MIRNA-497 INHIBITS PROLIFERATION, MIGRATION AND INDUCES APOPTOSIS OF GASTRIC CANCER CELLS BY NEGATIVELY REGULATING PBX3 EXPRESSION

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ABSTRACT

Background: *microRNA (miR) is an important factor in regulating human genes, and its abnormal expression is considered to be a significant factor in the progression of various tumors.*

Objective*: To explore the relationship between miR-497 expression and stomach cancer (SC) and its effect on the biological behavior of cancer cells. Methods: miR-497 expression in SC tissues and cells was detected, and its correlation with clinical characteristics and survival of SC patients was analyzed. miR-497 and PBX3 over-expression or inhibited vector were established and transfected into SC cells, and whether the biological behavior of SC cells changed after transfection was observed. Dual luciferase report was utilized for analysis of the relationship between miR-497 and PBX3.*

Results: miR-497 was reduced in SC tissues and cells. Its low expression was correlated with tumor size ≥ 5 cm, high stage (III+IV), lymph node metastasis and poor survival rate. Increasing miR-497 could inhibit SC cell proliferation, invasion and induce apoptosis, while increasing PBX3 could reverse the changes in SC cell biological behavior caused by miR-497 increase.

Conclusion*: miR-497 is down-regulated in SC and is related to the better prognosis of patients. In addition, miR-497 can inhibit SC progression by negatively regulating PBX3.*

Keywords: *miR-497, stomach cancer, PBX3, biological behavior*

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Introduction

Stomach cancer (SC) is a common malignant tumor and the third major cause of death of malignant tumors in the world[(1)]. According to statistics, in 2015, China alone had 679,100 new SC cases and 498,000 deaths[(2)]. SC is characterized by high metastasis and high recurrence, which makes treatment difficult[(3)]. Though great progress has been made in treatment strategies, the prognosis of patients is still unsatisfactory[(4)]. With the clinical application of targeted drugs such as apatinib, SC treatment has been changed[(5)]. Therefore, looking for the potential target of cancer treatment has become a hot spot of current research.

MicroRNA (miR) is a kind of non-coding small molecule RNA that can decrease target mRNA expression by binding to 3' untranslated region (3'-UTR), thus triggering mRNA degradation or translation inhibition[(6)]. MiR, as a significant factor regulating human genes, can be involved numerous biological activities, such as apoptosis, proliferation, differentiation and metabolism[(7,8)]. A variety of miRs can play two distinct roles of promoting cancer or inhibiting cancer in tumors, affecting the occurrence and development[(9-11)].

MiR-497 is located on human chromosome 17p13.1. There is a lot of evidence to prove that miR-497 plays a key role in tumor progression. For example, miR-497 can target BDNF and mediate PI3K/AKT to inhibit thyroid cancer progression[12]. MiR-497 can negatively regulate SMAD7 and inhibit oral squamous cell carcinoma metastasis[13]. In addition, miR-497 can also inhibit the growth and invasion of non-small cell lung cancer cells via targeted inhibition of SOX5 expression[14]. These results show that miR-497 is a promising target for tumor treatment. However, its role in SC and related mechanisms have not been fully elucidated.

This paper analyzes the expression and function of miR-497 in SC through clinical research and basic experiments, aiming at finding potential therapeutic targets for SC.

Methods and data

Clinical sample collection

Totally 92 surgical patients diagnosed with SC from November 2013 to January 2016 were recruited in this study, including 44 males and 48 females, with a mean age of 61.2±7.8 years. Cancer tissues and adjacent tissues of patients were collected during the surgery, and the samples were frozen for 5 minutes in liquid nitrogen and stored in a -80°C refrigerator. Inclusion criteria: Patients whose age were older than 18 years old, patients whose SC was confirmed by pathological examination, patients all completed a 3-year survival follow-up survey, patients all signed informed consent forms. Exclusion criteria: patients with other tumors, patients with incomplete clinical data, patients received treatment for cancer previously. The research was approved by the ethics association of our hospital.

Cell source and treatment

SC cell lines (BGC-823, MGC-803, SGC-7901) and normal gastric mucosa cell GES-1 were all purchased from ATCC cell bank (Virginia, USA). Cells were placed in DMEM (Gibco, USA) comprising 10% fetal bovine serum (Gibco, USA), and cultured at 37°C in an incubator (Thermo, USA) with 5% CO2. Cell passage was performed when their growth reached 70%-80%.

Cell transfection: miR-497 over-expression vector (miR-497-mimic), PBX3 inhibited vector (si-PBX3) and corresponding blank vector (miR-NC, si-NC) were constructed. Subsequently, transfection was conducted with Lipofectamine™ 2000 kit (Invitrogen, USA). After 6 hours, transfected cells were transferred to 10% FBS medium for culture.

qRT-PCR

Total RNA was extracted using TRIzol kit (Invitrogen, USA) and its purity, concentration and integrity were determined with UV spectrophotometer and agarose gel electrophoresis. Reverse transcription adopted TaqMan™ Reverse RT Kit (Invitrogen, USA). PCR amplification was conducted by the aid of PCR amplification kit (Takara Bio, Japan), and the procedures were as follows: 10 μL SYBR qPCR Mix, 0.8 μL upstream primers, 0.8 μL downstream primers, 2 μL cDNA product, 0.4 μL 50×ROX reference dye, and supplemented with 20 μL water. PCR reaction conditions were as follows: 95°C for 60 s, 95°C for 30 s, 60°C for 40 s, with 40 cycles in total. $2^{-\Delta\Delta ct}$ CT was used for data analysis. U6 was taken as the internal reference of miR-497, and the primer sequence was follows: miR-497 upstream: 5'-CAGCAGCACACUGUGGUUUGU-3', downstream: 5'-AAACCACAGUGUGCUGCUGUU-3', U6 upst5' -UUCUCCGAACGUGUCACGUTT-3', downstream: 5' -ACGUGACACGUUCGGAGAATT-3'.

Western blot detection

The cultured cells were lysed with RIPA buffer (Thermo Scientific, USA), and BCA kit was applied for protein concentration determination (Thermo Scientific, USA). Electrophoresis was performed and then protein was transferred to PVDF membrane, which was then washed with TBST solution twice. PVDF membrane was transferred to 5% skim milk powder, sealed for 2 h, and washed three times using TBST solution. PBX 3 (1: 500) and β-catenin (1: 500) (Abcam, USA) primary antibody were added to seal for a whole night at 4°C. The primary antibody was removed through washing membrane, added with horseradish peroxidase labeled goat anti rabbit secondary antibody (Abcam, USA) 1:1000 for one-hour incubation under 37°C, and then washed 3 times with PBS, with 5 min each. Excess liquid was absorbed. ECL was used for illumination and development in a dark room. The protein bands were scanned for gray value anlysis in Quantity One software.

Dual luciferase reporter

PBX3-3'UTR wild type (Wt) and PBX3-3'UTR mutant (Mut) were constructed. They were transferred to the downstream of luciferase reporter gene to sequence and identify the constructed

plasmid. The plasmid with correct sequencing was co-transfected with miR-497-mimics or miR-NC into targeted cells by Lipofectamine™ 2000 kit. Determination of luciferase activity was conducted using a dual luciferase reporter gene detection kit (Solarbio, Beijing).

Cell apoptosis detection

0.25% trypsin was applied for digestion. After that, 100 μL binding buffer was put into to prepare a suspension of 1*106 /mL. Ten μ AnnexinV-FITC /PI (Shanghai Yeasen Biotechnology Co., Ltd.) was successively added and incubated at indoor temperature for 5 min away from light. Flow cytometry (FCM) BD CantoII was applied for analysis, and calculation of apoptosis rate was conducted.

Cell proliferation detection (CCK-8)

CCK-8 (Beyotime Biotechnology, Shanghai, China) was adopted for determination. Cells were collected 24 hours after transfection, adjusted to 4*106 cells, and inoculated on 96-well plates. Then CCK solution (10 μL) was put into each well for two-hour culture under 37°C. The OD value of cells was measured under 490 nm absorbance with enzyme reader.

Cell invasion detection

Cell invasion ability was tested using Transwell chamber (Corning, USA). Cells were adjusted to 5*104 and inoculated to a 6-well plate and inoculated to the upper compartment which was added with DMEM nutrient solution (200 μL). The lower compartment was added with DMEM (500 mL, comprising 20% FBS), cultured for 48 h under 37°C. Cells that failed to penetrate the upper compartment were removed, rinsed for 3 times with PBS, fixed 10 min with paraformaldehyde, and rinsed for 3 times with sterile water. After drying, the cells were dyed with 0.5% crystal violet and its invasion was observed with microscope.

Statistical analysis

SPSS21.0 wasutilized for statistical analysis of the data collected in this study, and GraphPad7 software package was used to draw the required images. Enumeration data comparison between the two groups adopted chi-square test or Fisher' s precise test. Measurement data comparison between the two groups adopted independent sample t test. One-way analysis of variance was applied for comparison among groups, and verified by tukey hsd method.

Comparison among multiple time points expression were analyzed by Repeated Measures, qualified by Bonferroni. Survival of patients was plotted using K-M survival curve, and Log-rank test was used for analysis. When $P<0.05$, there was statistical differences.

Results

Expression and diagnostic value of miR-497 in KOA

According to qRT-PCR detection, miR-497 was decreased in SC tissues and cells. Patients were grouped according to the median expression value of miR-497 in tissues (low expression group and high expression group). The correlation of miR-497 with clinical pathological data and survival rate of patients was analyzed, showing that miR-497 low expression was related to tumor size ≥ 5 cm, high stage (III+IV), lymph node metastasis and poor survival rate of patients. This suggested that miR-497 may be involved in SC development. As shown in Table 1 and Figure 1.

Factors		miR-497		χ^2	p value
		Low expression (n=46)	High expression (n=46)		
Gender					
	Male (n=44)	24 (52.17)	20 (43.48)	0697	0.404
	Female (n=48)	22 (47.83)	26 (56.52)		
Age					
	<60 years old (n=31)	13 (28.26)	18 (39.14)	1.216	0.270
	≥ 60 years old (n=61)	33 (71.74)	28 (60.87)		
Tumor size					
	≥ 5cm (n=38)	25 (54.35)	13 (28.26)	4.802	0.011
	<5cm (n=54)	21 (45.65)	33 (71.74)		
TMN staging					
	Grade I+II (n=60)	24 (52.17)	36 (78.26)	6.900	0.009
	Grade III+VI (n=32)	22 (47.83)	10 (21.74)		
Lymph node metastasis					
	Metastasis (n=36)	23 (50.00)	13 (28.26)	6.071	0.033
	Without metastasis (n=56)	23 (50.00)	33 (71.74)		
Differentiation					
	Low differentiation (n=35)	21 (45.65)	14 (30.43)	2.260	0.133
	Middle+high differentiation (n=57)	25 (54.35)	32 (69.57)		

Table 1: miR-497 and clinicopathological data.

miR-497 can inhibit SC progress

In order to explore the function of miR-497 in SC progression, SGC-7901 cells were transfected with miR-NC, miR-497-mimics, CCK-8, Transwell and flow cytometry separately to observe the changes of cell biological behavior after transfection. The results revealed that compared with miR-NC group, miR-497 expression increased in miR-497-mimics group, with reduced cell proliferation and invasion ability, and elevated cell apoptosis.

This indicated that miR-497 can inhibit SC progress. As shown in Figure 2.

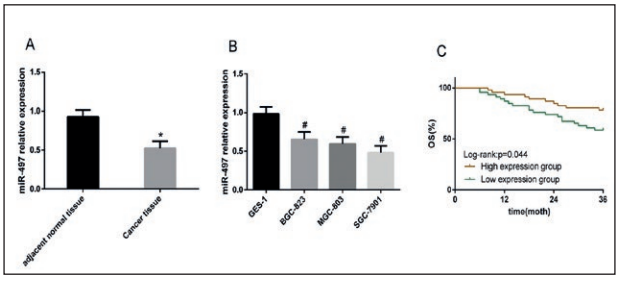

Figure 1: Expression and diagnostic value of miR-497 in SC.
***A**, miR-497 was down-regulated in SC tissues. **B**, miR-497 was down-regulated in SC cells. **C**, miR-497 low expression was correlated with better survival rate of patients. Notes: Compared with adjacent normal tissues, * represents p<0.05. Compared with GES-1 cells, # represents p<0.05.*

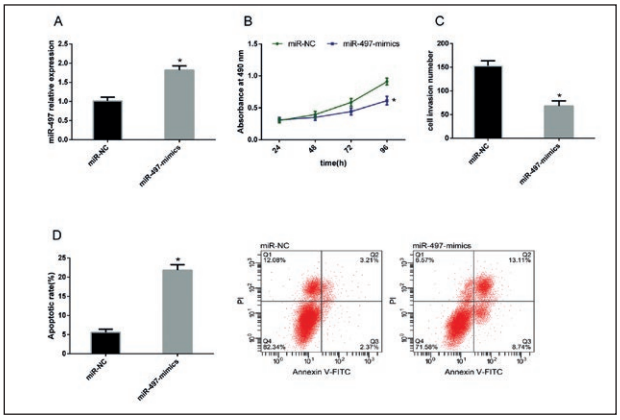

Figure 2: miR-497 can inhibit SC progress.
***A**, Compared with miR-NC group, miR-497 in cells of miR-497-mimics groups increased. **B**, Compared with miR-NC group, proliferation ability of miR-497 in cells of miR-497-mimics groups decreased. **C**, Compared with miR-NC group, invasion ability of miR-497 in cells of miR-497-mimics groups decreased. **D**, Compared with miR-NC group, apoptosis rate of miR-497 in cells of miR-497-mimics groups increased. Note: Compared with miR-NC group, * represents p<0.05.*

PBX3 can be negatively regulated by miR-497

It is known to all that miR can affect the biological behavior of cells via regulation of target gene expression. In order to further investigate the mechanism of miR-497 in SC, the potential binding sites between PBX3 and miR-497 were found through the target gene prediction website Targetscan7.2. By WB detection of PBX3 in miR-NC group and miR-497-mimics group, it was found that PBX3 was down-regulated in the latter. Subsequently, dual luciferase report was used to verify the relationship between PBX3 and miR-497. The results indicated that transfection of miR-497-mimics could inhibit PBX3-3'UTR Wt luciferase activity, without affecting PBX3-3'UTR Mut luciferase activity, suggesting that PBX3 can be negatively regulated by miR-497. As shown in Figure 3.

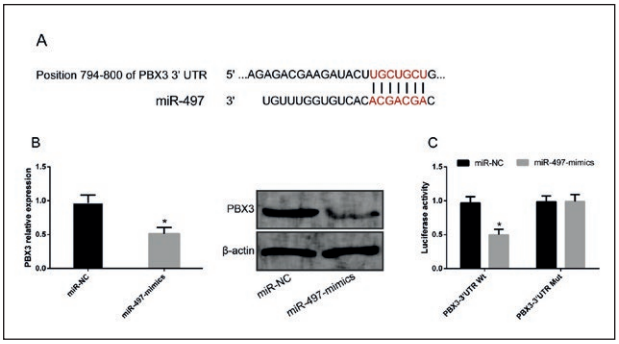

Figure 3: PBX3 can be negatively regulated by miR-497.
***A**, There were potential binding sites between BX3 and miR-497. **B**, PBX3 in cells of miR-497-mimics group was down-regulated when compared with miR-NC group. **C**, Transfection of miR-497-mimics could inhibit PBX3-3'UTR Wt luciferase activity, without affecting PBX3-3'UTR Mut luciferase activity. Note: Compared with miR-NC group, * represents p<0.05.*

Elevating PBX3 can weaken the inhibitory effect of miR-497 on SC progression

In order to explore whether miR-497 can inhibit SC progression through negative regulation of PBX3, SGC-7901 cells were transfected with sh-PBX3 and miR-497-mimics simultaneously, and were compared with cells transfected with miR-NC and miR-497-mimics.

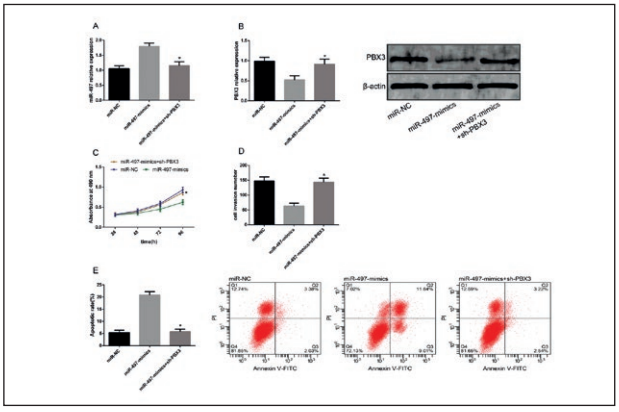

Figure 4: Elevating PBX3 can weaken the inhibitory effect of miR-497 on SC progression.
***A/B**, Compared with miR-497-mimics group, miR-497 in miR-497-mimics+sh-PBX3 group decreased, while PBX3 increased. **C/D/E**, Compared with miR-497-mimics group, miR-497-mimics+sh-PBX group 3 had enhanced cell proliferation and invasion abilities, and decreased cell apoptosis rate. Note: Compared with miR-NC group, * represents p<0.05.Note: Compared with miR-NC group, * represents p<0.05.*

According to the results, compared with miR-497-mimics group, miR-497 in miR-497-mimics+sh-PBX3 group was decreased, with increased PBX3, cell proliferation and invasion, and decreased

cell apoptosis rate, indicating that increasing PBX3 can effectively weaken the inhibition effect of miR-497 on SC progression. As shown in Figure 4.

Discussion

In recent years, there has been increasing evidence that miR can participate in the progression of SC. This paper analyzed the expression and function of miR-497 in SC through clinical research and basic experiments, and found that miR-497 decreased in SC tissues and cells, and miR-497 low expression was correlated with tumor size ≥ 5 cm, high stage, lymph node metastasis and poor survival rate. What's more, miR-497 can block SC cell proliferation and invasion and promote cell apoptosis, which is in connection with its negative regulation of PBX3.

As an important regulator of human genes, abnormal expression of miR is considered to be a vital factor in the development and progression of various tumors (16,17). Therefore, miR is also considered as a promising target for tumor treatment. This study explored the expression and role of miR-497 in SC, and the results showed that miR-497 decreased in SC tissues and cells, and miR-497 low expression was correlated with tumor size ≥5cm, high stage, lymph node metastasis and poor survival rate, which suggests that miR-497 may be bound up with the progress of SC. Therefore, in this paper, miR-497 expression in SC cells was increased to observe the impacts of such treatment on cell biological behavior, so that the function of miR-497 in SC could be explored. It could be seen form the results that miR-497 up-regulation can suppress the proliferation and invasion of cancer cells and promote apoptosis, and miR-497 is a tumor suppressor gene in SC, which is similar to the research results of Li et al[18]. miR-497 also acts as a tumor suppressor gene in other tumors[12,13,19], indicating that miR-497 has great potential value in cancer treatment.

It is well known that miR can be involved in cell biological behavior regulation by regulating target genes. With the purpose of exploring the molecular mechanism of miR-497 regulating SC proliferation, invasion and apoptosis, miR target gene prediction software was adopted, and PBX3 was found to be one of miR-497 target genes. PBX3 is an important member of PBX family, which can regulate transcription of downstream target genes through interaction with homeobox, thus enhancing its DNA binding affinity[20]. In tumors, PBX3 is often abnormally elevated and plays a role in promoting cancer. For example, PBX3 can promote invasion and metastasis by inducing epithelial-mesenchymal transformation of SC[21]. In cervical cancer, PBX3 can promote the proliferation of cancer cells through AKT signaling pathway, and is a marker of prognosis of the disease[22].

Previous studies have found that multiple miRs can participate in tumor progression by regulating PBX3. For example, miR-129-5p can target PBX3 to block proliferation, invasion and induce apoptosis in pancreatic cancer cells[23]. MiR-320 can inhibit the growth of glioma cells via targeting PBX3[24]. MiR-495 cam negatively regulate PBX3 and play a role in inhibiting cancer in human melanoma[25]. Therefore, we believe that miR-497 can regulate the biological behavior of SC cells through regulating PBX3. The results of this study elaborated that up-regulation of miR-497 can reduce PBX3 in SC cells, transfection of miR-497-mimics can inhibit PBX3-3'UTR Wt luciferase activity without affecting luciferase activity PBX3-3'UTof R Mut, indicating that PBX3 can be negatively regulated by miR-497. In addition, it is also found that increasing PBX3 can effectively reduce the inhibitory effect of miR-497 on SC progression. These results can prove that miR-497 can inhibit lung cancer progression by negatively regulating PBX3. We found that miR-497 is down-regulated in SC and is related to the better prognosis of patients. In addition, miR-497 can inhibit SC progression by negatively regulating PBX3. However, there are still some deficiencies in this article. First, tumor formation in nude mice was not conducted, and it is unclear whether miR-497 can directly affect the tumor formation effect. Secondly, whether miR-497 can play a role in SC through other target genes has not been explored. These deficiencies will be improved in the following research.

To sum up, miR-497 is down-regulated in SC and is related to the better prognosis of patients. In addition, miR-497 can play a role in inhibiting SC progression by negatively regulating PBX3.

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