ORIGINAL ARTICLES

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MiR-224 promotes proliferation and migration of gastric cancer cells through targeting PAK4

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Although recent studies have shown the important role and overexpression of miR-224 in several tumors, its function in gastric cancer has not yet been defined. In the present study, we tried to confirm the result of microRNAs microarray and further investigated the functions of miR-224 in gastric cancer, and tried to find new downstream targets of miR-224. In this study, the level of miR-224 was measured in gastric cancer cells with the normal human gastric epithelial cell. The effects of miR-224 of on proliferation, migration, and target protein expression were evaluated by CCK8 assay, colony assay, transwell migration assay, western blotting. In addition, luciferase reporter plasmid was constructed to demonstrate the direct target of miR-224. Overexpression of miR-224 was detected in the gastric cancer cells, especially in SCG-7901. Exogenous miR-224 expression promoted the proliferation and migration of gastric cells and abrogating expression of miR-224 suppressed proliferation, and migration of SCG-7901 cells in vitro. Luciferase assays revealed that miR-224 directly targeted the 3'UTR of p21-activated kinase 4 (PAK4). The present study provides an experimental foundation for miR-224 as a potential tumor suppressor that may decrease PAK4 expression to inhibit gastric cancer cells and that in the future, targeting of this miRNA may provide a novel strategy for the diagnosis and treatment of patients with this lethal disease.

1. Introduction

Gastric cancer (GC) is a malignant disease associated with a poor prognosis. It is responsible for the second highest cancer-related mortality rate with five-year survival rates of only 20-30% (Guo et al. 2014). Despite the studies that have been conducted in previous years, the molecular mechanism of gastric cancer has yet to be elucidated. Recently, more and more miRNAs, oncogenes and tumor suppressor genes have been confirmed to be closely associated with GC, but the specific molecular mechanisms on the proliferation, migration and invasion of the cancer cells have been still under investigation. Previous studies have shown that cell externalization is an initial sign of malignant transformation (Kabbage et al. 2013). Telomerase, which is responsible for telomere lengthening, is a key factor in the processes of cell externalization and malignant transformation. It is activated in approximately 90% of cancer cells, including gastric cancer cells (Du et al. 2015).

MicroRNAs are ~22 nucleotide (nt) non-coding RNAs that function as sequence-specific regulators of gene expression through translational repression and/or transcript cleavage (Chen et al. 2014). More and more studies have shown that microRNAs play key roles in cellular processes of differentiation, proliferation, apoptosis and metabolic homeostasis (Pandey et al. 2008). MicroRNAs profiles have shown that there was much microRNAs expression variation across the different subtypes and stages of carcinogenesis, with data indicating that they may play vital roles in the initiation and progression of human malignancies (Liu 2013). In cancer tissues, microRNAs appear to be dysregulated such that those with tumor-suppressor activity are abrogated, while those that are overexpressed may function as oncogenes promoting proliferation, migration and invasion, and repressing apoptosis respectively (Wu et al. 2016; Wang et al. 2016). Recently, it has been demonstrated that miR-224 was dysregulated in many tumors such as breast cancer (Labbozzetta et al. 2015), liver cancer (Kowalik et al. 2015), and colorectal cancer (Zhu et al. 2015). In addition, it also involved in regulating the migration and invasion during carcinogenesis (Guo et al. 2015). Taken together, these observations suggest that miR-224 may play a vital role in the initiation and progression of cancers.

In the present study, we reported that miRNA-224 could indirectly enhance PAK4 expression, which triggers tumor metastasis. We hope that this study can supplement previous data in describing how PAK4 might enhance the malignant activity of gastric cancer cells.

2. Investigations and results

2.1. MiR-224 is up-regulated in human gastric cancer lines

To investigate whether miRNA-224 was upregulated in gastric cancer lines, we evaluated its levels in three gastric cancer lines (MKN-45, BGC-823, SCG-7901) as well as the normal human gastric epithelial cell, GES-1. As shown in Fig. 1, we determined a high level of miR-224 in gastric cancer cells, especially SCG-7901.

2.2. MiR-224 promoted proliferation of human gastric cancer cells

To confirm the hypothesis that miR-224functions as a potential promoter in gastric cancer cell proliferation, we determined the transfection efficiency at 72 h post-transfection (Fig. 2A and 2B). As shown in Fig. 2C, treatment with miR-224m significantly stimulated the growth of SCG-7901 cells compared with miR-224m NC and control (Fig. 2C, $p < 0.01$). By contrast, the SCG-7901 cells which were transfected with miR-224i caused a significant decrease in proliferation compared with the control group (Fig. 2D, $p < 0.01$). Consistent with the results in CCK8 assay, the results of colony showed that miR-224 could significantly increase the numbers of SCG-7901 (Fig. 3).

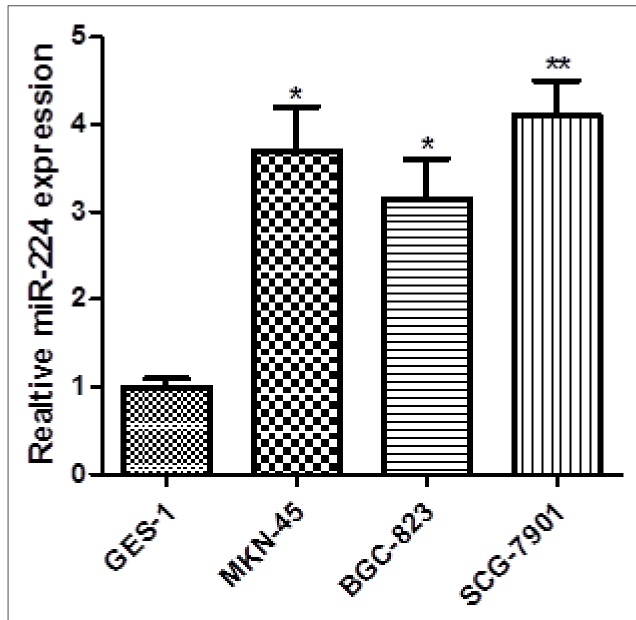
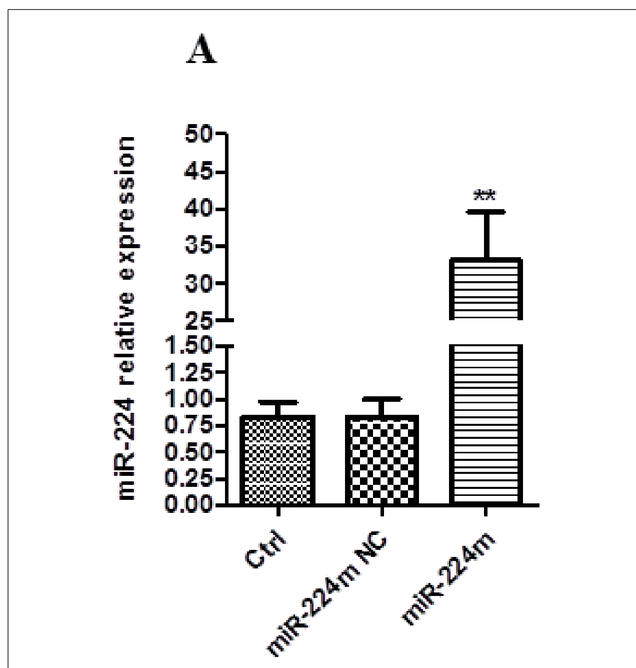


Fig. 1: MiR-224 is significantly up-regulated in gastric cancer cell lines. Relative miR-224 expression levels in gastric cancer cell lines and GES-1 normal human gastric epithelial cells (*p<0.05, **p<0.01).

2.3. MiR-224 enhanced the migration of human gastric cancer cells

Next, we explored the roles of miR-224 in the regulation of gastric cancer cell mobility by using transwell migration assay. As shown in Fig. 4, migration was significantly promoted in miR-224 overexpressed SCG-7901 cells. The number of migrated cells of SCG-7901 cells transfected with miR-224m was significantly increased



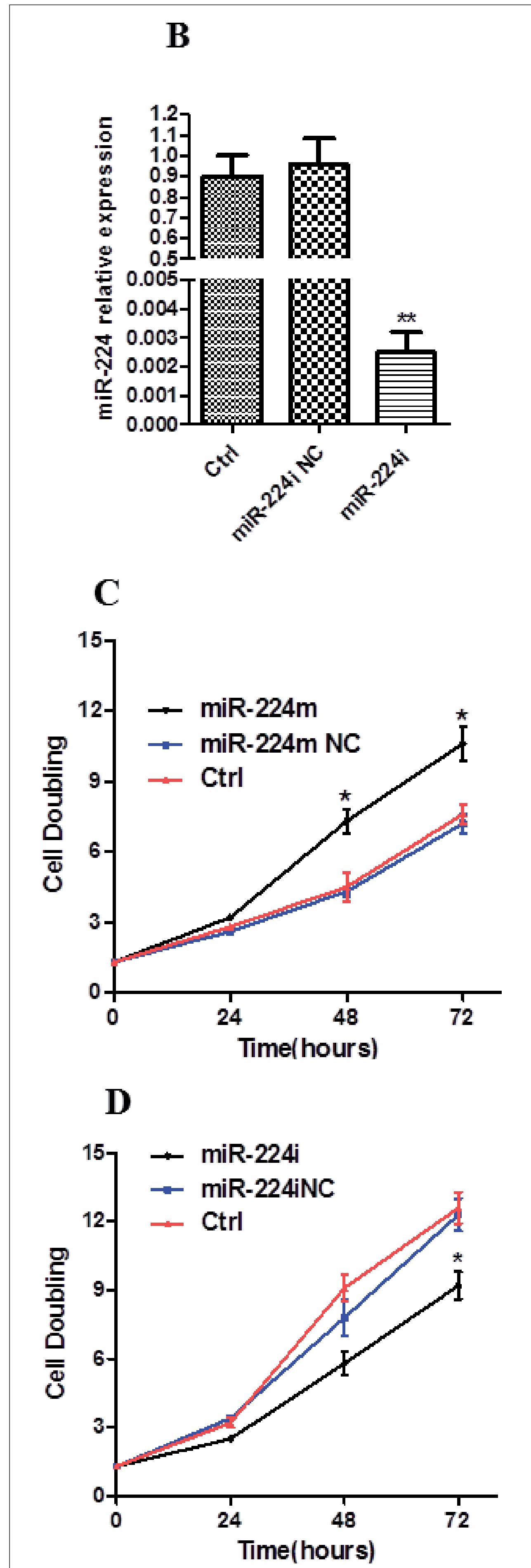


Fig. 2: Analysis of transfection efficiency and cell proliferation after differential treatment. A. Transfection efficiency of SCG-7901 cells was measured at 72 h after differential transfection by qRT-PCR. Bars show mean ± SD. **p<0.01 vs. miR-224m NC, control. B. Transfection efficiency of SCG-7901 cells was measured at 72 h after differential transfection using qRT-PCR. Bars show mean ± SD. **p<0.01vs. miR-224i NC, control. C. CCK8 analysis of the growth of three groups of SCG-7901 cells. *p< 0.05 vs. miR-224 NC, control. D. CCK8 analysis of the growth of three groups of SCG-7901 cells. *p< 0.05 vs. miR-224 NC, control.

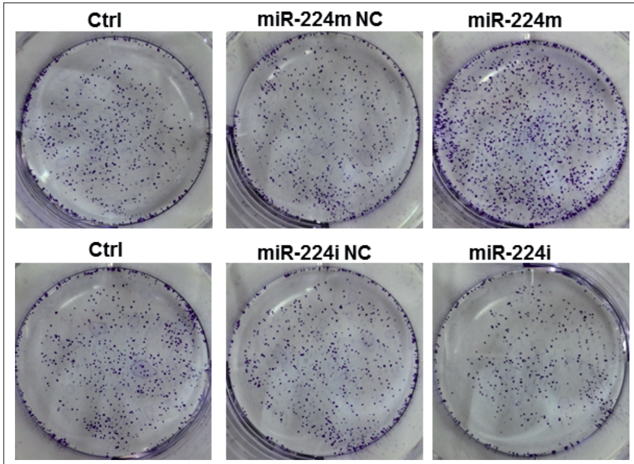


Fig. 3: Effect of miR-224 on cell proliferation of SCG-7901 cells after differential treatment in vitro colony assay. Ectopic miR-224 expression in SCG-7901 gastric cancer cells significantly increased the cell colony formation numbers.

compared to the miR-224m control group. The number of migrated cells of SCG-7901 cells transfected with miR-224i was markedly reduced as compared with miR-224i NC or blank. According to these results, miR-224 plays a functional role in mediating migration in SCG-7901 cells and suggest a mechanism by which overexpression of miR-224 may contribute to metastasis of gastric cancer cells.

2.4. *MiR-224 negatively regulated the expression of PAK4*

To search for potential targets of miR-224 that may promote proliferation and migration ability of cells, PAK4 was found among potential targets of miR-224 predicted using three publicly available databases, TargetScan, Pictar and miRanda. The 3'UTR of PAK4 carries a binding site for miR-224 (Fig. 5A), suggesting that PAK4 mRNA might be a direct target of miR-224. Then, we evaluated the effect of miR-224 on the expression of PAK4 protein in SCG-7901 cells transfected with miR-224m (or miR-224i) using western blotting. Transfection with miR-224m could significantly downregulate of PAK4 in SCG-7901 cells compared with miR-224m NC or the control group, whereas the cells treated with miR-224i obviously enhanced expression of PAK4 compared with miR-224i NC or the control group (Fig. 5B).

To verify that the putative miR-224 binding site in the 3'UTR of PAK4 is responsible for regulation by miR-224, we constructed vectors containing wild-type or mutant 3'UTR of PAK4 directly fused to the downstream of the firefly luciferase gene. As shown in Fig. 5C, miR-224 significantly decreased the relative luciferase activity of wild-type PAK43'UTR, whereas the reduction of the luciferase activity with mutant PAK4 3'UTR was not as sharp as that observed in the wild-type counterpart, suggesting that miR-224 could directly bind to the 3'UTR of PAK4.

Taken together, these findings suggested that PAK4 might act as a direct downstream target for miR-224 in gastric cancer cells.

3. Discussion

Evidence suggests that miRNAs may contribute to cancer pathogenesis, as they may serve as oncogenes or anti-oncogenes during

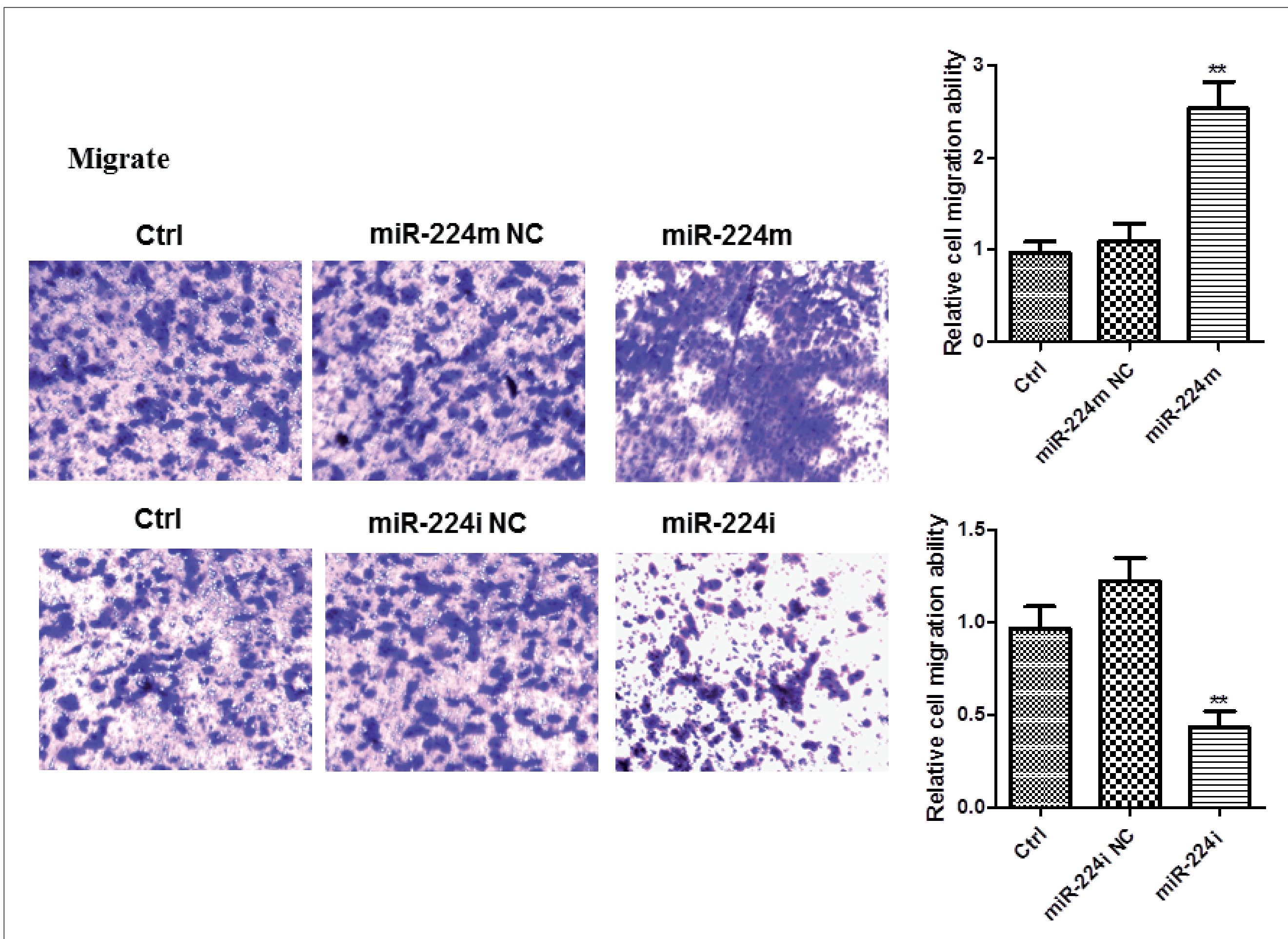


Fig. 4: MiR-224 enhanced the migration ability of SCG-7901 cells using transwell migration assay. SCG-7901 cells were seeded in transwell filter. Migrated cells on the lower surface of the transwell filter were stained and counted after 24 h. Photographs were taken at 24 h postmigration (magnification, 100×). Bars show mean ± SD. All experiments were repeated three times.

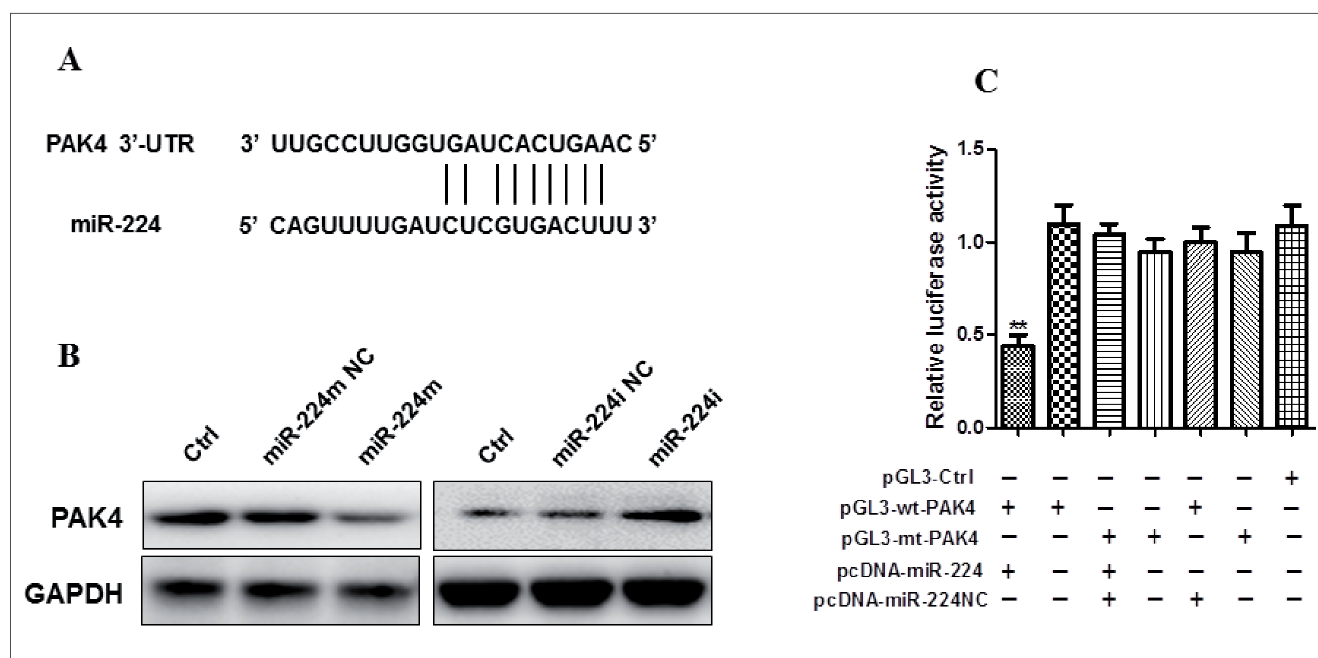


Fig. 5: MiR-224 negatively regulated PAK4 protein levels in SCG-7901 cells. A. Putative binding sites of miR-224 in the PAK4 3'UTR predicted by TargetScan. B. PAK4 protein levels were measured in SCG-7901 cells at 72 h post-transfection by western blot assays. (C) Co-transfection with pcDNA-miR-224 and pGL3-wt-PAK4 reduced the luciferase activity (** $p<0.01$), whereas co-transfection with pcDNA-miR-224 and pGL3-mt-PAK4 did not reduce the luciferase activity.

carcinogenesis (Chen et al. 2015). However, the precise role each miRNA plays in malignant progression is unknown. Overexpression of miR-224 has been observed in many cancers such as liver cancer, lung cancer, and some other solid tumors (Chen et al. 2015). Furthermore, miR-224 also plays a vital role in promoting the development of precursor B-cell acute lymphoblastic leukemia (Song et al. 2014). These reports suggested that miR-224 might be associated with tumorigenesis of other types of human tumors as well. Consistent with these published findings, we also found that miR-224 was significantly overexpressed in gastric cancer cells compared to the normal human gastric epithelial cell using microRNAs microarray and demonstrated that the miR-224 expression level displayed a positive correlation with the cell invasive potential, suggesting that miR-224 may act as an oncogene in gastric cancer.

The p21-activated kinase (PAK) family consists of six serine/threonine kinases (PAK1-6), which are indicated to be involved in a variety of cellular functions, including cell proliferation, migration and cytoskeletal organization (Kelly and Chernoff 2012). Among them, PAK4, a specific receptor of Cdc42 and Rac, plays roles in normal cellular functions, and is involved in tumorigenesis by promoting proliferation, migration, invasion, and metastasis of cancer cells, including prostate, ovarian, breast, colon and gastric cancer (Dart and Wells 2013). In this study, we validated the PAK4 as a miR-224 target and demonstrated that overexpressing miR-224 *in vitro* increased gastric cancer cell mobility. In addition, the PAK4 expression level was inversely correlated with the cell invasive potential. Collectively, these studies have shown that PAK4 plays a significant role as a general inhibitor of cell motility. In conclusion, the present study demonstrated that miRNA-224 could promote gastric cancer cell proliferation via targeting PAK4. Further studies are required to fully understand the roles and mechanisms of miRNA-244 and PAK4 in gastric cancer cell line SCG-7901, which will be beneficial for the development of therapeutic strategies for GC.

4. Experimental

4.1. Cell culture and transfection

MKN-45, BGC-823, SCG-7901, GES-1 cell lines were obtained from American Type Culture Collection and were maintained routinely in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin a humidified atmosphere of 5% CO_2 at 37 °C. Cells were seeded in 6-well plates at 70-80% confluence and grown overnight before transfection. Transfection of SCG-7901 cells with miR-224 inhibitors (miR-224i) or and miR-224 inhibitors negative control (miR-224i NC), miR-224 mimics (miR-224m) or miR-224 mimics negative control (miR-224m NC) using the lipfectamine2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

4.2. RNA isolation and qRT- PCR

Total RNA was isolated from cultured cells with using Trizol reagent (Invitrogen, CA, USA). QRT-PCR analyses for microRNAs were performed using TaqMan microRNA assays (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Expression of U6 was used as an endogenous control. Data were processed using $2^{-\Delta\Delta CT}$ method.

4.3. Cell proliferation assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK8) according to manufacturer's instructions. Cells at 12 h post-transfection were seeded in 96-well plates at 2500 cells/well. Then, 10 µl of CCK8 solution was added to the culture medium, and incubated for additional 3 h. The absorbance was determined at 450 nm wavelength. Average OD values were used to estimate the number of cells of each group.

4.4. Wound healing assay

Cells (6×10^6 per well) were seeded in 6-well plates 12 h after transfection and allowed to adhere for 24 h. Confluent monolayer cells were scratched by a 200 µl pipette tip and then washed three times with 1× PBS to clear cell debris and suspension cells. Fresh serum-free medium was added, and the cells were allowed to close the wound for 48 h. Photographs were taken at 0, 24, and 48 h at the same position of the wound.

4.5. Transwell migration assay

The ability of cell migration was performed by transwells with 8µm pore size (Corning Costar Corp, USA). Cells (2×10^5) were resuspended in 200 µl of serumfree medium and seeded on the top chamber of each insert. 500µl RPMI1640 with 10% fetal bovine serum were added into the bottom chamber. The cells were allowed to migrate for 24 h at 37 °C in a humidified incubator with 5% CO_2. The cells attached to the lower surface of membrane were fixed with 95% absolute alcohol and stained with crystal violet for 20 min at room temperature. Cells that had invaded through the membrane were manually counted at 100x magnification from ten different fields of each filter. The mean of triplicate assays for each experimental condition was analyzed.

4.6. Western blotting analysis

Cultured cells were lysed in RIPA buffer with 1 % PMSF, and isolated protein was loaded onto a SDS-PAGE mini-gel and transferred on PVDF (Millipore) membranes. The membranes were incubated with specific primary antibodies (1:1000) in 4 °C overnight after blocking in skim milk. Next the blotted membranes were incubated with HRP-conjugated anti-rabbit IgG (1:5000) antibodies at room temperature for

2 h. The signals were visualized by enhanced chemiluminescence (ECL kit, Pierce Biotechnology),) and visualized with Quantity One software 4.6.2.

4.7. Construction of reporter plasmids and luciferase assay

For the luciferase assays, the potential miR-224 binding site in the 3'-UTR of PAK4 mRNA was predicted by TargetScan, Pictar and miRanda. The 3'-UTR of wild-type (wt) and mutant (mt) PAK4 mRNA was cloned and inserted into the downstream of luciferse gene in pGL3/luciferase vector (Promega, Madison, WI, USA) and the resulting vectors were termed pGL3-wt-PAK4 and pGL3-mt-PAK4, respectively. The SCG-7901 cells were cultured in 24-well plates and co-transfected with miR-224 mimics and pGL3-wt-PAK4 or pGL3-mt-PAK4 using lipofectamine 2000. After 48 h, the cells were collected and luciferase activity was assessed using a Dual-Luciferase Reporter Assay kit (Promega Corporation).

4.8. Statistical analysis

In all the experiments, data were derived from at least three independent experiments and analyzed using one-way ANOVA for 3-group comparisons and t tests for 2-group comparisons. All statistical analyses were performed using SPSS 13.0 software package. The probability of $p < 0.05$ was considered to be statistically significant.

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