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MiR-493 suppresses the proliferation and invasion of gastric cancer cells by targeting RhoC

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ABSTRACT

Objective(s): MiRNAs have been proposed to be key regulators of tumorigenesis, progression and metastasis. However, their effect and prognostic value in gastric cancer is still poorly known.
Materials and Methods: Gastric cancer cell lines were cultured. Tissue samples obtained from 36 gastric cancer patients were used for quantitative real-time PCR (qRT-PCR) analysis. The tissue microarrays (TMAs) consisted of 126 cases of gastric carcinoma that were used for *In situ* hybridisation (ISH). Lentivirus plasmids were co-transfected into 293FT cells. Cell migration was examined using wound-healing assays. Statistical analyses were performed using SPSS16.0 software.
Results: In this study, we found that the expression levels of miR-493 were strongly down-regulated in gastric cancer and were associated with clinical stage and the presence of lymph node metastases. Moreover, miR-493 might independently predict OS and RFS in gastric cancer. We further found that up-regulation of miR-493 inhibited the proliferation and metastasis of gastric cancer cells, *in vitro* and *in vivo*. In addition, miR-493 directly targeted RhoC, which resulted in a marked reduction of the expression of mRNA and protein. This effect, in turn, led to a decreased ability of growth, invasion and metastasis in gastric cancer cells.
Conclusion: Taken together, our findings demonstrate that miR-493 is important for gastric cancer initiation and progression and holds promise as a prognostic biomarker to predict survival and relapse in gastric cancer. It is also a potential therapeutic tool to improve clinical outcomes in this disease.

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Introduction

Gastric cancer is one of the most common malignancies and is the second leading cause of cancer mortality worldwide (1). Nearly half of gastric cancer occurs in China with an overall 5-year survival rate of approximately 20% (2), most of which are diagnosed in advanced stages losing the opportunity for radical surgery. Lack of early detection and limited treatment options contribute to its bad prognosis. Therefore, identification of novel mediators of invasion and metastasis, in addition to novel biomarkers of gastric cancer progression, is crucial to improve patient outcome (3, 4).

MiRNAs are 19–24 nucleotides in length and regulate gene expression by imperfect base-pairing with complementary sequences located mainly. Hence, miRNAs represent one of the major regulatory families of genes in eukaryotic cells, and work by inducing translational repression and transcript degradation (5). MiRNAs have been reported to be significantly involved in tumorigenesis and progression, acting as either oncogenes or tumor suppressors (6-8). Emerging studies have reported that a group of miRNAs is commonly dysregulated in gastric cancer. For example, miR-429, miR-148a, miR-20b, miR-195, miR-378, miR-101 and miR-200b/c (8-12) were reported to be always down-regulated, while miR-19a, miR-301a, miR-544, miR-18a, miR-125b and miR-181b (13-18) were over-expressed. Since these specific miRNAs regulate different target genes which are involved in various signaling pathways and biological processes, gastric cancer could be complicated diseases with multiple genes being dysregulated (6).

Recently, Ueno *et al* reported that miR-493 was significantly down-regulated in bladder cancer as shown by miRNA microarray analysis and decreased cell motility and migration ability (19). miR-493 induction during carcinogenesis blocks metastatic settlement of colon cancer cells in the liver (20).

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Thus, we investigated the effect of miR-493 on the carcinogenesis and progression of gastric cancer and their prognostic significance.

In the present study, we found that miR-493 expression is down-regulated in 36 stomach tumour specimens, as well as gastric cell lines, by quantitative RT-PCR analysis. miR-493 expression was detected by in situ hybridisation on tissue microarrays, and the association between miR-493 levels and clinicopathologic factors and prognosis were analysed. Our results indicated that decreased miR-493 correlates with advanced clinical stage, lymph node metastases and poor clinical outcomes. Additionally, we observed that miR-493 suppresses gastric cancer growth and metastasis, *in vitro*.

Materials and Methods

Cell culture

The gastric epithelial cell line GES-1 was purchased from Beijing Institute for Cancer Research (Beijing, China). The gastric cancer cell lines SGC-7901, HGC-27, AGS MKN-45, MGC-803, BGC-823 and MKN-28 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cells were maintained at 37 ℃ in an atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% foetal bovine serum, penicillin and streptomycin (Gibco BRL, NY, USA). All transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA).

Clinical samples

All tissue samples used in the present study were collected from Hunan Provincial Tumour Hospital (Changsha, Hunan, China). Written informed consent was obtained from all the participants (4). The study was approved by the Ethics Committee of the University of South China Health Authority. Collection and use of tissues followed the procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration. Tissue samples obtained from 36 gastric cancer patients were used for quantitative real-time PCR (qRT-PCR) analysis. Resected cancerous tissues (Tumour) and paired matched normal gastric tissues (Normal) were immediately cut and stored in RNAlater (Ambion). The tissue microarrays (TMAs) consisted of 126 cases of gastric carcinoma. All data, including age, sex, histological grade, tumour size, invasion depth, and lymph node metastasis were obtained from clinical and pathological records.

Quantitative RT-PCR analysis (qRT-PCR)

Total RNA was extracted from cells or tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and RT reactions were performed using miR-493 special prime. The specific stem–loop RT primers for miR-493 were purchased from Ribobio co., Ltd (Guangzhou, China). Real-time PCR was performed using a standard protocol from the SYBR Green PCR kit (Toyobo, Osaka, Japan). Fold change was determined as 2-ΔΔCt. The Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The ΔCt was calculated by subtracting the Ct of snRNA U6 from the Ct of the miRNA of internal control. The ΔΔCt was calculated by subtracting the ΔCt of the reference sample (paired non-tumorous tissue for surgical samples) from the ΔCt of each sample. U6 snRNA primer, 5'-ATTGGAACGATACAGAGAAGATT-3' and 5'-GGAACGCTTCACGAATTTG-3'.

In situ hybridisation

Tissue microarray slides were deparaffinised and rehydrated. The miR-493 miRCURYTM LNA custom detection probe (Exiqon, Vedbaek, Denmark) was used for in situ hybridization (ISH). The sequence 5’-3’ (enhanced with LNA) was TCAGGAACTGCCTTTCTCTCCA with digoxigenin (DIG) at the 5’ and 3’ ends. Hybridization, washing, and scanning were carried out according to the manuals and protocols provided by the Exiqon life science department. The intensity of staining was scored from 0 to 4, while the extent of staining was scored from 0 to 100%. Relative expression was calculated by multiplying the two scores. The slides were analysed by two independent pathologists.

Overall survival (OS) and disease-free survival (DFS)

DFS was defined as the interval between surgery and the date of diagnosis of the first recurrence or the date of the last follow-up. OS was calculated from diagnosis to the date of death for any causes, while patients who were alive were censored at the date of last follow-up visit.

Lentivirus production and infection

Lentivirus plasmids were co-transfected into 293FT cells (Invitrogen) with pLP1, pLP2, and pLP/VSVG (Invitrogen), and virus containing supernatants were prepared according to manufacturer’s instructions. As for lentivirus infection, cells were incubated with virus-containing supernatants in the presence of 6 ug/ml polybrene. Infected cells were selected in the presence of 2 ug/ml puromycin to generate two paired stable monoclonal cell lines (a stable cell line expressing miR-493, MKN-45 -miR-493 and control stable cell line, MKN-45-control). For infection of GFP-expressing viruses for miRNA expression, flow cytometry analyses (FacsCalibur, Becton Dickinson) were performed to make sure that 90% of cells were infected.

Cell migration and invasion assays (8)

Cell migration was examined using wound-healing assays. An artificial ‘‘wound’’ was created on a confluent cell monolayer, scratching assay was treated by 10 μg/ml mitomycin C for 2 hr, and

Table 1. Analysis of the correlation between expression of miR-493 in primary gastric cancer and its clinicopathological parameters

Viable	Cases	miR-493		
		low	high	*P*- value
Age (years)				
＜60	73	40	33	0.192
≥60	53	34	19	
Gender				
Male	70	44	26	0.274
Female	56	30	26	
Histological grade [a]				
well and moderate	32	23	9	0.060
Poor and Other	94	51	43	
T stage				
T1-T2	71	37	34	0.062
T3-T4	55	37	18	
TNM stage				
I-II	51	24	27	0.022
III-IV	75	50	25	
Lymph node metastasis				
Present	88	59	29	0.004
Absent	38	15	23	

[a] Well differentiated adenocarcinoma (Well), Moderately differentiated adenocarcinoma (Moderate), Poorly differentiated adenocarcinoma (Poor), Other histological type (Other)

photographs were taken using an inverted microscope (Olympus, Tokyo, Japan) at 24 hr. As for cell invasion assay, cells were seeded onto the basement membrane matrix present in the insert of a 24-well culture plate (EC matrix, Chemicon, Temecula, CA) and fetal bovine serum was added to the lower chamber as a chemo-attractant. After 48 hr, the non-invading cells and EC matrix were gently removed with a cotton swab. Invasive cells which located on the lower side of the chamber were stained with Crystal Violet, counted and imaged.

Western blot analysis

Protein concentration in the lysates was measured with the Protein BCA Assay Kit (Bio-Rad), and 20 mg of protein mixed with 2× SDS loading buffer was loaded per lane. The proteins in the lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Next, the membranes were incubated for 12 hr at 4 ˚C with an antiserum containing antibodies against Rhoc, IGF1R, FZD4 and β-actin purchased from Cell signaling Technology. A peroxidase-conjugated secondary antibody and ECL western blotting detection reagents were used to visualise the target proteins (ECL New England Biolabs). Then, proteins were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nihon-BioImage Ltd.). An anti-β-actin antibody was used as a protein loading control.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Comparisons between groups were done by t-test and x2 test. All differences were statistically significant at the level of $P \leq 0.05$. Statistical analyses were performed using SPSS16.0 software.

Results

miR-493 is down-regulated in gastric cancer

First, a set of human gastric cancer cell lines was analyzed to assess the expression profile of miR-493 in gastric cancer using qRT-PCR (Figure 1A). Compared with the non-malignant gastric cell line GES-1, seven gastric cancer cell lines showed reduced miR-493 expression, especially the MKN-45 cells. We also compared miRNA-493 expression levels in a series of 36 pairs of gastric cancer tissues and their matched adjacent tissues. Among the 36 patients with gastric cancer, approximately 75% ($P<0.01$, 27 out of 36 patients) of tumours revealed a large reduction of miR-493 levels (2.6-fold) relative to adjacent non-tumour tissues (Figure 1B).

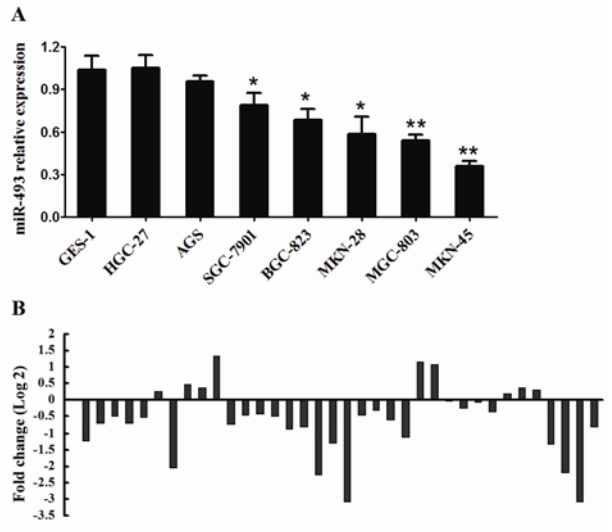

Figure 1. Analysis of the correlation between the expression of miR-493 in primary gastric cancer and its clinicopathological parameters

miR-493 expression level was frequently down-regulated in human gastric cancer. (A) Relative expression of miR-493 in seven cell lines derived from gastric cancer and one non-malignant gastric cell line (GES-1) was determined by qRT-PCR. The error bars represent the standard deviations (SD) from triplicates of one representative experiment. **P*<0.05 and ***P*<0.01. (B) miR-493 expression was detected in 36 gastric cancer patients by qRT-PCR

Decreased miR-493 correlates with advanced clinical stage, lymph node metastases and poor clinical outcomes

To further verify the results concerning the biological role of miR-493 in gastric cancer, we employed in situ hybridisation to evaluate miR-493 levels in 126 gastric tumour tissues in a tissue microarray (TMA). Of note, the miR-493 level inversely correlated with clinical stage and lymph node metastasis (*P*=0.022 and *P*=0.004, respectively) (Table. 1). However, neither miR-493 levels in gastric cancer patients correlated with age, gender, tumour size, cell differentiation or invasion depth. Our results suggest that miR-493 could play critical roles in carcinogenesis and progression of gastric cancer. To further analyze the significance of miR-493 in terms of clinical prognosis, Kaplan-Meier survival analysis was performed using patient overall survival and relapse-free survival. The results demonstrated that patients with low miR-493 expression had shorter mean months of OS (*P*=0.003) (Figure 2A) and RFS (*P*=0.013) (Figure 2B) than did patients with high miR-493 expression.

MiR-493 inhibited gastric cancer cell proliferation and invasion in vitro

Because miR-493 levels were down-regulated in gastric cancer and were associated with the clinical stage, lymph node metastasis and clinical outcome, we evaluated the effect of miR-493 overexpression on gastric cancer cell phenotype. Since MKN-45 with

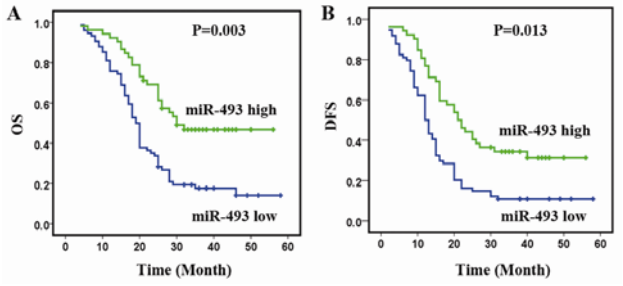

Figure 2. Survival curves of OS (A) and DFS (B) according to miR-493 expression. Low miR-493 expression was correlated with worse outcome

relatively low basal expression of miR-493 (Figure 1A), stable ectopic overexpression cell subsets MKN-45/miR-493 and their paired control cells were constructed. qRT-PCR analysis showed that the transfection were successful (data not shown). We determined that overexpression of miR-493 in MKN-45 cells markedly attenuated cell proliferation. The proliferation assay showed that ectopic expression of miR-493 in MKN-45 markedly attenuated cell proliferation compared with control cells (Figure 3A). Moreover, the expression of miR-493 significantly inhibited the capacity of cells for invasion (Figures 3B and C). These data demonstrate miR-493 functions as a tumour suppressor in gastric cancer.

Identification of miR-493 target genes and their effect on proliferation and invasion of gastric cancer cells

On the basis of the observation showing that miR-493 affects cell proliferation and invasion, we searched for target genes of miR-493 related to migration. Among these candidate target genes, three known genes (Rhoc, FZD4 and IGF1R) which had been proved as target genes of miR-493 (19, 20) attracted our attention. To verify if miR-493 directly targets and inhibits aforementioned genes, we firstly checked the target genes expression level of mRNA and protein in MKN-45 cells infected with miR-493 or mock lentivirus. The results indicated that miR-493 obviously reduced the expression of mRNA and protein of Rhoc but not FZD4 and IGF1R (Figures 4A and B). Rhoc has been reported as a relative gene to cancer metastasis and growth in massive studies. In light of the findings, we hypothesized that Rhoc might phenocopy the effects of miR-493 in gastric cancer cell lines. To confirm this presumption, we constructed siRNA fragments targeting Rhoc to knock down the expression of Rhoc. The effects of Rhoc knockdown on cell growth and invasion were evaluated in MKN-45 cells using the invasion and proliferation assays. The data indicated that Rhoc knockdown resulted in a reduction in cell proliferation and invasion (Figures 4C and D).

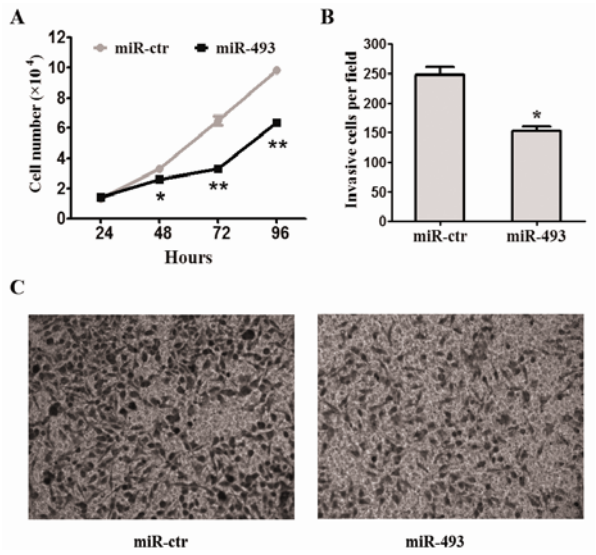

Figure 3. MiR-493 inhibited cell proliferation and invasion in gastric cancer. (A) The growth of MKN-45 cells infected with miR-493 lentivirus or control lentivirus was assayed. Data represent the means±SEM from three independent experiments. **P*<0.05, ***P*<0.01. (B) The invasion assay of MKN-45 cells infected with miR-493 lentivirus or control lentivirus. MiR-493 lentivirus inhibited the cells invation. Data represent the means±SEM from three independent experiments. **P*<0.05. (C) Representative images of the assays are shown. Original magnification: ×200

Discussion

Until now, a handful of studies have identified specific miRNAs involved in human tumorigenesis and tumor progression (3, 8, 21). Therefore, we believe more effort should be made, not only towards the identification of relevant miRNAs but also to identify the specific mechanisms through which they accomplish their specific functions, particularly with regard to the oncogenesis of different types of tumors (16, 22, 23). In this study, we used qRT-PCR and ISH to show that miR-493 was frequently down-regulated in gastric cancer, and 75% (27 out of 36) of the gastric cancer had 2.6-fold reduced expression of miR-493 as compared to their corresponding non-tumorous tissues. Intriguingly, we found that lower expression of miR-493 tended to have more advanced TNM stage (stage I/II vs. stage III/IV, *P*=0.004), suggesting that low expression of miR-493 is associated with gastric cancer progression. Kaplan-Meier survival analyses revealed that patients whose primary tumours displayed low expression of miR-185 had a shorter OS and RFS in gastric cancer. Further studies showed that overexpression of miR-493 suppressed gastric cancer cell proliferation and invasion, *in vitro*. The data from the current study suggests that miR-493 is important for gastric cancer initiation and progression and that down-regulated miR-493 contributes to lymph node-metastasis and tumor progression in gastric cancer patients.

As the next step, we explored the possible targets of miR-493 in lung cancer cells. Rhoc, FZD4 and

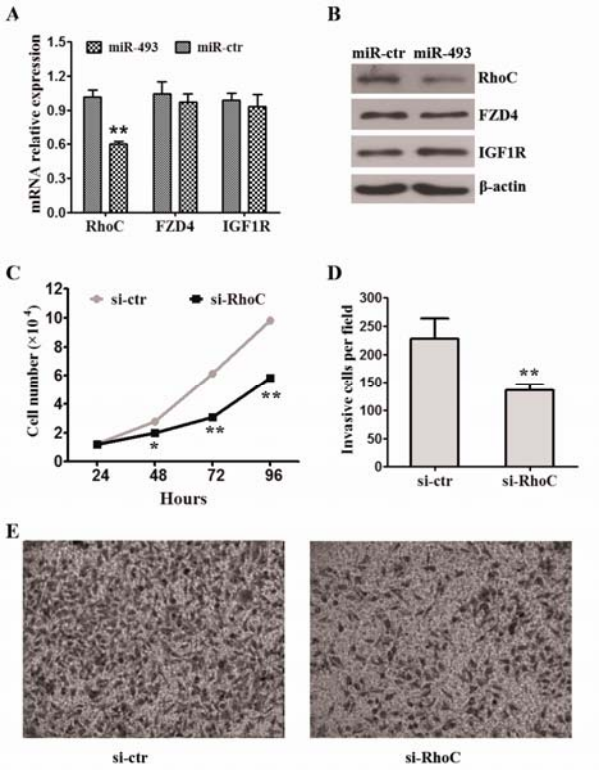

Figure 4. MiR-493 inhibited the capability of invasion of gastric cancer *in vitro* by targeting Rhoc. (A) miR-493 strongly reduced the expression of Rhoc but not FZD4 and IGF1R in mRNA level. The error bars represent the standard deviations (SD) from triplicates of one representative experiment. ***P*<0.01. (B) miR-493 strongly reduced the expression of RhoC but not FZD4 and IGF1R in protein level. (C) Knockdown of Rhoc inhibited the cells proliferation. Data represent the means±SEM from three independent experiments. **P*<0.05, ***P*<0.01. (D) Knockdown of Rhoc inhibited the cells invasion. Data represent the means±SEM from three independent experiments. **P*<0.05. (E) Representative images of the assays are shown. Original magnification: ×200

IGF1R were identified as the regulated target gene in previous studies (19, 20). Thus, we examined the effects of miR-493 on the expression of Rhoc, FZD4 and IGF1R. The data indicated that miR-493 dramatically reduced the expression of mRNA and protein of RhoC, but not FZD4 and IGF1R. It is possible that the discrepancies in the functions of miR-493 in different types of cancer may reflect differences in target genes. To confirm the role of RhoC in miR-493 inducing the inhibition of cell proliferation, we constructed siRNA fragments targeting RhoC to knock down the expression of RhoC. The data indicated that RhoC knockdown resulted in a reduction in cell proliferation and invasion. Rho family proteins regulate multiple cellular functions including motility and invasion through regulation of the actin cytoskeleton and gene expression. RhoA and/or RhoC are key players that regulate the metastatic activity of malignant tumor cells (24). RhoC expression correlates positively with metastasis in a number of cancer types, including melanoma (25), lung cancer (26)

and gastric cancer (27). RhoC deletion did not affect breast cancer growth in a mouse model, but metastasis was specifically impaired (28). Interestingly, we found that knockdown Rhoc partly inhibited the proliferation and invasion of MKN-45 cells.

In summary, we observed down-regulation of miR-493 in gastric cancer cells and tissues. We further found that miR-493 as an important tumor suppressive miRNA which inhibits cell proliferation and invasion by blocking RhoC in gastric cancer. Our findings demonstrated that miR-493 is important for gastric initiation and progression and could be considered as a potential therapeutic to suppress gastric cancer invasion.

Conclusion

Our findings demonstrated that miR-493 is important for gastric cancer initiation and progression and holds promise as a prognostic biomarker to predict survival and relapse in gastric cancer. It is also a potential therapeutic tool to improve clinical outcomes in this disease.

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