

The Prognostic Value and Biological Function of MicroRNA-491-5p for Gastric Cancer

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Abstract

Background: Gastric Cancer (GC) is one of the deadliest cancers in China. And, it can be regulated by MicroRNAs (miRNAs) generally. miR-491-5p function as a tumor suppressor in different types of cancer, but we still don't know the role of miR-491-5p in GC.

Methods: RT-PCR was used to detect the level of miR491-5p and its clinical value, functional experiments including CCK-8 assay and transwell assay were performed. Furthermore, the underlying mechanism was explored through qRT-PCR and western blotting. The function of miR-491-5p was also identified in vivo.

Results: miR-491-5p was related with TNM stage, patients with high miR-491-5p had better prognosis. We found that high level of miR-491-5p caused a weak cell proliferation, migration and invasion abilities. In order to explore the role of miR-491-5p in vivo, we set a xenograft mouse model, and found that high level of miR-491-5p suppressed tumor growth. Moreover, we found that miR-491-5p regulate the tumor development through regulate the expression of EMT (Epithelial-Mesenchymal Transition), cell adhesion genes and IFITM2.

Conclusions: miR491-5p is an important prognostic bio-marker, which can offer help for diagnosis and treatment of GC patients, and miR-491-5p function as a tumor suppressor in GC both in vitro and in vivo.

Keywords: miR-491-5p; Gastric Cancer; EMT

Introduction

Background

Gastric Cancer (GC) is an aggressive and deadly malignancy. Surgery and Endoscopic Mucosal Resection (EMR) with or without adjuvant systemic chemotherapy are the primary treatments for gastric cancer. Currently, there are no effective drugs for gastric cancer. Despite a steady decline in overall morbidity and mortality over the past few decades, most of patients diagnosed with GC are already in an advanced-stage so that the 5-year survival rate is less than 15%. Due to its high mortality and geographical distribution, gastric cancer has become an important public health problem in China. Therefore, it is urgent to further explore the gastric cancer progress and develop mechanisms [1].

MicroRNAs (miRNAs) have been shown to be oncogenes in kind of cancer. To the contrary, it also had been reported as a tumor suppressor in some other kind of cancer. In GC, miRNAs have their own unique expression profile, abnormal expression of miRNAs are associated with abnormal histological subtypes and cause a progression of cancer. miR-491-5p function as a tumor suppressor in kind of cancer that regulate the abilities of cell proliferation, migration, invasion and associated with apoptosis even chemo resistance activities. However, the function of miR-491-5p in GC is not very clearly. Some research reported that miR-491-5p is low expressed in GC, and cause a progression of gastric cancer through regulated Wnt3a/beta-catenin signaling. However, the role of miR-491-5p in GC, particularly metastatic gastric cancer is still not very clearly.

In this research, we are going to find out the function of miR-491-5p in gastric cancer and the mechanism of miR-491-5p function as a tumor suppressor in gastric cancer.

Methods

Patients

This study retrospectively recruited 80 cases of gastric cancer patients that received curative gastrectomy in the First Affiliated Hospital of Anhui Medical University. The inclusion criteria were: 1) All patients with GC were confirmed histo-pathologically; 2) The clinical data of the patients were complete; 3) The patients could be followed up; 4) no other treatment, such as chemo radiotherapy and interventional therapy before surgery. We collected information of patients through the record room of our hospital. This study was approved by the ethics committee of the First Affiliated Hospital of Anhui Medical University, and all patients signed the preoperative informed consent [2].

PCR

Total RNA from gastric cancer tissues and adjacent tissues were extracted on ice by using TRIzol[®] (Thermo Fisher Scientific). After that, RNA concentration was quantified by measuring the absorbance at 260/280 nm. CDNA was synthesized using miRNA First Strand cDNA Synthesis (Stem Loop Method) Kit (Sangon Biotech) according to the manufacturer's instructions. qRT-PCR was performed using an AceQ[®] Pcr, R SYBR[®] Green Master Mix ((Vazyme Biotech) on an ABI StepOne

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Plus PCR System (Applied Biosystem). The PCR cycle was as follows: Pretreatment at 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec and 60°C for 30sec, and finally 95°C for 15 sec, 60°C for 60sec, and 95°C for 15 sec. All data were quantified using the 2- $\Delta\Delta$ Ct method and normalized using GAPDH. The U6 as endogenous controls to detect miR-491-5p expression.

Cell Culture

The GC cell line AGS were purchased from Cosmo Bio Company (Tianjing, China). The cell line was cultured with DMEM-H supplemented with 10% FBS at 37°C with 5% CO₂.

Cell Transfection

Human GC cell line AGS was maintained in DMEM-H containing 10% FBS and grown in 5% CO₂ at 37°C. A total of 1.0 k × 10⁶ cells were seeded into 10cm cell culture dish in 24 h before transfection, so that the cells can grow into 70-80% cell density when transfection. First, prepare A and B transfer solution in a sterile 1.5 ml centrifugal tube (A solution: miRNA mimics (20 pmol, 30 μ l) in 100 μ l Opti-MEM; B solution: Lipofectamine 2000 Reagent in 100 μ l Opti-MEM), gently mixed and let stand at room temperature for 5 min. Add liquid droplets B to liquid A and mix it gently. Stand at room temperature for 20 min. Second, add the mix transfer solution into the dish, make total volume to 5 ml with Opti-MEM, shake the cell culture dish to make it evenly distributed, and then culture it in a cell incubator at 37°C and 5% CO₂. After 4 hours, each dish was changed into 5 ml of DMEM-H culture medium with 10% FBS, and the culture was continued in a cell incubator containing 5% CO₂ at 37°C.

MTT Assay

AGS cells were seeded into a 96-well plate in 24 h after transfection, 3000 cells/well. MTT was used to test the cell viability at 24 h, 48 h, 72 h. We added 10 μ l MTT solution with a concentration of 10 mg/mL into each well, continue to culture for 4 h. Carefully suck away the original culture solution, add 100 μ l DMSO to each well, and then oscillate in the shaking bed for 10 min to dissolve the blue and purple crystalline formazan. On the enzyme-linked immune detector measured at the wavelength of 490 nm absorbance value of each hole [3].

Migration Assays

For cell migration assays, completely discard the cell culture solution of each group, wash the cells with PBS, add digestive juice (0.02% EDTA, 0.25% trypsin) for digestion after each well, and conduct the Transwell experiment. 4 × 10⁵ AGS cells were plated in chamber insert, serum-free medium is put on the top of the insert, the bottom of the plate to join DMEM medium containing 20% FBS. Cells were incubated for 72 h at 37°C. Remove the Transwell chamber, wipe off the cells on the top of the insert with cotton swabs, and gently wash off the remaining cells with PBS. The mixed solution was prepared with methanol and glacial acetic acid according to 3:1, and the cells on the opposite side of the Transwell chamber were fixed for 30 min. Cells with crystal violet blue staining for 15 minutes, and then washed it with double-distilled water (ddH₂O). Data are shown as mean \pm S.D. from three independent experiments [4].

Invasion Assays

For cell invasion assays, completely discard the cell culture solution of each group, wash the cells with PBS, add digestive juice (0.02% EDTA, 0.25% trypsin) for digestion after each well, and conduct the Transwell experiment. Dissolve matrigel gel, add 50 μ l of gel to each chamber, 4 × 10⁵ AGS cells were plated in chamber insert, serum-

free medium is put on the top of the insert, the bottom of the plate to join DMEM medium containing 20% FBS. Cells were incubated for 72 h at 37°C. Remove the Transwell chamber, wipe off the cells upside of the insert with cotton swabs, and gently wash off the remaining cells with PBS. The mixed solution was prepared with methanol and glacial acetic acid according to 3:1, and the cells downside of the Transwell chamber were fixed for 30 min. Cells with crystal violet blue staining for 15 minutes, and then washed it with double-distilled water (ddH₂O). Data are shown as mean \pm S.D. from three independent experiments.

Cell Colony Formation Assay

200 cells of AGS were plated in per 12-well plates well, and cultured in DMEM with 10% FBS for 14 days. PBS was used to wash the cells twice, and then fixed with methanol for approximately 10 minutes. After fixed, wash the cells twice with PBS again. Cells with crystal violet blue stain for 30 minutes, and then washed it with double-distilled water (ddH₂O). The numbers of the colonies were counted under the naked eye [5].

Western Blot Assay

Cells was washed with PBS for once, and then put 300 μ l RIPA into each well, incubate in a shake bed at 4°C for 30 min. BSA was used to measure the concentration of the protein. Separate the protein in each sample with 10% SDS polyacrylamide gel. After that, transfer the protein into PVDF film. Blocking with 1 × Blotto for 2 h, incubate with 1:500 ITGB3 primer antibody at 4°C overnight. Washed with 1 × TBST for 5 min four times, incubate with the second antibody at room temperature for 1.5 h. Washed with 1 × TBST for 5 min four times, Western Lightning TM Chemi luminescence Reagen was used to develop.

HE Staining

Clinical samples were soaked in xylene for 10 min, and then soaked for another 10 min after replacing xylene. And then soak in gradient ethanol (100%, 95%, 85%, 70% and 50%) for 30 second to 1 min respectively. Wash twice with PBS, each time for 5 min. stained with heamatoxylin for 2 min, wash with tap water. Hydrochloric acid alcohol was differentiated three times, each time for 2 seconds, and wash with tap water. Soaked with gradient ethanol (50%, 70%, 85%, 95%, 100%) to dehydration. Soak the tissue slices in xylene for 10 min, and soak them for another 10 min after replacing xylene. Add 1-2 drops of neutral gum and tilt the cover glass from one end to prevent bubbles. Observation with microscope, take photos with 3 visual fields, 200 × [6].

Immunohistochemistry

4% Para Form Aldehyde (PFA) fixed the clinical samples, and then embedded in paraffin. Immuno histochemical staining of the samples was performed using N-cadherin, IFTTM2, β -catenin, E-cadherin, vimentin primary antibodies.

In Vivo Xenograft Study

The animals used in this study were obtained from the company of Beijing Hua kang. To the side of the subcutaneous implanted into nude mice AGS cells, we measure the nude mice weight once a week. And after experiment, these animals were killed by the injection of Barbiturate. Then the tumors were dissected, weighed and photographed.

Statistical Analyses

The differences between two groups were tested by student's t-test, all statistical analyses used the SPSS 17.0 software. Chi-square test or Fisher exact test were used to investigate categorical variables.

Univariate analysis was performed for all variables, and then the variables of $P < 0.05$ were selected for multivariate Cox regression analysis. The survival curves were analyzed by Kaplan-Meier method and compared by the log-rank test in each group. Download the target gene of miR-491-5p from ENCORI database <http://starbase.sysu.edu.cn/>, Enrichment analysis using GO and KEGG, implemented in R with the packages clusterProfiler, enrichplot and ggplot2, $p < 0.05$ means meaningful. When $* p < 0.05$, we think it has statistical significance [7].

Results

The correlation of miR-491-5p with clinical information

They showed the correlation between miR-491-5p with clinical characteristics, the level of miR-491-5p was related with TNM stage ($p < 0.05$).

The prognostic value of miR-491-5p

The K-M survival curve (supplementary) showed that patients with high miR-491-5p had better prognosis compared with low level of miR-491-5p, besides multivariate analysis got that TNM stage and miR-491-5p were independent risk factors of survival of gastric cancer patients.

miR-491-5p Inhibits Gastric Cancer Cells Proliferation and Metastasis

It has been reported that miR-491-5p is low expressed in gastric cancer, and perhaps function as a tumor suppressor. In order to find out the function of miR-491-5p in gastric cancer, we transfected miR-491-5p into AGS cells and then observed the change of cells proliferation and metastasis abilities. The results of MTT assay shows that the cells transfected with miR-491-5p mimics have a low cell activity than control. The inhibitor of miR-491-5p we named it ASO- miR-491-5p treated can increase the cell activity. The same result can be got in 24 h, 48 h and 72 h. Transwell assay was used to test the invasion and metastasis changes of AGS cells. As expected, transfection with the miR-491-5p mimics decrease the invasion and metastasis abilities of AGS cells, and the miR-491-5p inhibitor significantly increase the invasion and metastasis abilities. To further test the effect of miR-491-5p on AGC cells growth, we performed colony formation assay. Cell transfected miR-491-5p showed decreased colony formation, and the simultaneous miR-491-5p inhibitor increased colony formation compared with the control cells [8].

miR-491-5p Effectively Down Regulates Genes associated with EMT, Cell Adhesion and IFITM2

EMT plays an important role in cancer metastasis. At first, it can cause the down regulation of E-cadherin, so that leading to loss of cell-cell contact. Secondly, it could upregulate N-cadherin, Vimentin, which mediate the mesenchymal phenotype of cells. It has been reported that β -catenin can be mediated by E-cadherin. When E-cadherin is down regulated, β -catenin become more active, so that it trans located to the nucleus and activated the expression of target genes including IFITM2 family members that promote tumor initiation and progression.

To test the association between miR-491-5p and EMT associated genes expression, we performed a qRT-PCR assay. The result shows that high miR-491-5p level related to low expression of N-cadherin, β -catenin, Vimentin and IFITM2 and high expression of E-cadherin. When we used the miR-491-5p inhibitor ASO- miR-491-5p, the expression of these genes can be recovery. The result shows that miR-491-5p can directly down regulated the expression of the EMT associated and cell adhesion gene in transcription level. Moreover, the

western blot results were consistent with the RT-PCR result.

miR-491-5p Suppresses the Growth Of AGS Xenograft Tumors

Based on our *in vitro* results, we further investigated the inhibitory potential of miR-491-5p on GC growth *in vivo*. To the side of the subcutaneous implanted into nude mice AGS cells. We measure the nude mice weight once a week, and there were no big difference between mimics NC and miR-491-5p mimics group. It means that the tumor growth don't have effect on mice growth. At the endpoint of the assay, we remove the tumor and weight it. As expected, the tumors in miR-491-5p mimics group are not as big as mimics NC group. The miR-491-5p mimics transfect decrease the growth of GC cells *in vivo*.

miR-491-5p Regulates Genes associated With EMT, Cell Adhesion And IFITM2 *In Vivo*

H&E staining were performed to observe the tumor histomorphology in mice. In miR-491-5p mimics group, the cells show less nuclear heterogeneity than control group. To further confirm the result that miR-491-5p can down regulate the genes associated with EMT, cell adhesion and IFITM2 *in vivo*, we used western blot to test the level of these protein expression. N-cadherin, β -catenin, Vimentin and IFITM2 were low expressed in miR-491-5p mimics group, E-cadherin was high expressed in miR-491-5p mimics group. Moreover, the immune histochemical results confirm the result. In total, miR-491-5p could down regulate the genes associated with EMT, cell adhesion and IFITM2 *in vivo*.

The GO and KEGG of Targeted Gene of miR-491-5p

A total of 340 target genes of miR-491-5p were obtained from the ENCORI database, and GO and KEGG analysis of target gene were performed. As shown, in the GO function analysis, The top three in the Biological Process (BP) are primary neural tube formation, tube formation and neural tube formation, the top three in Cellular Component (CC) are focal adhesion, cell-substrate junction and uropod, and the top three in Molecular Function (MF) are GTPase regulator activity, transcription coregulator activity and nucleoside-triphosphatase regulator activity. KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis showed that Human papillomavirus infection, Insulin signaling pathway and Endocrine resistance were the top three [9].

Discussion

There is increasing evidence that miRNA can play a proliferating or anti-proliferating role in physiological conditions, while abnormal expression of miRNA can cause a lot of healthy problems, including cancer. miRNAs dysregulation has been identified as a marker of cancers. And also found the miRNAs expression changes in gastric cancer. Recently discovered miRNA miR-491, is down regulated in several types of cancer, including breast cancer, oral squamous cell carcinoma, hepatocellular carcinoma, pancreatic, glioblastoma, ovarian cancer and hepatocellular carcinoma. miR-491-5p has been shown to be a mature form of miR-491 that induce apoptosis and inhibit the proliferation of pancreatic, ovarian, breast cancer and colorectal cells, as well as the migration and invasion of glioma and breast, oral squamous cell. It means that miR-491-5p play as a tumor suppressor.

In clinical, miR-491-5p was related with TNM stage, it can reflect the progression of gastric cancer. Patients with high miR-491-5p had better prognosis compared with low level of miR-491-5p, besides multivariate analysis got that TNM stage and miR-491-5p were independent risk

factors of survival of gastric cancer patients, this indicated miR491-5p is an important prognostic bio-marker, which can offer help for diagnosis and treat of gastric cancer patients.

It has been reported that miR-491-5p not only inhibit the proliferation, invasion and migration but also induced cell apoptosis and suppressed tumor growth; In estrogen-receptor- α -positive breast cancer, miR-491-5p functions as a tumor suppressor through mediate JMJD2B; In oral squamous cell carcinoma, miR-491-5p and GIT1 function as modulators and biomarkers of invasion and metastasis. Our data shown that increasing expression of miR-491-5p cause a low cell proliferation, migration and invasion abilities. In order to confirm the conclusion, we established a xenograft mouse model, and the result shows that miR-491-5p overexpression suppressed tumor growth. In summary, miR-491-5p functions as a tumor-inhibitory in gastric cancer in vitro and *in vivo*.

EMT has to obvious characteristics, one is that acquisition of a mesenchymal phenotype and the other is loss of epithelial characteristics. These two characteristics have closely related to tumor metastasis and tumor recurrence. There are some EMT molecular markers, the level of N-cadherin, E-catenin and Vimentin suggest the state of EMT. Cell-cell adhesion defines cellular and tissue morphogenesis so that it is a fundamental biological process in multicellular organisms. During normal tissue homeostasis, signals transmitted by adhesion between cells and to the underlying Extra Cellular Matrix (ECM) are closely related to gene regulation. WNT signaling activation and EMT-associated changes have closely relate with the displacement of β -catenin from adherens junctions in normal and cancer cells. Inter Feron (IFN)-Induced Trans Membrane Protein (IFITM) family has at least three members, IFITM1, IFITM2 and IFITM3. IFITMs have been reported could control the cell cycle, and then function as tumor suppressors, while the conserved cluster of differentiation 225 domains is important for the function. It has been reported that IFITM2 acted as a pro-apoptotic gene in a p53-independent manner in colon cancer. In this study, we found out that miR-491-5p function as a tumor-inhibitory in gastric cancer, and the molecular mechanism is that it direct targeting EMT, cell adhesion genes, including N-cadherin, β -catenin, E-cadherin, Vimentin and IFITM2.

From the GO and KEGG analysis of target gene of mir491-5p, KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis showed that Human papillomavirus infection, Insulin signaling pathway and Endocrine resistance was the top three, Human papillomavirus infection was related with the occur of gastric cancer, then we will explore the mechanism of mir491-5p with gastric cancer in the future [10].

Conclusion

In conclusion, we demonstrated that miR-491-5p can down regulate the cell viability, invasion, metastasis and clone formation abilities of GC cells; miR-491-5p can also down regulate the GC associated protein expression and inhibit the tumor grow in mouse model. Our data suggest that miR-491-5p function as a tumor suppresser in GC via direct targeting EMT, cell adhesion genes, including N-cadherin, β -catenin, Vimentin and IFITM2.

Declaration Section

Abbreviations

GC: Gastric Cancer; EMR: Endoscopic Mucosal Resection; EMT: Epithelial-Mesenchymal Transition

Ethics Approval and Consent to Participate

This study was approved by the Institute Research Ethics Committee of The First Affiliated Hospital of Anhui Medical University, Hefei, China.

Consent for Publication

Not applicable

Availability of Data and Materials

Due to ethical restrictions, the raw data underlying this paper are available upon request to the corresponding author.

Competing Interests

The authors disclose no competing interests. There are no financial and non-financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial or any other) to declare in relation to this.

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Author's Contributions

PQL and LXZ carried out the main work and contributed equally. They designed this study, drafted this manuscript and revised this work. BCD helped them, AMX performed the study and participated in this work. All authors read and approved the final manuscript.

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