), which are key transcriptional factors in the G0–G1 phase. The results showed that there was a reduction on expression level of CDK2 as well as the phosphorylation of the cyclin D1 at threonine 286 in the cells transfected by miR-338-3p. Similarly, we also provide evidence that miR-338-3p-induced PI3K–AKT pathway plays an important role in the regulation of the Bcl-2 and Bax family, as previously shown (35).

In addition, RNAi-mediated knockdown or overexpression of P-Rex2a was also able to control xenograft tumor formation in vivo (36). The evidence of cancer xenograft models in our study has already been shown, but studies to date have shown only changes in tumor size when cells were transfected by miR-338-3p and control vector, and then injected to produce the tumor model. Such studies were also performed for prostate cancer (37), hepatocellular carcinoma (38), and pancreatic cancer (39). These results also confirmed the tumor-suppressive role of miR-338-3p in gastric cancer.

In conclusion, we investigated the role of miR-338-3p, its target gene P-Rex2a, and their potential implications on the process of gastric cancer. These data suggest that miR-338-3p may be a novel tumor suppressor that blocks the growth of gastric cancer cells through PTEN/PI3K signaling pathways by targeting P-Rex2a. Our findings provide evidence that miR-338-3p could be a useful tool in the future control of gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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