STUDY ON THE MECHANISM OF MIR-1254 PROMOTING PROLIFERATION AND MIGRATION OF GASTRIC CANCER BY TARGETING NKD1

Wei Zhang, Wensheng Li, Shizhang Liu, Huitong Liu, Jiyuan Shi, Keke Duan, Bingqiang Xu*
Shaanxi Provincial People's Hospital, the Third Affiliated Hospital of Xi'an Jiaotong University, No.256 Youyi West Road, Beilin District, Xi'an, Shaanxi, 710068, China

ABSTRACT

Objective: *To investigate the effect of miR-1254 on proliferation and migration of gastric cancer cells.*

Methods: *The expression of miR-1254 in gastric cancer tissues and cells was investigated by TCGA database analysis and real-time quantitative PCR. After overexpression or knockout of miR-1254 in gastric cancer cells, the effect of miR-1254 on the proliferation of gastric cancer cells was detected by CCK-8 assay, and the effect of miR-1254 on the migration of gastric cancer cells was measured by scratch method. The binding sequence of miR-1254 and NKD1 3'UTR was predicted by TargetScan software, and the regulatory effect of miR-1254 on NKD1 was further verified by luciferase reporter gene detection. The effect of miR-1254 on the expression of NKD1 in gastric cancer cells was determined by western blot assay. The expression of NKD1 in tumor tissues and normal tissues was analyzed by immunohistochemistry method, and the effect of over-expression of NKD1 on the proliferation and migration of gastric cancer cells was investigated. Finally, rescue experiments were used to further verify whether miR-1254 affects the proliferation and migration of gastric cancer cells by regulating NKD1.*

Results: *Compared with normal tissues and normal gastric epithelial cells, the expression of miR-1254 in human gastric cancer tissues and gastric cancer cells was increased ($P < 0.01$). Over-expression of miR-1254 in SGC7901 cells can enhance the proliferation and migration of gastric cancer cells ($P < 0.01$). Moreover, the inhibition of miR-1254 expression in AGS cells can decrease the proliferation and migration ability of gastric cancer cells ($P < 0.01$). MiR-1254 can combine with NKD1 3'UTR region, thus reducing the expression of NKD1. Over-expression of NKD1 could inhibit the proliferation and migration of gastric cancer cells ($P < 0.01$), and at the same time, it could weaken the enhancement of proliferation and migration of gastric cancer cells induced by over-expression of miR-1254 ($P<0.01$).*

Conclusion: *MiR-1254 is capable of promoting proliferation and migration of gastric cancer cells by inhibiting the expression of NKD1.*

Keywords: *Gastric cancer, miR-1254, proliferation, migration, NKD1.*

DOI: 10.19193/0393-6384_2019_6_467

Received *November 30, 2018;* ***Accepted*** *February 20, 2019*

Introduction

Gastric cancer is an epithelial malignant tumor. Dietary structure, food storage, acquisition of fresh agricultural products and helicobacter pylori (HP) are the main factors for the occurrence and development of gastric cancer[1]. Therefore, in-depth exploration of the pathogenesis of gastric cancer is crucial to the development of new therapeutic methods.

The role of miRNAs in tumor progression has been extensively studied since the discovery of miRNAs in 1993. MiRNA is a non-coding single-stranded RNA of 19-24 nt, which can regulate about one third of mammal gene expression at the post-transcriptional level. MiRNAs inhibit protein translation or degradation of target mRNA by binding to 3'UTR of targeting mRNA[2]. A large number of reports have shown that miRNA can regulate the occurrence and development of gastric cancer, such as miR-137, miR-421 and miR-1[3-5].

MiR-1254 has carcinogenic activity in a variety of cancers. For example, it promotes the proliferation of lung cancer by targeting SFRP1[6]. In breast cancer, miR-1254 directly targets RASSF9 and plays a pro-cancer role[7]. The level of miR-1254 in serum of patients with lung cancer is significantly increased, which can be used as a biomarker of early lung cancer[8]. In this study, we investigated the role of miR-1254 in the proliferation and migration of gastric cancer cells, and found for the first time that miR-1254 can promote the proliferation and migration of gastric cancer cells by targeting NKD1.

Materials and methods

Tissue samples and sources

This research plan was approved by the Medical Ethics Committee of our hospital. Sixteen patients with gastric cancer who underwent surgical treatment in our hospital from March 2017 to September 2018 were selected as the subjects. Appropriate amount of gastric cancer lesion samples were taken, some paracancer tissues were taken more than 5 cm from the cancer edge, and all samples were preserved in liquid nitrogen. All the patients signed informed consent, did not receive radiotherapy and chemotherapy before operation, and were diagnosed as gastric cancer by imaging, pathological and clinical symptoms.

Cell culture and transfection

Human gastric cancer cell line MGC-803, AGS, MKN-28, SGC7901 and normal gastric epithelial cell line GES-1 were cultured in RPMI-1640 medium (Hyclone, USA) containing 10% fetal bovine serum (Sijiqing, China). The cells were cultured in an incubator at 37°C with 5% CO2. When the cells grown to the logarithmic growth phase, transfection experiments were carried out in the SGC7901 or AGS cells. The above cells were inoculated in 6-well plate with 2 × 105 cells per well and cultured overnight. The cells were transfected with miR-1254 non-homologous sequence, miR-1254 mimics, miR-1254 inhibitor and NKD1 over-expression plasmid by Lipofectamine 2000 (Invitrogen, USA) to form miR-NC group, miR-1254 over-expression group, miR-1254 low expression group and NKD1 over-expression group, respectively.

Real-time quantitative PCR (RT-qPCR)

The total RNA of tissues and cells in each group was extracted by Trizol RNA extraction reagent. According to the instructions of reverse transcription kit (PrimeScript™ RT reagent Kit, TaKaRa), the quantitative PCR reaction (SYBR® Premix Ex Taq™ II, TaKaRa) was carried out. The Ct value of U6 was used as the internal reference and the relative expression level of miRNA was calculated by $2^{-\Delta\Delta Ct}$.

Cell proliferation test

The cells were collected after transfection for 24 h, evenly laid in a 96-well plate with 2×10^3 / wells, and the cells were cultured under normal conditions. The cell proliferation ability was tested in accordance with the specification of the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) after 24, 48 and 72 h of culturing. 10 μL of CCK-8 solution was added to cells in each well and cultured at 37°C for 1 h, then the absorbance of cells was detected at 490 nm.

Scratch experiment

The cells were seeded on a 6-well plate, and after growth to fusion, a scratch was made on single-layer membrane of the cell with a suction head, and the cells were washed three times with a medium free of serum. The cells were cultured for 24 h, followed by observation and photographing.

Luciferase reporter gene detection

The 3'URT region or mutant 3'URT region of NKD1 was cloned into psiCHECK2 vector and simultaneously transfected with miR-1254 mimics or inhibitor in gastric cancer cells. Luciferase activity was determined by luciferase report detection kit (Beyotime, China) after transfection for 48 h.

Western blot

After the cells in each group were collected, the cells were lysed by RIPA reagent and the total protein was extracted. The protein concentration was determined by BCA method. SDS-PAGE electrophoresis was performed after the same amount of the protein was sampled. After the protein was separated, it was transferred to the PVDF membrane, and the primary antibodies of anti-NKD1, anti-GAPDH (CST, USA) was diluted with TBST buffer until the working concentration, then the protein was incubated overnight. The second antibody labeled with HRP was incubated at room temperature for 2 h, ECL developing was performed, Bio-rad gel imaging system was used for exposure and the images were analyzed.

Immunohistochemical analysis

The tissue samples were fixed in 10% formaldehyde solution, embedded in paraffin, inactivated with endoperoxidase, 50 μL of the primary antibody was added and incubated overnight at 4 °C. After adding the secondary antibody, DAB coloring was carried out, the samples were lightly redyed by hematoxylin, dehydrated by gradient ethanol, and the film was sealed for observation under the microscope.

Statistical analysis

GraphPad Prism software was used for statistical analysis. The experimental results were

expressed as mean ± standard deviation ($\bar{x}$±s) and t test was used for comparison between groups. P < 0.05 indicated the difference was statistically significant.

Results

High expression of miR-1254 in gastric carcinoma tissues and cells

The statistical results of TCGA database showed that the expression levels of miR-1254 in gastric cancer tissues were significantly higher than that in normal tissues (P < 0.01). The results of RT-qPCR showed that the level of miR-1254 in tumor tissue was significantly higher than that in normal tissue (P < 0.01) (Fig. 1B). The expression of miR-1254 in different gastric cancer cell lines was significantly higher than that in normal gastric epithelial cells (Fig. 1C), and the difference was statistically significant (P < 0.01). MiR-1254 over-expression was performed in the SGC7901 cell line according to the RT-qPCR result, and the expression of miR-1254 was inhibited in the AGS cell (Fig. 1D). The above cell material was provided for subsequent functional verification.

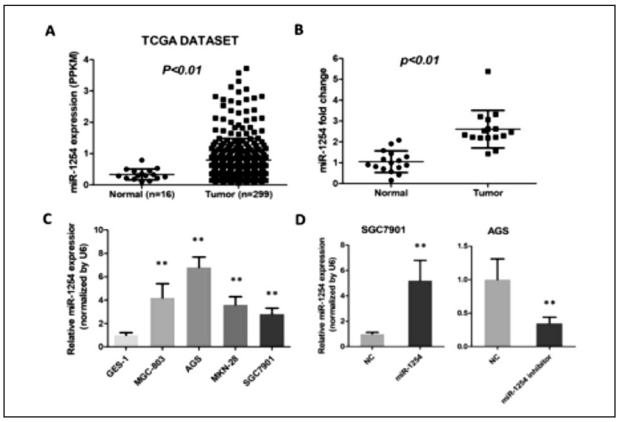

Fig. 1: The expression of miR-1254 in gastric cancer tissues and cells. A. The expression of miR-1254 in gastric tumor tissues and normal stomach tissues. Date was downloaded from TCGA dataset. B. The level of miR-1254 in gastric cancer tissues and normal gastric tissues. C. miR-1254 expression in gastric cancer cells and normal gastric epithelial cell. D. The effect of miR-1254 overexpression in SGC7901 or knockdown in AGS. **p<0.01.

MiR-1254 promotes proliferation and migration of gastric cancer cells

The results of CCK-8 assay showed that the proliferation ability of SGC7901 cells increased significantly after over-expression of miR-1254 at 48 h and 72 h (P < 0.01) (Fig. 2A).

After inhibiting the expression of miR-1254 in AGS cells, the proliferation ability of AGS cells decreased significantly (P < 0.05, P < 0.01) (Fig. 2B). The results of scratch test showed that the cell mobility of SGC7901 cell line transfected with miR-1254 mimics was remarkably higher than that of cell line transfected with miR-NC (Fig. 2C). The migration ability of the AGS cells transfected with miR-1254 inhitor decreased (Fig. 2D).

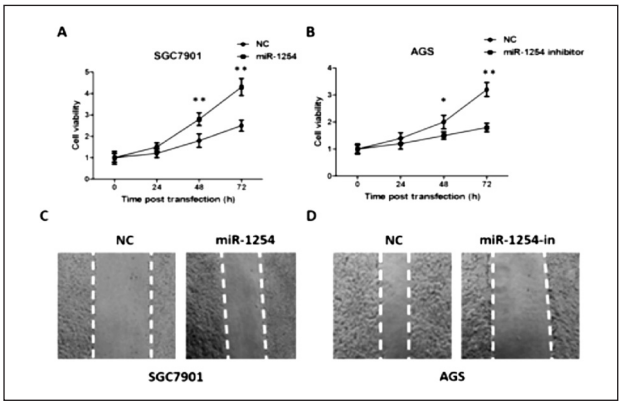

Fig. 2: The effect of miR-1254 on cell proliferation and migration. A. Effect of miR-1254 on proliferation of SGC7901 cells. B. Effect of miR-1254 on proliferation of AGS cells. C. Effect of miR-1254 on migration of SGC7901 cells. D. Effect of miR-1254 on migration of AGS cells. *p<0.05, **p<0.01.

MiR-1254 promotes proliferation and migration of gastric cancer by targeting NKD1

The specific binding sequence between miR-1254 and NKD1 3'UTR region was predicted by TargetScan software analysis (Fig. 3A). The results of Western blot confirmed that in SGC7901 cells, the over-expression of miR-1254 could reduce the expression of NKD1, while in the AGS cells, inhibition of miR-1254 expression could promote the expression of NKD1 (Fig. 3B). After the NKD1 3'UTR sequence was cloned into luciferase reporter gene plasmid, the results showed that the over-expression of miR-1254 could significantly decrease luciferase activity (P < 0.01) (Fig. 3C). At the same time, the inhibition of miR-1254 increased the activity of luciferase (P < 0.01) (Fig. 3D).

NKD1 can inhibit the proliferation and migration of gastric cancer

Immunohistochemical analysis of tumor tissues and paracancerous tissues in patients with gastric cancer indicated that the expression of NKD1 protein in tumor tissues was significantly lower than that in normal tissues (Fig. 4A).

After over-expression of NKD1 in AGS cells (Fig. 4B), the proliferation ability of AGS cells decreased significantly ($P < 0.01$) (Fig. 4C), and the mobility of AGS cells also decreased ($P < 0.05$) (Fig. 4D).

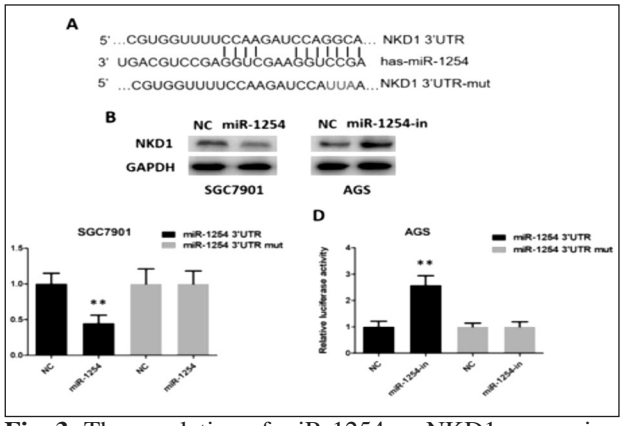

Fig. 3: The regulation of miR-1254 on NKD1 expression in gastric cancer. A. The predicted binding site of miR-1254 on 3'UTR of NKD1 using TargetScan software. B. The effect of miR-1254 on the expression of NKD1. C. Analysis of the luciferase activity of psiCHECK-2-NKD1 3'UTR and mut vector in SGC7901 cells by miR-1254 over-expression. D. Analysis of the luciferase activity of psiCHECK-2-NKD1 3'UTR and mut vector in AGS cells by miR-1254 inhibition. **p<0.01.

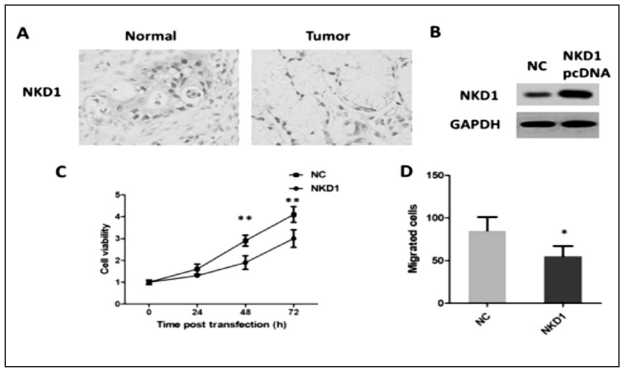

Fig. 4: Effect of NKD1 expression on proliferation and migration of gastric cancer cells. A. The expression of NKD1 in normal tissues and gastric cancer tissues. B. The expression of NKD1 analyzed by Western blot assay. C. The effect of NKD1 on the proliferation of AGS cells. D. The effect of NKD1 on the migration of AGS cells. *p<0.05, **p<0.01.

Over-expression of NKD1 reduced promoting effect of proliferation and migration by miR-1254

MiR-1254 and NKD1 were over-expressed in AGS cells at the same time (Fig. 5A) and the results found that NKD1 over-expression could weaken the proliferation (Fig. 5B) and migration (Fig. 5C) induced by miR-1254 over-expression. The above results further confirmed that the regulation of miR1254 on proliferation and migration of gastric cancer cells was achieved by NKD1 protein.

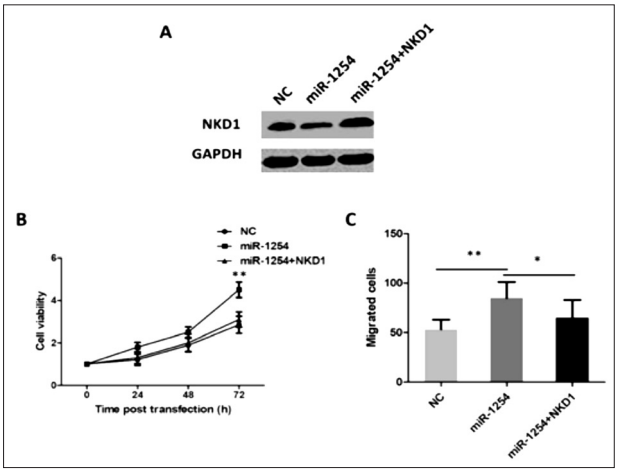

Fig. 5: The effect of miR-1254 on proliferation and migration in gastric cancer cell via targeting NKD1. A. The over-expression of miR-1254 and NKD1 on the regulation of NKD1 protein. B. Effects of miR-1254 and NKD1 over-expression on the proliferation of AGS cells. C. Effects of over-expression of miR-1254 and NKD1 on migration of AGS cells.

Discussion

The causes of gastric cancer are varied, there is no complete therapeutic tool for gastric cancer, and the pathogenesis of gastric cancer has not yet been studied. With the progress of science, it has been gradually found that miRNA plays an important role in the development of cancer and is abnormally expressed in all stages of tumorigenesis[9]. Therefore, the down-regulation of some miRNA expression in the process of tumor development may be used as a method to inhibit tumor development[10].

In this study, we compared the TCGA database and analyzed the tumor tissues and paracancerous tissues of patients with gastric cancer. It was found that miR-1254 was highly expressed in gastric cancer tissues. The expression of miR-1254 was further investigated in a variety of gastric cancer cells, it was found that miR-1254 was also highly expressed in a variety of gastric cancer cells, and the over-expression of miR-1254 in gastric cancer cells promoted the proliferation and migration of gastric cancer cells, and the inhibition of miR-1254 could reduce the proliferation and migration of gastric cancer cells. It is confirmed for the first time that NKD1 is the target of miR-1254 and NKD1 is a tumor inhibitor. For example, NKD1 can inhibit the invasion of non-small cell lung cancer and is a good prognostic factor[11]. NKD1 inhibits the expression and activity

of Rac1, thereby inhibiting the metastasis of liver cancer[12]. NKD1 is a favorable prognostic factor in patients with invasive ductal carcinoma of breast[13]. The role of NKD1 in the progress of gastric cancer has also been confirmed. HOXA11 can inhibit the proliferation, migration, invasion and induce apoptosis of gastric cancer cells by up-regulating NKD1 expression[14]. In this study, we further confirmed that the expression level of NKD1 in gastric cancer tissues was lower than that in normal tissues, and the over-expression of NKD1 could inhibit the proliferation and migration of gastric cancer cells. Meanwhile, the results of rescue experiment showed that NKD1 could weaken the effect of miR-1254 over-expression on gastric cancer cells, which suggested that NKD1 could inhibit the proliferation and migration of gastric cancer cells. Although we found that miR-1254 promotes the proliferation and migration of gastric cancer cells and the level of miR-1254 was positively correlated with tumor metastasis, in vivo experiments were also required to further confirm the role of miR-1254 in the occurrence and development of gastric cancer.

In conclusion, it was proved in this study that miR1254 was highly expressed in gastric cancer tissues and cells, which could targeted inhibit NKD1 expression, thus promoting the proliferation and migration of gastric cancer cells.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA A Cancer Journal for Clinicians, 2019; 69: 7-34.
2) He W, Zhao S, Zhang C, Vincent MS, Zhang B. QuickRNASeq: Guide for Pipeline Implementation and for Interactive Results Visualization. Methods Mol Biol 2018; 1751: 57-70.
3) Du Y, Chen Y, Wang F, Gu L. miR-137 plays tumor suppressor roles in gastric cancer cell lines by targeting KLF12 and MYO1C. Tumour Biol 2016; 37: 13557-13569.
4) Feng X, Deng S, Zhang D, Liu N, Wang K, et al. miR-199a modulates cisplatin resistance in ovarian cancer by targeting Hif1α. Onco Targets Ther 2017; 10: 5899-5906.
5) Han C, Zhou Y, An Q, Li F, Li D, et al. MicroRNA-1 (miR-1) inhibits gastric cancer cell proliferation and migration by targeting MET. Tumour Biol 2015; 36: 6715-6123.
6) Li H, Yang T, Shang D, Sun Z. miR-1254 promotes lung cancer cell proliferation by targeting SFRP1. Biomed Pharmacother 2017; 92: 913-918.
7) Li B, Chen P, Wang J, Wang L, Ren M, et al. MicroRNA-1254 exerts oncogenic effects by directly targeting RASSF9 in human breast cancer. Int J Oncol 2018; 53: 2145-2156.
8) Peng H, Wang J, Li J, Zhao M, Huang SK, et al. A circulating non-coding RNA panel as an early detection predictor of non-small cell lung cancer. Life Sci 2016; 151: 235-242.
9) Si W, Shen J, Zheng H, Fan WM. The role and mechanisms of action of microRNAs in cancer drug resistance. Clin Epigenet 2019; 11: 25.
10) Lin S, Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer 2015; 15: 321-333.
11) Hou LK, Pei L, Ma YS, Jia CY, Yu F, et al. MicroRNA-125a-3p downregulation correlates with tumorigenesis and poor prognosis in patients with non-small cell lung cancer. Oncol Lett 2017; 14: 4441-4448.
12) Li J, Zhang S, Hu Q, Zhang K, Jin JB, et al. The NKD1/Rac1 feedback loop regulates the invasion and migration ability of hepatocarcinoma cells. Sci Rep 2016; 6: 26971.
13) Zhao ZM, Yost SE, Hutchinson KE, Li SM, Yuan YC, et al. CCNE1 amplification is associated with poor prognosis in patients with triple negative breast cancer. BMC Cancer 2019; 19: 96.
14) Bronchud MH, Tresserra F, Zantop BS. Epigenetic changes found in uterine decidual and placental tissues can also be found in the breast cancer microenvironment of the same unique patient: description and potential interpretations. Oncotarget 2018; 9: 6028-6041.

Corresponding Author:
Bingqiang Xu
E-mail: pkuj0j@163.com
(China)