JBUON 2021; 26(2): 483-489
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Expression of miR-614 in gastric cancer and its effect on invasion and proliferation of gastric cancer cells HGC-27

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Summary

Purpose: *To investigate the expression of miR-614 in serum of gastric cancer patients and its effect on invasion and proliferation of gastric cancer cell line HGC-27.*

Methods: *Thirty patients with gastric cancer from May 2016 to November 2018 comprised the research group, and 30 healthy people undergoing physical examination in the same period comprised the control group. The expression of miR-614 in tissues and miR-614 in HGC-27 cell line was detected by qRT-PCR, miR-614-mimics was transfected into HGC-27, while miR-614-mimics group, blank control group and negative control group were established respectively. Cell invasion was detected by Transwell method, and CCK-8 method was used to detect the effect of miR-614 transfection on the proliferation of HGC-27 cells on the 1st, 2nd, 3rd and 4th day.*

Results: *The expression of miR-614 in the research group was significantly lower than in the control group ($p<0.05$). The number of cells passing through the invasion microvessel membrane in miR-614-mimics group was significantly lower than in negative control group and blank control group ($p<0.05$) 24 h after transfection. On the 3rd and 4th day, the cell proliferation of miR-614-mimics group was significantly lower than in blank group and negative control group ($p<0.05$).*

Conclusion: *In conclusion, miR-614 is lowly expressed in gastric cancer patients and inhibits the invasion and proliferation of gastric cancer cell line HGC-27.*

Key words: *gastric cancer, miR-614, HGC-27 cell, invasion, proliferation*

Introduction

Gastric cancer is the fifth most common cancer in the world [1], and it is also a common malignant tumor of digestive tract in China. In recent years, with the increasingly serious aging of the society, the morbidity of elderly gastric cancer patients has increased rapidly, which seriously threatens the survival and quality of life of the elderly [2]. At present, conventional treatment with drugs and surgery, as well as radiotherapy and chemotherapy are mostly used clinically [3,4] and treatment strategies have made progress, e.g. hyperbaric oxygen therapy can improve melatonin-induced apoptosis of gastric cancer cells [5]. However, the lesions had metastasized when the patients visited the hospital for physical examination. The survival rate of gastric cancer patients is not significantly improved, and the prognosis of metastatic gastric cancer patients is poor (5-year survival rate <10%) [6]. Therefore, it is necessary to further study the pathogenesis of gastric cancer and find targeted molecular therapeutic targets to provide new therapeutic options for gastric cancer patients.

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Received: 15/09/2020; Accepted: 22/10/2020

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MicroRNA (miRNA/miRs) are a class of endogenous non-coding RNA molecules, which can inhibit the expression or translation of target genes by combining with complementary sequences of the 3' untranslated region (3-UTR) of target messenger RNA transcript (mMiR) [7]. MiRs can regulate the expression of human protein coding genes [8]. Some studies demonstrated that up-regulation or down-regulation of miRs might play a key role in cancer progression by affecting proliferation and apoptosis of cancer cells [9]. In previous studies, miR-614 over-expression in ovarian cancer promoted cell proliferation and inhibited cell apoptosis by targeting PPP2R2A2A [10]. We suspected that miR-614 over-expression might play a carcinogenic role in cancer, but there is little research on the role of miR-614 in gastric cancer, which is worthy of study.

This study explored the relationship between miR-614 expression in gastric cancer patients and clinicopathological parameters of gastric cancer, as well as the effect of miR-614 on the proliferation and invasion of gastric cancer cells, providing more effective biological basic data for clinical early prediction, diagnosis and treatment of this malignancy.

Methods

General data

From May 2016 to November 2018, 30 patients with gastric cancer comprised the research group, and another 30 healthy people undergoing physical examination comprised the control group. There were 17 males and 13 females in the research group, aged 37-62 years on average (52.61±13.35). In the control group, there were 16 males and 14 females, aged 35-65 years on average (49.28±14.48).

Inclusion criteria were as follows: gastric cancer patients confirmed by preoperative or intraoperative pathology, patients who did not receive any radiotherapy, chemotherapy or immunotherapy before the operation, patients that were operated in our hospital, and patients with complete clinical data. Exclusion criteria were as follows: patients with severe liver and kidney dysfunction, systemic blood diseases, mental diseases, other malignant tumors, neurological diseases and systemic autoimmune diseases. This study was approved by the ethics committee of our hospital, and all subjects signed a fully informed consent.

Sample collection

Altogether 5 ml of fasting venous serum of patients in the two groups were collected and analyzed in the morning after admission. Then, it was coagulated and allowed to stand for 30 min, centrifuged and at 3500 $r \cdot min^{-1}$ for 5 min. The serum was taken from a 1.5 mL centrifuge tube and stored in a refrigerator at -80°C for testing.

Cell culture

Human gastric cancer cell line HGC-27 was purchased from the Cell Research Institute of Shanghai Academy of Sciences, China. HGC-27 cells were cultured in DMEM medium (GIBCO-BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS), penicillin (100 U/mL)/streptomycin (100 U/mL) in a cell incubator with 5% CO_2 and 37°C constant temperature saturated humidity. When the cell confluence reached about 80%, 0.25% trypsin was used for passage. Cells were divided into three groups, namely miR-641-mimics group, negative control group and blank control group.

Transient transfection of cells

Cells in logarithmic growth phase were inoculated into 6-well plates with cell density of 5×10^5 cells/mL per well, and they were transfected after culturing until the confluence reached 80-85%. The transfection step was carried out according to the instruction manual of Lipofectamine 2000 (Invitrogen, Waltham, USA, 11668027). Altogether 2μL Lipofectamine 2000 and 5nmoL miR-614 mimics (miR-641-mimics group) or their corresponding unrelated control sequences (negative control group) were fully mixed by volume 1:50 and then added into serum-free medium for transfection. The blank control group was not transfected. After 6h of transfection, phosphate buffer solution (PBS) was used for washing 3 times, and the normal culture solution was replaced and placed in a 5% CO_2, 37°C cell constant temperature incubator for 48h. qRT-PCR was used to verify the transfection efficiency of cells.

qRT-PCR detection

Total RNA was extracted from serum and cultured cells by Trizol extraction kit (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of RNA were detected by Nano-Drop2000 ultraviolet spectrophotometer (Beijing Keyu Xingye Technology Development Co., Ltd., China). According to Takara reverse transcription kit (Invitrogen, Carlsbad, CA, USA), RNA was reverse-transcribed into cDNA, and the synthesized cDNA was stored at -20°C for later use. The primers were designed and synthesized by GenePharma Pharmaceutical Technology Co., Ltd., Shanghai, China. The reaction was carried out on ABI PRISM 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). PCR amplification cycle conditions were as follows: 90°C for 5 min, 90°C for 5 s, 60°C for 30 s, 72°C for 5 s, with a total of 40 cycles, each sample was repeatedly detected 3 times and the relative expression of genes was expressed by $2^{-\Delta CT}$ calculation.

Cell invasion detected by Transwell method

BD Matrigel melt overnight at 4°C in advance, 100 μL diluted matrigel glue (the ratio of matrigel to serum-free medium was 1: 5) was spread on the surface of each Transwell upper chamber, 50 μL per well, and solidified for standby in 37°C incubator for 4-5 h. Cells in logarithmic growth phase were digested with 0.25% EDTA trypsin, washed 3 times in serum-free medium, counted, prepared into cell suspension, and adjusted

at cell density 2×10^5. Each upper chamber was washed once with serum-free medium, and treated according to experimental groups. Each group was provided with 3 multiple wells, and 100 μL of cell suspension was taken and added into Transwell chamber. Altogether 500 μL of conditioned medium containing 20% FBS was added to the lower chamber and cultured in an incubator for 24 h. The chamber was taken out, washed twice with PBS, fixed with 5% glutaraldehyde, and dyed for 30 min at 4°C after crystal violet was added. Then it was washed twice with PBS; the cells that did not pass through the upper layer of the chamber were wiped off with wet cotton swabs. Using an inverted microscope, the number of cells was observed, and the average was calculated.

Cell proliferation detected by CCK-8 method

The cells were diluted into a cell suspension at a density of 5×10^5 cells/mL, and 100 μL was aspirated and inoculated into 96-well plates. Each group was made with 3 multiple wells, and the cells were 1×10^3 per well. The cells were incubated in an incubator at 37°C. 100 ml of CCK-8 solution was added to each well at 24h, 48h, 72h and 96h. After 1 h, the optical density (OD) of each well was detected at 450nm wavelength on Elx-800 ELISA detector (BioTek, Winooski, VT, USA), and all samples were repeatedly measured for 3 times and averaged.

Statistics

SPSS20.0, and GraphPad Prism 6 (Shenzhen APS-GO Network Co., Ltd., China) were used for statistical analyses. The measurement data were expressed by mean±standard deviation, and the comparison between groups was performed with t-test of independent samples. Repeat measures analysis of variance (ANOVA) was used for data comparison at multiple time points, one-way (ANOVA) was used for comparison among multiple groups of mean numbers, and LSD-t test was used for the following two comparisons. $P<0.05$ was considered as statistically significant difference.

Results

Comparison of general data

No significant difference was found in age, gender, BMI, nationality, smoking, drinking and exercise between the control group and the re-

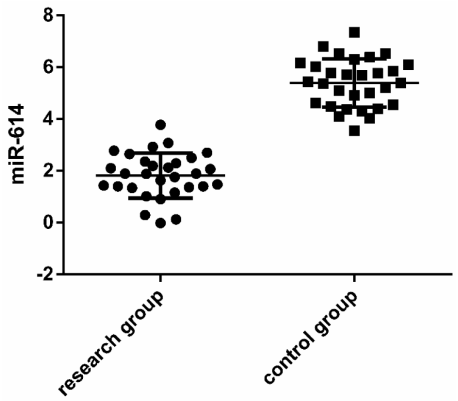

Figure 1. Expression of miR-614 in serum of patients in the two groups. qRT-PCR showed that the relative expression of miR-614 in the research group was significantly lower than in the control group, with statistically significant difference ($p<0.05$).

Table 1. Comparison of clinical data

	Control group (n=30) *n (%)*	*Research group (n=30)* *n (%)*	x^2 *or t*	*p*
Age, years	49.28±14.48	52.61±13.35	0.926	0.358
Gender			0.067	0.795
Male	16 (53.33)	17 (56.67)		
Female	14 (46.67)	13 (43.33)		
BMI, kg/m^2	23.17±2.34	23.46±1.86	0.531	0.597
Nationality			0.111	0.738
Han	24 (80.00)	25 (83.33)		
Minority	6 (20.00)	5 (16.67)		
Smoking history			0.277	0.598
Yes	13 (43.33)	11 (36.67)		
No	17 (56.67)	19 (63.33)		
Drinking history			0.071	0.790
Yes	12 (40.00)	11 (36.67)		
No	18 (60.00)	19 (63.33)		
Exercise habits			0.341	0.559
Yes	7 (23.33)	9 (30.00)		
No	23 (76.67)	21 (70.00)		

search group (P>0.050), proving that patients in the two groups were comparable, as shown in Table 1.

Expression of miR-614 in serum of patients in the two groups

qRT-PCR detection results showed that the relative expression of miR-614 in the serum of patients in the research group and the control group were 2.38±0.79, 5.34±1.03, respectively, in other words, the relative expression of miR-614 in the research group was significantly lower than in the control group, with statistically significant difference (P<0.05), as shown in Figure 1.

Relationship between the expression of miR-614 in serum of patients in the research group and its clinicopathological features

Analysis of clinicopathological data and miR-614 expression in serum of patients identified that miR-614 expression was not related to age, gender and tumor location (P>0.05), but related to average tumor diameter (cm), pathological typing, lymph node metastasis and TNM staging (P<0.05), as shown in Table 2.

Transfection efficiency of miR-614

We transfected HGC-27, and qRT-qPCR test results revealed that the relative expression of miR-614 in HGC-27 blank control group was 1.47±0.39, which was not significantly different from that of negative control group (1.46±0.41), and was significantly lower than in miR-614-mimics group (8.47±1.38; p<0.005), proving that transfection was effective, as shown in Figure 2.

Effect of miR-614 on invasion of HGC-27 cells

Transwell experiment results demonstrated that the number of cells penetrating through the microporous membrane in the transfected miR-614-mimics group, negative control group and blank group was 52.35±12.34, 89.14±14.32 and 90.23±15.34, respectively, 24h after transfection. The number of cells penetrating through the microporous membrane in the invaded cell in the transfected miR-614-mimics group was significantly lower than in the negative control group and blank control group, with significant statistical difference (p<0.005). There was no significant difference in the proliferation between blank control group and negative control group, as shown in Figure 3.

Table 2. Relationship between expression of miR-614 in serum and clinicopathological characteristics of patients in the research group

	Number n (%)	*Relative expression of miR-614*	*t*	*p*
Age (years)			0.042	0.966
≤60	11 (36.67)	2.38±0.71		
>60	19 (63.33)	2.37±0.56		
Gender			0.038	0.969
Male	17 (56.67)	2.38±0.73		
Female	13 (43.33)	2.37±0.68		
Tumor location			0.037	0.970
Gastric antrum	14 (46.67)	2.38±0.74		
Gastric body	16 (53.33)	2.37±0.72		
Average tumor diameter, cm			2.074	0.047
≤5	19 (63.33)	2.60±0.72		
>5	11 (36.67)	2.02±0.77		
Pathological typing			2.718	0.011
Highly differentiated	18 (60.00)	2.67±0.62		
Moderately and poorly differentiated	12 (40.00)	1.96±0.81		
Lymph node metastasis			2.274	0.030
Yes	19 (63.33)	2.18±0.71		
No	11 (36.67)	2.71±0.39		
TNM staging			3.184	0.003
I-II	17 (56.67)	2.69±0.48		
III-IV	13 (43.33)	1.98±0.74		

Effect of miR-614 on the proliferation of HGC-27 cells

There was no significant difference in cell proliferation among the three groups at the 1st and 2nd day (p>0.05). At the 3rd and 4th day, the cell proliferation of miR-614-mimics group was significantly lower than in the blank group and negative control group, with significant difference (p<0.05). There was no significant difference in cell viability between blank group and negative control group at each time point (p>0.05), as shown in Table 3 and Figure 4.

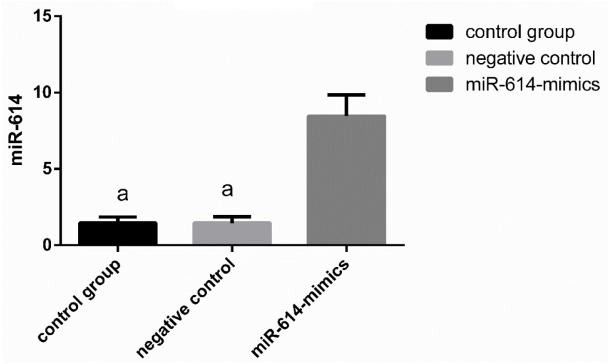

Figure 2. Expression of miR-614 in HGC-27 cells after transfection. qRT-PCR showed that the expression of miR-614 in miR-614-mimics group was significantly higher than in blank control group and negative control group, with statistically significant difference (p<0.05), while there was no significant difference between blank control group, negative control group and miR-614-mimics group (p>0.05).

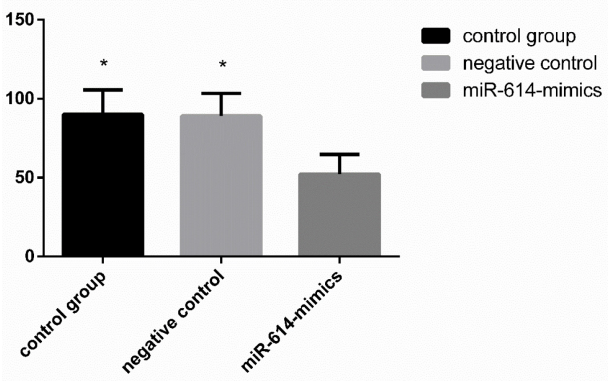

Figure 3. Comparison of invasive ability of HGC-27 cells in each group. Transwell experiment results showed that the number of cell membrane penetrations in miR-614-mimics group was lower than in negative control group and blank group, with statistically significant difference (p<0.05). There was no significant difference between blank group and negative control group (p>0.05) (*p<0.05).

Discussion

Gastric cancer is a clinically common malignant tumor of the digestive tract [11]. At present, the main methods of treatment for gastric cancer are surgery [12], chemotherapy [13] and radiotherapy [14]. Although surgical techniques and targeted drug therapy [15,16] are gradually maturing, the 5-year overall survival rate is less than 30% [17] because many patients are diagnosed as advanced and accompanied by lymph node metastasis [18]. The high mortality of gastric cancer patients is strongly linked to tumor recurrence and metastasis [19], while tumor recurrence and metastasis are bound up with abnormal gene expression, cell proliferation, invasion and migration [20]. Therefore, exploring the effect of genes on cell proliferation and invasion is helpful for the new

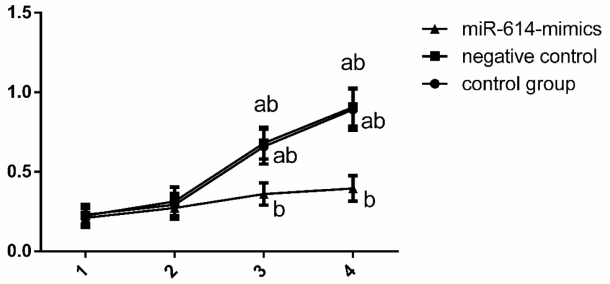

Figure 4. Comparison of HGC-27 cell proliferation in each group. CCK-8 test results showed that the cell proliferation of cells in the three groups had no significant difference at the 1st and 2nd day (p>0.05). At the 3rd and 4th day, the cell proliferation of miR-614-mimics group was significantly lower than that of blank group and negative control group, with significant difference (p<0.05). There was no significant difference in cell viability between blank group and negative control group at each time point (p>0.05); [a] means compared with miR-614-mimics group at the same time, P was less than 0.05, and [b] means the comparison between the same group, p was less than 0.05.

Table 3. Comparison of cell proliferation in each group

Time	*Transfection group*	*Negative control group*	*Blank group*	*F*	*p*
Day 1	0.211±0.06	0.225±0.07*	0.230±0.06*	0.072	0.931
Day 2	0.273±0.07	0.314±0.09*	0.293±0.07*	0.211	0.815
Day 3	0.361±0.07**	0.681±0.10*	0.659±0.11*	10.651	0.010
Day 4	0.396±0.08**	0.907±0.12*	0.892±0.13*	20.192	0.002

When * compared with * at the same time, p was over 0.05, and when * compared with ** at the same time, p was less than 0.05.

effective treatment of gastric cancer patients. Recent studies revealed that miRs were abnormally expressed in various malignant tumors [21]. Previous studies found that miR-614 expression was down-regulated in pancreatic cancer [22,23], and miR-614 expression in gastric cancer remains to be studied. Therefore, this article discussed miR-614 expression in gastric cancer and its effect on proliferation and invasion of gastric cancer cells, thus providing more possibilities for improving prognosis of gastric cancer and improving the patient survival.

Our study revealed that the level of miR-614 in gastric cancer patients was lower than in normal people, which suggested that miR-614 protein was usually highly expressed in gastric cancer cells. On this basis, our in-depth study of the relationship between miR-614 and clinicopathological data showed that miR-614 was related to mean tumor diameter (cm), pathological typing, lymph node metastasis and TNM staging ($p<0.05$). In order to investigate the expression of miR-614 in gastric cancer cells and its effect on proliferation and invasion of gastric cancer cells, we transfected HGC-27 cells. HGC-27 is a commonly used gastric cancer research cell line [24,25]. After transfection, the expression level of miR-614 in miR-614-mimics group was significantly higher than in negative control group and blank group, indicating successful transfection. We used Transwell experimental method to study its effect on invasion of gastric cancer cells. The experimental results showed that the number of cells in the miR-614-mimics group penetrating through the microporous membrane of the invasion chamber was significantly lower than in the negative control group and the blank control group, suggesting that the cell proliferation ability of the miR-614-mimics group was significantly inhibited after transfection, and the high expression of miR-614 could inhibit the invasion of gastric cancer cells. In addition, we further studied its effect on the proliferation of gastric cancer cells, and experimental results showed that the OD detection value of miR-614-mimics group was significantly lower than that of blank control group and negative control group in different time periods, suggesting that the cell proliferation ability was significantly reduced after transfection of miR-614-mimics. The high expression of miR-614 could inhibit the proliferation of gastric cancer cells, suggesting that miR-614 might become a potential option for targeted treatment of gastric cancer. This was different from our previous speculation that miR-614 was a tumor-promoting gene, and we think miR-614 might play different roles in different cancers. Afshar et al found that the expression of miR-614 in colorectal cancer patients was lower than in normal people through establishing an artificial neural network model and searching from GEO data set, and they found that RHOT1 was the most important target gene of miR-614 [26] through miR target prediction. What's more, Li et al pointed out that RHOT1 could induce proliferation and migration of pancreatic cancer cells [27]. At the same time, Lv et al investigated the effect and mechanism of miR-614 on proliferation and invasion of lung cancer cells, and found that the high expression of miR-614 in lung cancer cells inhibited the cell invasion and proliferation, which proved that puromycin-sensitive aminopeptidase (PSA) gene was a downstream target gene regulated by miR-614 [28]. Hence, we suspected that miR-614 could inhibit the tumorigenesis of cancer cells by targeting RHOT1 and PSA. At present, there are few reports on the role of RHOT1 and PSA in gastric cancer. In our follow-up study, we will conduct in-depth research on the biological functions of RHOT1 and PSA in gastric cancer cells.

This study confirmed the role of miR-614 in HGC-27 cells, and preliminarily discussed the mechanism of invasion and proliferation ability in HGC-27 cells. However, there are still some limitations in this research. Firstly, the migration and apoptosis of HGC-27 cell line by miR-614 has not been deeply evaluated. Secondly, we have not predicted and verified the target gene, and the regulatory mechanism is still unclear. These limitations need to be further supplemented in future research.

In summary, miR-614 expression is down-regulated in gastric cancer patients, and miR-614 over-expression inhibits the invasion and proliferation of gastric cancer cell line HGC-27, which may participate in the occurrence and development of gastric cancer.

Conflict of interests

The authors declare no conflict of interests.

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