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Original Article

The research of miR-301 in gastric cancer cell proliferation and apoptosis

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Abstract: We investigated the impact and mechanisms of miR-301 in gastric cancer cell proliferation and apoptosis, and looked forward to find a new frame of reference in the clinical diagnosis and treatment. 100 case gastric carcinoma collected from our hospital gastrointestinal surgery were as the observation group (group A); then collected 100 cases of carcinoma adjacent as the negative control group (group B) and 10 healthy adult normal gastric cell line (GES-1) as the healthy control group (group C). Used real-time PCR to analyze the miR-301 expression of the three groups, MTT assay detect cell proliferation, annexin V/PI staining detect cell apoptosis, TargetScans software predict target genes, then used luciferase reporter gene assay to verify the predicted target genes. Western blot was used to detect the predicted miR-301 target gene expression. The results showed that miR-301 expression from the observation group was significantly higher than the negative control group and the healthy control group ($P < 0.05$). The absorbance values measured showed that the observation group was significantly higher than the other two groups ($P < 0.05$). After transfection anti-miR-301, MGC-803 apoptosis index was significantly higher than (NC) group and control (Ctr) group ($P < 0.05$). MiR-301 expression was positively correlated with tumor size, TNM staging and Lauren type, and it had no correlation with gender, age, lymph node metastasis and tissue differentiation. Targetsscan software predicted the target gene of miR-301 may be PTEN, which was confirmed by luciferase reporter assay. Western blot showed that transfection of miR-301 mimic significantly inhibited the expression of PTEN in MGC-803 cells ($P < 0.01$), whereas transfection anti-miR-301 could significantly increase the PTEN expression ($P < 0.01$). MiR-301 upregulated in gastric cancer, high expression of miR-301 may be involved in the occurrence and development of gastric cancer by adjusting the target gene PTEN.

Keywords: miR-301, PTEN, gastric, proliferation, apoptosis

Introduction

Gastric cancer is a common phenomenon in China. However the cell proliferation mechanism is still not explicitly. Its development involves multiple factors, multiple genes, multiplestep process. miRNA is aclass of endogenous non-coding RNA which has a regulatory function found in eukaryotes, and it is about 20 to 25 nucleotides. Recent studies [1, 2] found that some miRNA abnormal expression is closely related to tumor, and it plays a role of oncogenes or suppressor genes in the development of tumors. The expression of miRNA associated with a variety of cancers, such as mir-125b-1, located at chromosome 11q24 fragile sites, and it is often missing in breast cancer, lung cancer, ovarian cancer, uterine cancer patient. To investigate the effect of miR-301 in gastric cancer cell proliferation and apoptosis, selected 100 cases of pathologically confirmed gastric cancer tissues, adjacent tissues and 10 healthy adults of normal gastric mucosa cell line (GES-1) in our hospital (Pathological diagnostic criteria from the national "863" major projects "gastric molecular typing and individualized treatment" research group [3]). Genomic DNA was extracted, to explore the relationship between micro RNA miR-301 expression and clinicopathological features in human gastric cancer cell lines and gastric carcinoma, reveals correlation between gastric cancer incidence and tiny RNA miR-301.

Materials and methods

Patients and tissue samples

Clinical gastric cancer tissues and adjacent cancer tissues were collected from 100 pati-

Table 1. Log-rank test univariate analysis

Factors	N	miR-301 expression values	*P*
Age			*P* = 21.2 > 0.05
≥ 60	53	5.65 ± 0.31	
< 60	47	5.71 ± 0.33	
Gender			*P* = 18.7 > 0.05
Male	62	5.39 ± 0.26	
Female	38	5.56 ± 0.21	
TNM stage			*P* = 0.02 < 0.05
Stage I~II	34	5.61 ± 0.38	
Stage III~IV	66	6.36 ± 0.51	
Tumor size			*P* = 0.04 < 0.05
≥ 5 cm	41	6.68 ± 0.59	
< 5 cm	59	5.16 ± 0.33	
Lauren classification			*P* = 0.01 < 0.05
Intestinal type	45	4.97 ± 0.45	
Diffuse type	55	6.33 ± 0.64	
Lymph node metastasis			*P* = 7.3 > 0.05
Have	27	5.15 ± 0.51	
No	73	5.33 ± 0.56	
Staging			*P* = 1.5 > 0.05
Poorly differentiated	41	4.68 ± 0.48	
Differentiated	33	4.55 ± 0.41	
Well-differentiated	26	4.61 ± 0.51	

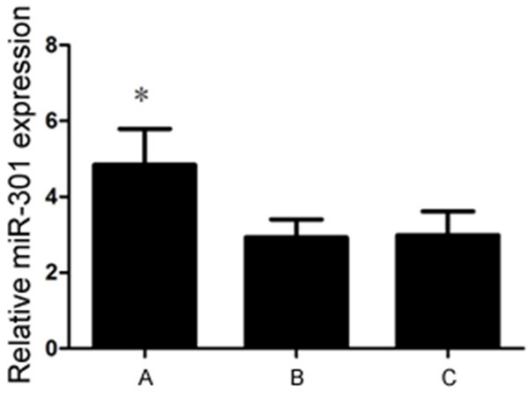

Figure 1. The relative amounts of miR-301; compared with the remaining group, *P < 0.05.

ents who had undergone surgeries and pathologically confirmed gastric cancer in our hospital during March 2013 to March 2014. The Gastric cancer tissues as the observation group, adjacent tissues distance > 3 cm of cancerous tissue as the negative control group, tissues were immediately frozen and stored in liquid nitrogen once taken. The study pact was approved by our institutional review boards for human studies, and informed consent was obtained from all patients. All specimens were confirmed after HE staining gastric pathology. 10 healthy adults of normal gastric cell line (GES-1) as the healthy control group, purchased from Shanghai Institute of Life Science Cell Bank. Gastric cancer patients inclusion criteria: all patients before surgery were not received radiotherapy and chemotherapy; aged 18 or above; no other primary tumors; no serious heart, liver and kidney dysfunction; no other serious chronic diseases. (**Table 1**).

Cell culture and ASO transfection

The sample cell (including gastric cancer tissues and cells) were seeded in medium containing 10% fetal bovine serum, using LipofectamineTM2000 transfection kit (Batch number: 201317) from the US Invitrogen Corporation strictly in accordance with transfection procedure. The observation group, the negative control group and the healthy control group were defined as miR-301 ASO group, ASO Ctr group and Ctr group after transfection.

Quantitative real-time PCR

RNA in the clinical tissue samples were isolated using analyzer 7500 (ABI, USA). cDNA was synthesized from the total RNA using Reverse Transcriptase, then quantified the expressions of the gene.

MTT assay cell proliferation

Cell proliferation was detected used the MTT assay kit (Lot: 201410210, KeyGEN Bio TECT, nanjing), in additionthe absorbance was measured at 450 nm wavelength after transfection 0, 24, 48, 72, 96 h, calculate the rate of cell proliferation.

Flow cytometer detecting apoptosis

Using FACS Canto flow cytometer (BD Biosciences USA) analyze cell apoptosis of miR-

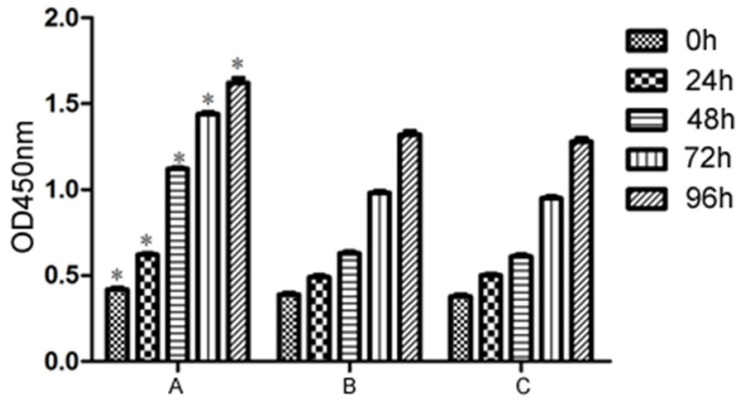

Figure 2. The absorbance values measured at 450 nm wavelength at 0, 24, 48, 72, 96 h, compared with the healthy control group, *P < 0.05.

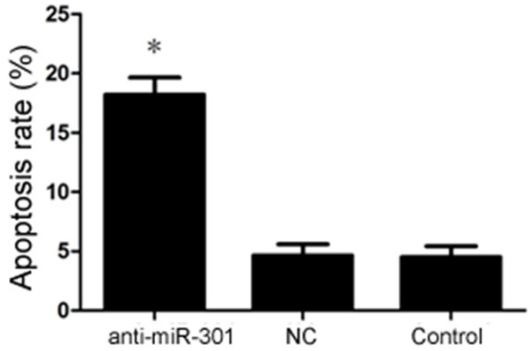

Figure 3. 3 Group apoptotic index; compared with the remaining group, *P < 0.05.

301 ASO group, ASO control group and the control group.

Bioinformatics prediction miR-301 target genes

By TargetScans target gene prediction software selected PTEN gene as a target of miR-301.

Luciferase reporter gene validation of target genes

TargetScans target gene prediction software prompted the possible action site was 412-418 sites of 3' end of the untranslated region (3'UTR) of PTEN mRNA in vitro synthesized DNA fragment containing the site and DNA fragment with mutants site, cloned to dual luciferase promoter vector pMIR. After co-transfected pMIR and miR-301 into MGC-803 cells, cultured 48 h, used luciferase assay kit detect luciferase activity.

Statistical analysis

Data were analyzed with statistical software SPSS19.0, the results of measurement data such as miR-301 expression, cell proliferation result and apoptotic index between the groups were compared with t test, the count data with χ^2 test, the univariate correlation analysis with the Log-rank test, and the significance level was set at $P < 0.05$.

Results

Expression of miR-301 in different groups

miR-301 expression in the observation group was significantly higher than that of the negative control group and the healthy control group ($P < 0.05$), miR-301 relative content among the three groups shown in **Figure 1**.

Cell proliferation results

With time, the proliferation of gastric cancer cells was significantly enhanced, the absorbance values measured at 450 nm wavelength at 0, 24, 48, 72, 96 h in the observation group was significantly higher than in the healthy control group ($P < 0.05$); However, there was no significant difference between the negative control group and health control group, absorbance values shown in **Figure 2**.

Apoptotic index

After transfection of miR-301 ASO, the miR-301 ASO group apoptosis index (18.21 ± 1.45) was significantly higher than that (ASO Ctr) group (4.66 ± 0.93) and control (Ctr) group (4.52 ± 0.91) ($P < 0.05$), apoptotic index shown in **Figure 3**.

Log-rank testsingle factor analysis

The expression of miR-301 was positively correlated with tumor size, TNM stage and Lauren classification. That was the larger tumor, TNM stage higher, MiR-301 expression was higher-with Lauren type was diffuse type, and it has no correlation with the patient's sex, age, lymph node metastasis and tissue differentiation degree; Log-rank test single factor analysis shown in **Table 1**.

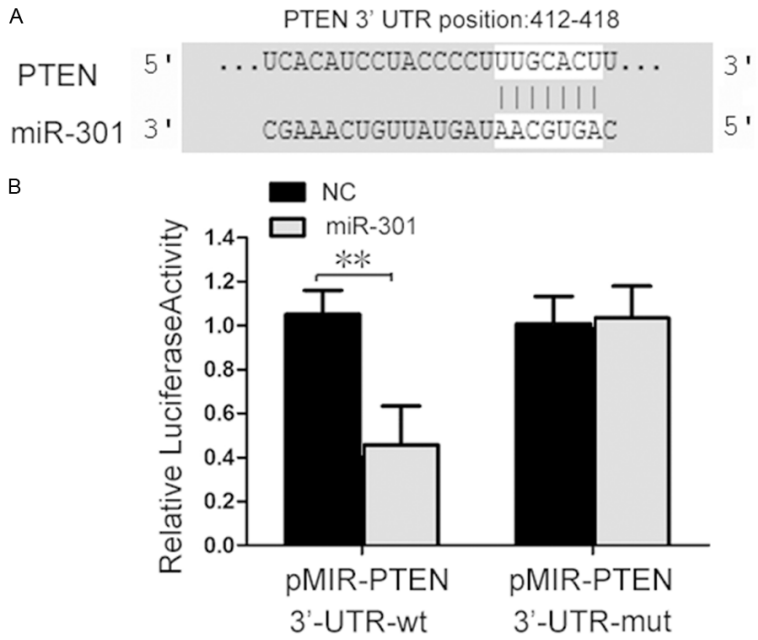

Figure 4. TargetScans target gene prediction software predicted miR-301 target genes. A. miR-301 predicted combined PTEN of 3'-UTR sites, compared with the NC group, **P < 0.01; B. Verify the interaction between miR-301 and the PTEN.

miR-301 target gene prediction

TargetScans target gene prediction software prompted the PTEN gene might be the target gene of miR-301. The predicted PTEN 3'-UTR target sites shown in **Figure 4A**. Luciferase reporter gene assay results showed that co-transfection of wild-type pMIR-PTEN 3'-UTR carrier and miR-301 significantly reduce luciferase activity (P < 0.01), while co-transfection of mutant pMIR-PTEN 3'-UTR carrier and miR-301 did not significantly change luciferase activity, which suggested a direct interaction between miR-301 and PTEN. As shown in **Figure 4**.

Correlation miR-301 expression and PTEN

As shown in **Figure 5**, transfection miR-301 mimic significantly inhibited MGC-803 cells PTEN expression (P < 0.01), whereas transfection anti-miR-301 can significantly increased the MGC-803 cells PTEN expression (P < 0.01).

Discussion

Cancer is a disease caused by multiple gene abnormalities, the role and influence of various genes and their expression products in cancer development process has been a hot research of molecular mechanisms. Gastric cancer in China is one of gastrointestinal cancer-prone in recent years, gastric cancer gene diagnosis and gene therapy has made obvious progress in fundamental research [4, 5]. The role of miRNA in cancer occurrence and development in recent years has become a hot topic, and has made great progress. Some studies show that it was more stable and effective put miRNA as target molecule than coding gene [9, 10].

There had several miRNA expression abnormal in gastric cancer, more and more evidence suggested that multiple miRNA associated with apoptosis [15, 16] could regulated apoptosis in the process of development and some other biological processes. Domestic WU et al [17, 18] found that the expression of miR-195 reduced in the tumor tissue relative to normal tissue. Studies had found that these miRNA regulated molecular level through regulating tumor associated target genes in the occurrence, development and metastasis of gastric cancer, then influenced gastric carcinoma development and prognosis [19, 20].

miR-301 was a recently discovered miRNA, there had a number of studies shown that it played an important role in the occurrence and development of tumors. Scholar Shi [6] reported miR-301 overexpression in breast cancer, and after inhibiting the expression of miR-301, cell proliferation, invasion and migration significantly reduced, indicated that miR-301 plays an important role in the occurrence and development of breast cancer. Lee [7] believed that miR-301 higher expression closely related to the invasion and metastasis in pancreatic cancer cell lines and tissues. Zhang et al [14] had pointed out that miR-301 upregulated in colon cancer, reduced miR-301 expression might inhibited cancer cell growth and promote apoptosis.

But about how the miR-301 expression affected gastric cancer cell proliferation and apopto-

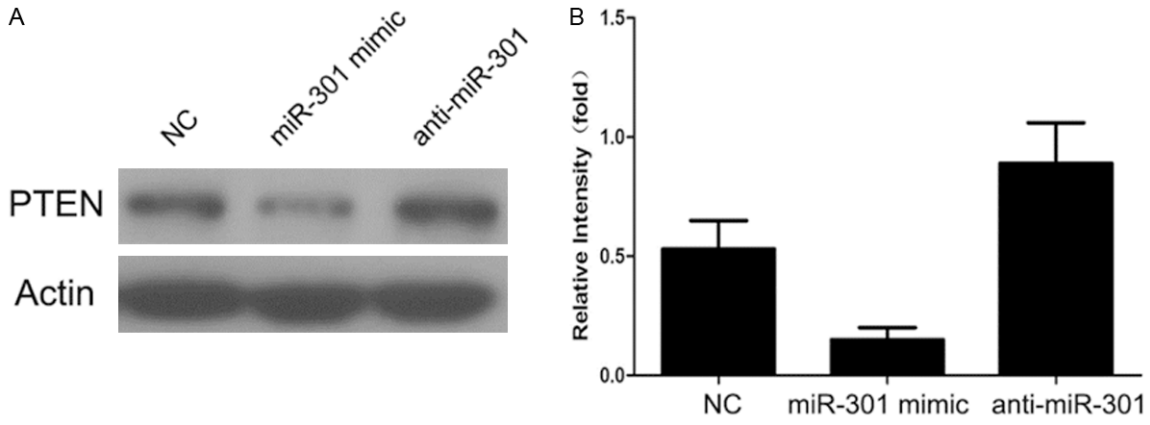

Figure 5. PTEN expression detected by Western blot.

sis, there has few reports. According to Wang et al [25] study, miR-301a has an important role in the development of gastric cancer, but the exact function and mechanism of the development of the gastric still unclear. It was a multifactorial involving multiple genes, complex multi-step process [12, 13]. Thus exploring miR-301 expression in gastric have potentially significance, it was possible to provide a new basis for the study of the mechanisms and the development of gastric cancer.

The study found that there are 63 cases in the observation group miR-301 expression was significantly higher than the control group, the absorbance values measured at 450 nm wavelength at 0, 24, 48, 72, 96 h, the observation group was significantly higher than the healthy control group suggested that at all times point, gastric cancer cell proliferation was significantly enhanced, miR-301 up-regulated in gastric cancer. Mishra et al [21, 22] proposed miRNA mutations (methylation or acetylation) lead to biology dysfunction, thought this was the expression mechanism how miRNA regulated tumor development and progression. In our study, Log-rank test showed that the expression of miR-301 was positively correlated with tumor size, TNM stage and Lauren classification, and it has no correlation with the patient's sex, age, lymph node metastasis and tissue differentiation degree. Shi [6] thought that miR-301 mediated tumor cell proliferation and invasion in human breast cancer, and it was a poor prognostic indicator. In this study, miR-301 upregulated in gastric cancer, suggested that miR-301 was also mediated the proliferation and invasion of gastric carcinoma tumor cells, In addition, the correspondence between the miR-301 expression and TNM staging of gastric cancer consistent with the above results. Therefore, miR-301 expression in gastric cancer may suggest a poor prognosis. Xu Ying [23] studies suggested that, in the head and neck carcinoma, with the deterioration of the tumor, mir-301a expression gradually increased, eventually promoted the head and neck squamous cell proliferation, so that the head and neck squamous cell cancerous and gradually worsen, conclusions of this study was similar to the above conclusions.

miRNAs play biological effect mainly by adjusting the downstream target genes, and therefore the role of downstream target genes determine the role of miRNAs. According to the literature, miR-301 adjusted the invasiveness of pancreatic cancer cells by regulating the expression of downstream target genes TP63 [24]. Meanwhile, Wang et al [25] studies have shown that in gastric cancer, miR-301 targeting regulate RUNX3 further regulate the proliferation and invasion of gastric cancer cells. We therefore interested in whether miR-301 could regulate other target genes then involved in the occurrence and development of gastric cancer. Accordingly, we used TargetScans target gene prediction software screened target genes of miR-301. Among target gene, PTEN played an important role in the human cancer [26, 27], and it was chosen as the target gene of interest. Finally, our results shown that transfection of miR-301 mimic significantly inhibited the expression of PTEN in MGC-803 cells ($P < 0.01$), whereas transfection anti-miR-301 can significantly increase the PTEN expression in MGC-803 cells ($P < 0.01$).

In summary, MiR-301 expression positively correlation with tumor size, TNM staging and Lauren type, and there has no correlation with gender, age, lymph node metastasis and tissue differentiation. MiR-301 upregulated in gastric cancer, high expression of miR-301 was likely played an important role on the development of gastric cancer. High expression of miR-301 may participate in gastric cancer progression by regulating target gene PTE, and it was possible to become a new target for gastric cancer gene regulation.

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Disclosure of conflict of interest

None.

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