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Original Article

Knockdown of miR-24 suppresses the proliferation, migration and invasion of gastric cancer cells and predicts a poor prognosis in gastric cancer

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Abstract: Objective: Gastric cancer (GC) is one of the leading causes of cancer death worldwide. MicroRNAs have recently emerged as key regulators of GC. In this study, we tried to investigate the effects of knockdown of miR-24 on the proliferation, migration and invasion of gastric cancer cells. Methods: A total of 28 GC tissue samples and matched non-tumor tissue samples were obtained from surgery patients at First Affiliated Hospital of Nanjing Medical University between 2013-2014. Human GC cell lines MGC803, AGS, BGC823 and SGC7901 were cultured. MiRNA mimics and their appropriate negative control were used to knock down the target gene. QRT-PCR, Western blot were used to test the expression of the gene and MTT assay, cell migration and invasion assay were adopted to evaluate the cells' proliferation, migration and invasion. Results: Results showed that miR-24 was up-regulated in GC tissues and cell lines. Knockdown of miR-24 remarkably suppressed cell proliferation of GC cell lines SGC7901. Moreover, knocking down on miR-24 also significantly suppressed migration and invasion of GC cells SGC7901. What's more, the results of viewing on patients showed the patients in the miR-24-positive group had a poorer prognosis than did the patients in miR-24-negative group. Conclusion: Taken together, miR-24 could suppress GC cells growth and motility. MiR-24 acts as a predictor of a poor prognosis in patients with GC.

Keywords: miR-24, gastric cancer, proliferation, migration, invasion

Introduction

Gastric cancer (GC) is one of the leading causes of cancer death worldwide. Because of its heterogeneity, GC has been an interesting model for studying carcinogenesis and tumorigenesis [1]. The carcinogenesis of GC is complicated, and it involves the dys-regulation of oncogenes and tumor suppressors [2]. Despite great advances in GC diagnosis and treatment, GC is still the second most frequent malignancy in global. The common molecular mechanism of GC remains poorly understood.

As an important part of epigenetic regulation, recent studies have shown that the expression of microRNAs (miRNAs) is closely related to the initiation, development, invasion, metastasis and prognosis of GC [3]. MicroRNAs (miRNA) are short non-coding RNAs with a length of 19-22 nucleotides that function as post-transcriptional regulators by directly cleaving target messenger RNA (mRNA) or translational repression [4-7]. It has been shown that many miRNAs are related to various types of cancer. For example, down-regulation of miR-200c is used by breast cancer stem cells to maintain their undifferentiated state [8]. MiR-520b can inhibit the proliferation of liver cancer cells through down-regulation of MEKK2 and cylindromatosis (Cyclin) D1 [9]. Moreover, miRNAs involved in the development and progression of GC have been widely explored [10]. Many studies have identified a number of miRNAs aberrantly expressed in GC [11]. Among them, miR-24 restrains GC progression by downregulating RegIV [12].

In this study, we validated the differential expression of miR-24 in GC and investigated the

Table 1. The mRNA levels of miR-24 and CEA in GC patient tissue samples and matched-non-tumor tissue samples

Cases	MiR-24 (GC tissues)	MiR-24 (matched tissues)	CEA (GC tissues)	CEA (matched tissues)
1	0.7986	0.1255	0.3569	0.01256
2	0.6694	0.0865	0.3845	0.03669
3	0.5415	0.1254	0.4123	0.01216
4	0.8566	0.2569	0.2965	0.00986
5	0.4125	0.0899	0.5147	0.02566
6	0.5893	0.1224	0.2986	0.01458
7	0.5745	0.0789	0.3789	0.01426
8	0.3647	0.1725	0.3426	0.02369
9	0.2415	0.0625	0.4756	0.03574
10	0.7145	0.1225	0.2956	0.01245
11	0.3458	0.0547	0.3412	0.04125
12	0.2369	0.0415	0.6142	0.01125
13	0.3489	0.0569	0.3969	0.01482
14	0.2147	0.0547	0.4755	0.03564
15	0.5699	0.0146	0.2145	0.01566
16	0.4589	0.1266	0.3796	0.06563
17	0.3256	0.0567	0.5471	0.03895
18	0.1479	0.0415	0.3564	0.04153
19	0.2569	0.0145	0.3984	0.02569
20	0.3415	0.0412	0.4896	0.05871
21	0.3694	0.0632	0.5994	0.04571
22	0.1478	0.0364	0.6423	0.01225
23	0.1255	0.0777	0.3654	0.03698
24	0.4586	0.0657	0.4745	0.04712
25	0.3145	0.0847	0.5745	0.01589
26	0.5147	0.0216	0.6142	0.02471
27	0.2698	0.0364	0.3956	0.04786
28	0.3475	0.0578	0.4961	0.01391

function of miR-24 in proliferation, migration and invasion.

Material and methods

Tissue samples

A total of 28 GC tissue samples and matched non-tumor tissue samples were obtained from surgery patients at First Affiliated Hospital of Nanjing Medical University between 2013-2014. The study was approved by Institutional Review Boards of the First Affiliated Hospital of Nanjing Medical University and informed consent was taken from all patients. All the tissues were histopathologically confirmed and kept at -80°C for further RNA extraction.

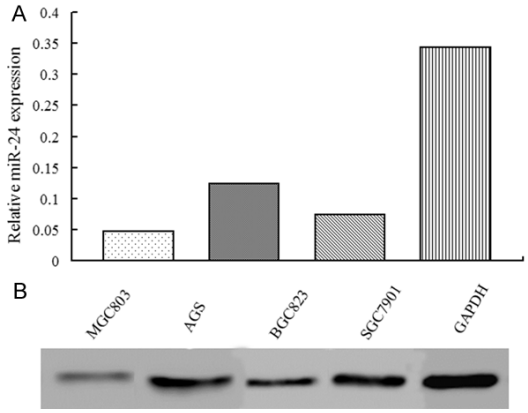

Figure 1. A. The mRNA level of miR-24 in GC cell lines MGC803, AGS, BGC823 and SGC7901 determined by RT-PCR. B. The protein expression of miR-24 in GC cell lines MGC803, AGS, BGC823 and SGC7901 determined by western blot assay.

Cell culture and transfection

Human GC cell lines MGC803, AGS, BGC823 and SGC7901 were obtained from the Chinese Academy of Sciences (Shanghai, China). They were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco-BRL, Grand Island, NY, USA). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. MiRNA mimics and their appropriate negative control (NC) were purchased from Guangzhou RiboBio (RiboBio, Guangzhou, China) and transfected into SGC7901 cells at a final concentration of 50 nmol/L by using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The infection efficiency was 100%. SGC7901 cells were harvested 24 h after transfection for cell proliferation and migration assays.

RNA isolation and qRT-PCR

Total RNA from tissues and cells were isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of the total RNA were evaluated using the ultraviolet spectrophotometer. Total RNA was stored at -80°C until analysis.

The specific primers of reverse transcription (RT) and polymerase chain reaction (PCR) from Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio, China) were used to amplify miRNAs. The amounts of PCR products were determined by

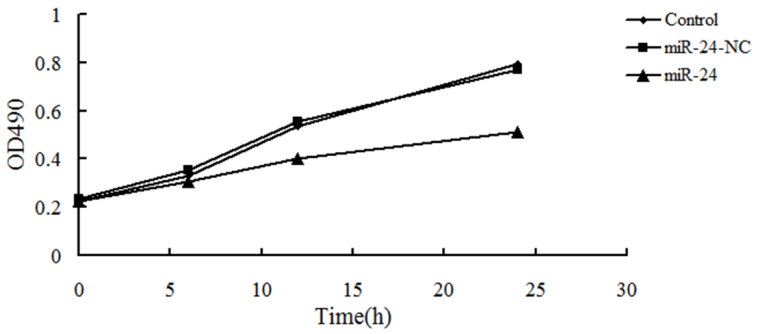

Figure 2. MiR-24 suppressed GC cell growth in vitro. Cell proliferation of SGC7901 cells transfected with miR-24 or control mimic was detected by MTT assays.

the level of fluorescence in emitted by SYBR Green (SYBR Premix Ex Taq™ II, TaKaRa). RT reaction was incubated at 42°C for 60 min followed by 70°C for 10 min. The qRT-PCR was performed on 7900HT real-time PCR system (Applied Biosystems) at 95°C for 10 s, 60°C for 20 s and then 60°C for 10 s. The melting analysis was added finally to assess the specificity of PCR products.

Western blot assay

Cells were washed twice with ice-cold PBS and then lysed in RIPA buffer with protease and phosphatase inhibitors (Beyotime Biotechnology, China). Protein concentrations were determined using a BCA protein assay kit (KeyGen Biotech). Equivalent amounts of total proteins (40 ug) were loaded onto a 10% SDS-polyacrylamide gel and then transferred onto polyvinylidine difluoride membranes. The transferred membranes were blocked for 1 h in 5% nonfat milk and then incubated with the primary antibodies for MGC803, AGS, BGC823 and SGC7901 (Santa Cruz Biotechnology, Santa-Cruz, CA, USA) at 4°C overnight. Membranes were washed three times with TBSH for 10 min and incubated with horseradish peroxidase-coupled secondary antibodies for 2 h at room temperature. The immunoreactive bands were visualized using enhanced chemiluminescence with ECL reagents (Pierce, Rockford, IL). Each assay was repeated three times and β-actin protein level was used as a control for equal protein loading.

MTT assay

Cells were seeded in 96-well plates (2×10^3/per well) 24 h after transfection. MTT (Roche Applied Science, Foster City, CA, USA) was added to each well and cells were further cultured for 4 h at 37°C. The reaction was stoppted by 150 uL DMSO and optical density at 490 nm was detected on a microplate reader.

Cell migration and invasion assay

For migration assay, 1×10^5 cells were plated onto 24-well MilliCell chambers (Millipore, Bedford, MA, USA) with an 8-um pore polycarbonate membrane. For invasion assay, 1.5×10^5 cells were plated pre-coated with 20 ug of Matrigel on the upper side. In both assays, the cells were plated in medium supplemented with 0.1% serum and the chambers were placed into 24-well plates with medium containing 10% serum. After 24 or 48 h, invaded cells on the lower membrane surface were fixed and stained with 0.1% crystal violet while cells that did not migrate or invade through the pores were removed by cotton swabs. Three random fields for each insert were counted and the results were averaged among three independent experiments.

Statistical analysis

All statistical analysis was performed using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation (S.D.) from at least three separate experiments. The differences between means were analyzed using the Student's t-test and Chi-square test. A p-value <0.05 was considered to indicate a statistically significant difference. Survival curves were estimated by the Kaplan-Meier method and compared by the log-rank test. A Cox proportional hazard model was used for univariate and multivariate survival analyses.

Results

The expression of miR-24 was upregulated in gastric cancer tissues

The mRNA levels of miR-24 and carcinoma embryonic antigen (CEA) in GC patient tissue samples and matched non-tumor tissue samples were determined by RT-PCR. Results showed miR-22 was significantly increased in GC

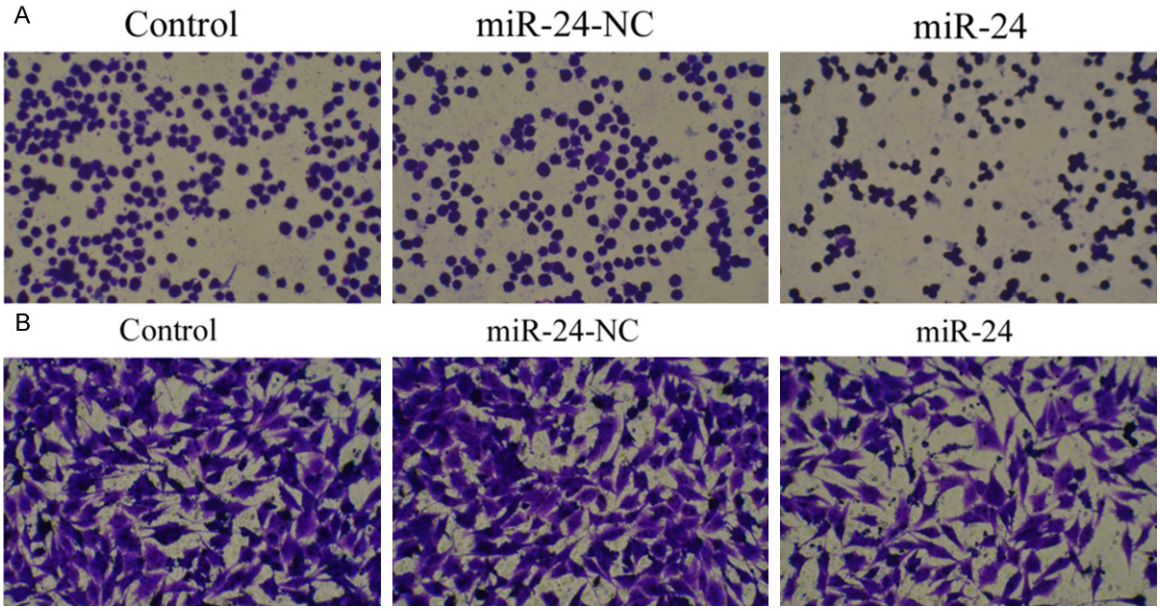

Figure 3. MiR-24 suppresses cell migration and invasion in vitro. A. Cell migration was measured by transwell assays 48 h after incubation. The number of migrated cells from three random areas of the membrane was counted using a light microscope. B. The knockdown of miR-24 inhibited cell invasion in SGC7901 cells compared with negative control and miR-NC group.

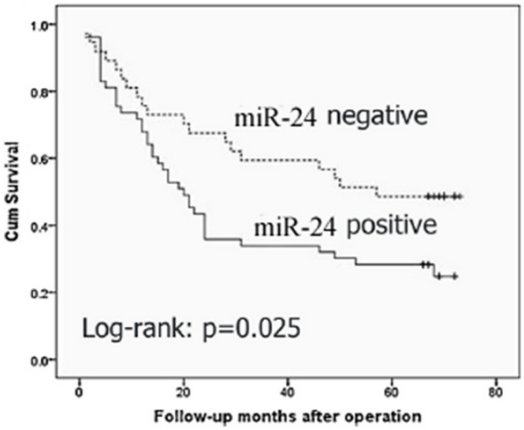

Figure 4. Overall survival curves of the patients with gastric cancer who underwent complete resection, according to the positive or negative expression of miR-24.

patient tissue samples compared with matched non-tumor tissue samples (**Table 1**). The expressional rate of miR-24 and CEA was 47% and 67%, respectively. Moreover, we found the expression of CEA was low when the expression of miR-24 was high.

Screening on GC cell lines

In this study, four GC cell lines MGC803, AGS, BGC823 and SGC7901 were prepared. To better explore the influence of miR-24 on GC, we used RT-PCR and western blot assay to screen GC cell lines in which the expression of miR-24 was much higher. The results of RT-PCR were shown in **Figure 1A**. As shown, the mRNA level of miR-24 in GC cell line SG-C7901 was significantly higher than in GC cell lines MGC803, AGS and BGC823. The results of western blot assay also showed the expression value of miR-24 in GC cell line SGC7901 was markedly higher than in GC cell lines MGC803, AGS and BGC823 (**Figure 1B**). Therefore, GC cell line SGC7901 was selected for further study.

Knockdown on miR-24 inhibit gastric cancer cell proliferation

To examine the role of miR-24 in the proliferation of GC cells, we conducted MTT assay with transfected with miR-24 or control mimic in SGC7901 cell lines. The results showed that knockdown of miR-24 significantly suppressed cell proliferation in SGC7901 cell lines (**Figure 2**).

Knockdown on miR-24 inhibit gastric cancer cell migration and invasion

To determine whether miR-24 could regulate human GC cell migration and invasion, we performed migration and invasion assays in GC

Table 2. Univariate and multivariate survival analysis of influencing factors

Variable	Univariate	Multivariate		
	p-value	*p*-value	HR	95% CI
Age	0.162	0.465	0.811	0.462-1.423
Grade I, II versus III	0.082	0.389	1.274	0.734-2.209
T1, T2 versus T3, T4	0.073	0.812	1.060	0.655-1.716
N stage	0.003[a]	0.001[a]	2.823	0.652-5.669
M stage	0.005[a]	0.236	1.923	0.652-5.669
pStage (I, II versus III, IV)	0.000[a]	0.675	1.344	0.338-5.348
TRAF6	0.025[a]	0.083	0.540	0.269-1.084

[a]*p*-value which less than 0.05

cell lines SGC7901. MiR-24 or control mimic were transiently transfected in these cells. As expected, knockdown of miR-24 significantly decreased the migration (**Figure 3A**) and invasion capability (**Figure 3B**) in SGC7901 cell lines. The observations indicated that miR-24 were positive regulators of GC migration and invasion.

Prognostic significance of tumor necrosis factor receptor-associated factor 6 (TRAF6) in gastric cancer

The patients in the miR-24-positive group had a poorer prognosis than did the patients in miR-24-negative group (P=0.025). The average survival time in miR-24-negative and-positive groups was 46.378 ± 4.735 and 31.481 ± 3.749 months, respectively. The 5-year survival rate in TRAF6-negative and-positive groups was 48.6% and 28.3%, respectively. The post-operative overall survival curves for the two groups are shown in **Figure 4**. Cox proportional hazards regression analysis was used to define biological markers with an independent predictive value with respect to survival, as shown in **Table 2**. The N stage acted as the only significant inde-pendent prognostic factor in GC. However, multivariate regression analysis did not support TRAF6 as an independent prognostic factor (P=0.083).

Discussion

Gastric cancer is the second common cause of cancer-related death worldwide. It is the main cause of cancer deaths in China [13]. It is widely accepted that miRNA dysregulation occurs in GC as well as other cancers and contributes to carcinogenesis and tumor progression [14]. Recently, numerous studies have reported the aberrant expression of miRNAs in a wide range of human cancers, and the aberrant expression of miRNAs contributed to carcinogenesis by inhibiting the expression of their target genes [15]. The direct link between miRNA function and carcinogenesis is supported by studies detecting the expression of miRNAs in clinical samples [16, 17].

MiR-22 has been found to be elevated in some cancers. In this study, we determined the expression of miR-24 in human GC tissues and investigated its possible roles in GC. Our results showed that expression of miR-24 was upregulated in GC patient tissue samples compared with matched non-tumor tissue samples. Moreover, the expression of CEA was low when the expression of miR-24 was high. Therefore, we speculated miR-24 may influence the growth of GC cells. To explore the influence of miR-24 on GC, we used RT-PCR and western blot assay to screen GC cell lines in which the expression of miR-24 was high. After screening, SGC7901 cell lines were selected to explore the relationship between miR-24 and the proliferation, migration and invasion ability of GC cells. SGC7901 cell lines were transfected with miR-24 or control mimic. Results showed knocking down on miR-24 remarkably suppressed cell proliferation of GC cell lines SGC7901. Moreover, decrease of miR-24 also significantly suppressed migration and invasion of GC cells SGC7901. MiR-24 could upregulate during terminal differentiation of multiple lineages and inhibit cell-cycle progression [18]. In cancer cells, it regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 [19]. miR-24 is consistently upregulated during terminal differentiation of hematopoietic cell lines into a variety of lineages [20]. It is also upregulated during thymic development to naive CD8 T cells [21] and during muscle and neuronal cell differentiation [22]. In glioma cells, miR-24-3p and miR-27a-3p promote cell proliferation via cooperative regulation of MXI1 [23]. Moreover, miR-24 enhances tumor invasion and metastasis by targeting PTPN9 and PTPRF to promote EGF signaling [24]. MiR-24 can regulate the proliferation and invasion of glioma by ST7L via β-catenin/Tcf-4 signaling [25]. What's more, miR-

34a can regulate cisplatin-induce GC cell death by modulating PI3K/AKT/survivin pathway [26].

View on patients showed patients with miR-24-positive expression had a poorer prognosis than did patients with negative expression (P=0.025). However, multivariate survival analysis with a Cox proportional hazard model showed that only advanced N stage was an independent predictor for patients' prognosis, and miR-24 was not an independent prognostic factor in GC (P>0.05).

Collectively, this study showed that miR-24 was decreased in GC tissue samples and cell lines, and knockdown of miR-22 substantially suppressed cell proliferation, migration and invasion of GC cells. Those suggesting that miR-24 might be a novel therapeutic strategy for the treatment of GC.

Disclosure of conflict of interest

None.

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