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MiR-708 regulates the biological behavior of childhood leukemia cells by binding to the 3'utr end of the target gene and reducing the level of the target gene

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ABSTRACT

This study was performed to analyze the biological behavior of childhood leukemia cells regulated by miR-708 by binding to the 3' UTR end of the target gene and reducing the level of the target gene. In this regard, human leukemia Jurkat cell lines were selected and divided into a control group, miR-708 overexpression group and miR-708 inhibition group. MTT assay was used to detect the cell proliferation inhibition rate, flow cytometry was used to detect the apoptosis rate and cell cycle change, the scratch test was used to detect the cell migration capacity, and Western Blot assay was used to detect the expression of CNTFR, apoptosis and JAK/STAT pathway related proteins. To verify the binding site of miR-708 and target gene CNTFR. The results showed that the cell proliferation inhibition rate, apoptosis rate, G1 phase ratio, Bax protein, and CNTFR protein in the miR-708 overexpression group were significantly lower than those in the control group at each time point, while the S phase ratio, Bcl-2 protein, cell migration ability, JAK3 and STAT3 protein were significantly higher than those in the control group ($P<0.05$). The results of the miR-708 inhibition group were contrary to those of the miR-708 overexpression group. The binding sites of miR-708 and CNTFR were predicted by TargetScan bioinformatics software. It was found that there were two binding sites of miR-708 and CNTFR, 394-400 bp and 497-503 bp respectively. In conclusion, miR-708 can reduce the expression of CNTFR by binding to the target gene CNTFR3' UTR, activate the JAK/STAT pathway to regulate apoptosis-related proteins, reduce apoptosis, and enhance the migration ability of leukemia cells.

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Introduction

Leukemia is a malignant clonal disease of the hematopoietic system. It is a malignant tumor characterized by a certain line of hematopoietic cells in the hematopoietic tissue stagnating at the primitive poorly differentiated stage and undergoing malignant clonal proliferation, manifested as clinically with anemia, bleeding, infection, and splenomegaly, systemic swollen lymph nodes, bone spurs and other symptoms (1). Currently, the etiology and mechanism of leukemia are not completely clear. Most studies believe that there is a certain connection between viral infection, physical and chemical factor stimulation, and genetic defects (2). With the in-depth research on the types and functions of miRNAs in recent years, their influence on the occurrence, development and prognosis of leukemia has become the focus of research by domestic and foreign researchers (3). Roberto et al. (4) found that miR-708 is abnormally expressed in children with leukemia, but its relationship with the occurrence and development of leukemia has not yet been fully clarified. Previous studies believe that miR-708 can inhibit the migration and invasion of hepatocellular carcinoma cells. The study by Huang et al. (5) showed that miR-708 can reduce the growth, migration and invasion of renal cell carcinoma cells. This experiment took the human peripheral blood leukemia Jurkat cell line as the research subject and aimed to analyze the effect of miR-708 on the biological behavior of childhood leukemia cells and its related mechanisms.

Materials and Methods

General information

Cell line: Human peripheral blood leukemia Jurkat cell line was selected, provided by the Cryogenics Laboratory of Shandong University.

Methods

The Jurkat cell line was selected, and the RPMI-1640 cell culture medium was used after resuscitation. 10% fetal bovine serum was added and incubated in a 37°C, 5% CO_2 incubator. The medium was changed after 24 hours, and then half of the medium was changed every 48 hours. The cells under the microscope were counted. When the cell density was 1×10^6/ml, inoculated into a 6-well plate and prepared for transfection. The cells were divided into the control group, miR-708 overexpression group and miR-708 inhibition group, miR-708 overexpression and miR-708 inhibitory infectious agents were selected, respectively dissolved in 50μL of RPMI-1640 serum-free medium, the medium in the original culture plate was discarded, fresh RPMI-1640 serum-free medium was added, the mixture was added to the corresponding wells of the culture plate, cultured in a 37°C, 5% CO_2 cell incubator, and subsequent experiments were conducted after transfecting the cells for 24 hours.

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Observation indicators

Cell proliferation inhibition rate of each group detected by the MTT method

Jurkat cells were taken 24h, 48h, and 72h after transfection to make a single cell suspension, inoculated into a 96-well cell culture plate at a density of 1×10^4 cells/mL, and placed in a cell incubator with saturated humidity, 37°C, and 5% CO_2 for 4 days. 20μLMTT solution was added into the culture well, the culture solution was aspirated and discarded after incubating for 4 hours. Then DMSO was added to each cell culture well at 150 μL/well, and a shake reaction was performed on a decolorizing shaker for 10 minutes. The absorbance of the solution in each well at a wavelength of 492nm was measured, and the cell proliferation inhibition rate was calculated.

Cell apoptosis rate was detected by Flow cytometry

Logarithmic growth cells were taken, the cells with 0.25% trypsin were digested to make a single cell suspension, centrifuged at 4°C for 5 min, and 100 μL binding buffer was added. 5μL Annexin V-FICT was added and incubated at room temperature for 10min in the dark, 1μL PI staining solution was added, incubated at room temperature for 5min in the dark, 400μL buffer was added, and the apoptosis rate was detected within 30 minutes.

Cell cycle changes detected by Flow cytometry

Logarithmic growth cells were taken, pipetted to make a single cell suspension, pre-cooled 75% ethanol solution was added, stored in a refrigerator at 4°C overnight, centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded. 0.5% propidium iodide dye solution was added and resuspended, incubated at 4°C for 20 min.

Cell migration ability detected by Scratch test

A marker pen was used to draw a partition on the back of a 6cm cell culture dish, and Jurkat cells were selected and transfected. After 24 hours, a line was drawn vertically in a 6-well plate with a 20μL pipette tip, and RPMI-1640 serum-free medium was added. Pictures were taken under a microscope and continued to incubate in a 37°C, 5% CO_2 incubator. Now was marked as the 0th hour; the floating cells were washed with PBS every 24h, the RPMI-1640 serum-free medium was replaced, and pictures were taken and recorded.

CNTFR, apoptosis and JAK/STAT pathway-related protein expression detected by the Western Blot method

After 24 hours of transfection, the cells were taken out, washed once with pre-cooled PBS, and lysed on ice for 30 minutes by adding the cell lysis buffer, transferred to an EP tube, centrifuged and the supernatant was taken to determine the protein concentration. The same amount of protein was taken, after electrophoresis in a 10% SDS-PAGE gel, was transferred the membrane to the PVDF membrane, blocked for 1 hour, the primary antibody was incubated at 4°C overnight. The membrane was washed 3 times with TBST, the secondary antibody was incubated, the membrane was washed after 1 hour and it was exposed.

Database and bioinformatics analysis

Three online analysis softwares of TargetScan were used to predict miR-708 target genes and the conservative binding sites of miR-708 and target genes CNTFR.

Statistical methods

The data in this study all used SPSS 20.0 software package for statistical data analysis. All measurement data conforming to the normal distribution were compared with ($x\pm s$). One-way analysis of variance was used for comparison among multiple groups, and the SNK-q test was used for pairwise comparison; the statistical result was statistically significant with $P<0.05$.

Results

Comparison of cell proliferation inhibition rate in each group

The cell proliferation inhibition rate of the miR-708 overexpression group at the same point was significantly lower than that of the control group; the cell proliferation inhibition rate of the miR-708 inhibition group at the same point was significantly higher than that of the control group, and the difference was statistically significant ($P<0.05$) (Table 1).

Comparison of cell apoptosis rate in each group

The apoptosis rate of the miR-708 overexpression group was significantly lower than that of the control group; the apoptosis rate of the miR-708 inhibition group was significantly higher than that of the control group, and the difference was statistically significant ($P<0.05$) (Table 2).

Comparison of cell cycle ratio in each group

The proportion of cells in the miR-708 overexpression group in the G1 phase was significantly lower than that in the control group, and the proportion in the S phase was significantly higher than that in the control group; the ratio of cells in the miR-708 inhibition group in G1 phase was

Table 1. Comparison of cell proliferation inhibition rate in each group ($\bar{x}\pm s$).

Groups	24h	48h	72h
The control group	4.05±0.46	4.05±1.05	4.16±1.05
miR-708 overexpression group	3.15±0.46[a]	2.75±0.85[a]	2.34±0.16[a]
miR-708 inhibition group	9.46±0.85[ab]	13.26±3.16[ab]	15.16±1.24[ab]
F	610.063	166.782	1082.891
P	<0.001	<0.001	<0.001

Note: a means [a]P<0.05 compared with the control group, b means [b]P<0.05 compared with the miR-708 overexpression group.

Table 2. Comparison of cell apoptosis rate in each group ($\bar{x}\pm s$)

Groups	Apoptosis rate
The control group	3.46±1.25
miR-708overexpression group	2.67±0.85[a]
miR-708 inhibition group	20.16±1.38[ab]
F	1397.372
P	<0.001

Note: a means [a]P<0.05 compared with the control group, b means [b]P<0.05 compared with the miR-708 overexpression group.

Table 3. Comparison of cell cycle ratio in each group ($\bar{x}\pm s$).

Groups	G2/M	S	G1
The control group	16.96±3.12	34.52±10.64	48.52±2.87
miR-708 overexpression group	16.80±10.26	40.52±10.56[a]	42.68±3.48[a]
miR-708 inhibition group	16.52±5.68	28.16±5.16[ab]	55.32±3.46[ab]
F	0.022	9.122	74.303
P	0.985	<0.001	<0.001

Note: a means [a]P<0.05 compared with the control group, b means [b]P<0.05 compared with the miR-708 overexpression group.

significantly higher than that in the control group, and the ratio in S phase was significantly lower than that in the control group, the difference was statistically significant (P<0.05) (Table 3).

Comparison of apoptosis-related proteins in each group

The Bax protein of the cells in the miR-708 overexpression group was significantly lower than that of the control group, and the Bcl-2 protein was significantly higher than that of the control group; the Bax protein of the miR-708 inhibition group was significantly higher than that of the control group, and the Bcl-2 protein was significantly lower than that of the control group. The difference was statistically significant (P<0.05) (Table 4).

Comparison of cell migration ability in each group

The cell migration ability of the miR-708 overexpression group was significantly higher than that of the control group; the cell migration ability of the miR-708 inhibition group was significantly lower than that of the control group, and the difference was statistically significant (P<0.05) (Table 5).

Table 4. Comparison of apoptosis-related proteins in each group ($\bar{x}\pm s$).

Group	n	Bax	Bcl-2
Control	6	1.11±0.03	0.89±0.06
miR-708 high expression	6	0.63±0.04[a]	1.37±0.05[a]
miR-708 inhibition	6	1.38±0.05[ab]	0.72±0.02[ab]

Note: a means [a]P<0.05 compared with the control group, b means [b]P<0.05 compared with the miR-708 overexpression group.

Comparison of JAK3/STAT pathway proteins of cells in each group

The CNTFR protein of the miR-708 overexpression group was significantly lower than that of the control group, and the JAK3 and STAT3 proteins were significantly higher than that of the control group. The CNTFR protein of the miR-708 inhibition group was significantly higher than that of the control group, and the JAK3 and STAT3 proteins were significantly lower than that of the control group. The difference was statistically significant (P<0.05) (Table 6).

Verification of miR-708 and CNTFR binding site

The binding sites of miR-708 and CNTFR were predicted by TargetScan bioinformatics software, and it was found that there are two binding sites between miR-708 and CNTFR, located at 394-400bp and 497-503bp, respectively.

Discussion

White blood cells are a kind of malignant tumor that threaten people's lives and health, which are common in adolescents and children under the age of 18 (6). With the continuous development of immunology, molecular biology and genetics technology in recent years, the continuous improvement of clinical diagnosis and individualized treatment plans, the success rate of treatment for children with leukemia has shown an increasing trend year by year. However, 30% of leukemia children still relapse in the bone marrow and central nervous system, which not only seriously affects the life quality of the children, but also brings a heavy economic burden to the family and society (7,8).

miRNA is a small non-coding RNA, which can degrade the expression of target gene mRNA or inhibit the translation of mRNA by binding to the 3'UTR or 5'UTR end of its corresponding target gene, thereby realizing the regulation of target gene expression level (9). Previous studies have shown that miRNAs play an important role in the differentiation of hematopoietic stem cells into mature blood

Table 5. Comparison of cell migration ability in each group ($\bar{x}\pm s$).

Group	n	0h	24h	48h
Control	6	110.32±10.36	186.37±11.20	197.33±11.52
miR-708 high expression	6	113.05±15.31	231.45±10.41[a]	281.10±10.64[a]
miR-708 inhibition	6	109.35±12.35	143.39±11.74[ab]	173.40±12.81[ab]

Note: a means [a]P<0.05 compared with the control group, b means [b]P<0.05 compared with the miR-708 overexpression group.

Table 6. Comparison of JAK3/STAT pathway proteins of cells in each group ($\bar{x}\pm s$).

Group	n	CNTFR	JAK3	STAT3
Control	6	1.01±0.02	1.03±0.02	0.91±0.03
miR-708 high expression	6	0.72±0.01[a]	1.38±0.03[a]	1.29±0.04[a]
miR-708 inhibition	6	1.33±0.02[ab]	0.76±0.02[ab]	0.68±0.06[ab]

Note: a means [a]P<0.05 compared with the control group, b means [b]P<0.05 compared with the miR-708 overexpression group.

cells (10). miRNA has the ability to regulate the qualitative differentiation of hematopoietic stem cells, and there are different types of miRNA expression in different blood cells. miR-708 plays different roles in a variety of cancers (11). Li et al. (12) showed that ectopic expression of miR-708 prohibited cell proliferation and invasion in gastric cancer. Feng et al. (13) found that miR-708 can induce apoptosis of osteosarcoma cells and inhibit cell migration by targeting ZEB1. All of the above shows that miR-708 is involved in the occurrence and development of malignant tumors. Recent studies have found that miR-708 is significantly abnormally expressed in leukemia patients, so we speculate that miR-708 may be involved in the biological behavior of leukemia. In this experiment, the simultaneous point proliferation inhibition rate of the miR-708 overexpression group was significantly lower than that of the control group, while the miR-708 inhibition group was the opposite. The proportion of cells in the miR-708 overexpression group in the G1 phase was significantly lower than that in the control group, and the proportion in the S phase was significantly higher than that in the control group, miR-708 inhibition group is the opposite, suggesting that miR-708 can increase the proliferation ability of leukemia cells by regulating changes in the cell cycle of leukemia cells.

Apoptosis and migration are important biological behaviors of malignant tumor cells, which are jointly involved in the occurrence and development of leukemia. Bcl-2 is a common anti-apoptotic gene in the body, which can inhibit apoptosis and improve cell viability (14). Bax is a pro-apoptotic gene, the ratio of the two changes together to regulate the process of cell apoptosis (15). In this experiment, the apoptosis rate of the miR-708 overexpression group was significantly lower than that of the control group, while the miR-708 inhibition group was the opposite. The Bax protein of cells in the miR-708 overexpression group was significantly lower than that of the control group, and the Bcl-2 protein was significantly higher than that of the control group. The miR-708 inhibition group was the opposite. miR-708 can reduce the apoptosis of leukemia by regulating apoptosis-related proteins, which is similar to the results of Wang et al. (16). In this experiment, the cell migration ability of the miR-708 overexpression group was significantly higher than that of the control group, while the miR-708 inhibition group was the opposite, suggesting that miR-708 can enhance the migration ability of leukemia cells and promote the occurrence and development of leukemia.

CNTFR is a ganglion factor receptor, which can bind to Leukemia inhibitory factor (LIF) and regulate the JAK/STAT pathway to play an important role in the occurrence and development of leukemia (17). Activation of the JAK/STAT pathway can enhance the proliferation ability of cells, and inhibiting the expression of JAK3 can induce apoptosis of leukemia cells (18). In this experiment, the CNTFR protein of the miR-708 overexpression group was significantly lower than that of the control group, the JAK3 and STAT3 proteins was significantly higher than the control group, and the miR-708 inhibition group was the opposite. The binding sites of miR-708 and CNTFR were predicted by TargetScan bioinformatics software, and it was found that there are two binding sites between miR-708 and CNTFR, located at 394-400bp and 497-503bp, respectively, suggesting that the involvement of miR-708 in the biological behavior of leukemia cells may be related to the activation of JAK/STAT pathway related proteins by binding to CNTFR3'UTR.

In conclusion, miR-708 can reduce the expression of CNTFR by binding to the 3'UTR end of the target gene CNTFR, further activate the JAK/STAT pathway to induce cell proliferation, regulate apoptosis-related proteins, reduce cell apoptosis, and enhance the migration ability of leukemia cells.

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