Cellular and Molecular Biology

Original Research

Effect of FUT3 gene silencing with miRNA on proliferation, invasion and migration abilities of human KATO-III gastric cancer cell line

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Abstract: This study investigated the effects of FUT3 gene expression inhibition with miRNA on the proliferation, invasion and migration abilities of KATO-III cells. KATO-III cells were transfected with plasmid pcDNA™6.2-GW/EmGFP-FUT3-miR(FUT3-miRNA) and negative control plasmid in mediation of liposome, respectively, using untransfected cells as blank controls. Forty-eight hours after transfection, FUT3 mRNA levels were tested by RT-PCR. Levels of sLeA proteins were assayed by Western blot. The effects of FUT3-miRNA on the proliferation, invasion and migration of KATO-III cells were determined by CCK8 testing and Transwell assays, respectively. Results indicate that the transfection of FUT3-miRNA may down-regulate sLeA protein expression on the surface of KATO-III cells, and significantly inhibit cell proliferation (p<0.05). As compared to the negative and blank control groups, the number of invasion and migration cells in the FUT3-miRNA group decreased significantly (each p<0.05). Experimental results indicate that the miRNA expression vector which targets the FUT3 gene can effectively inhibit the proliferation, migration and invasion abilities of KATO-III cells.

Key words: FUT3 gene, miRNA, sLeA, gastric cancer, proliferation, invasion, migration.

Introduction

Gastric carcinoma is the most common malignant tumor to appear in the human digestive tract, and which demonstrates various methods of invasion and metastasis. Lewis antigen is a group of carbohydrate antigens which present in the glycan structure of glucolipid and glycoprotein on cell surface (1). As a member of the Lewis antigen family, high concentrations of sialyl Lewis A antigen (sLeA) has been reported on the surface of many tumor cells, and correlated to the proliferation, differentiation, adhesion and invasion of malignant tumors (2,3). There has also been significant evidence that sLeA may be associated with the metastatic potential of cancer cells in pancreatic and colon tumors significantly (1,4). In one research, sLeA was served as an important prognostic factor for disease recurrence or survival among patients with cancers (5).

The Lewis enzyme FUT3, which has α1,3 and α1,4-Fucosyltransferase activity, is the key enzyme involved in the synthesis of sLeA antigen. While increased levels of FUT3 have been detected in gastric tumors (6), FUT3 is identified as the target gene to investigate new gene therapy for gastric cancer patients.

This study employed FUT3 as a target gene to silence. The recombinant miRNA plasmid used in the experiment was previously constructed earlier in a laboratory (7). The inhibition of FUT3 expression by miRNA is hypothesized to alter the sLeA expression pattern on the cell membrane of gastric cancer cells and reduce the invasion and migration capacities of the cells. This study investigates whether FUT3-miRNA is able effectively to down-regulate the proliferation, invasion and migration abilities of KATO-III cells.

Materials and Methods

Design of FUT3 specific miRNA oligonucleotide and construction of FUT3-miRNA recombinant plasmid

The recombinant miRNA plasmid used in the experiment was constructed earlier in a laboratory. According to the FUT3 gene sequence (NM-000149), RNAi miRNA software was used to synthesize the corresponding Oligo DNA. Two sections of the target sequence were selected to investigate the target site with the best silencing effect; the two selected sequences were 5'-AACC-CATACAGTGAATCCATT-3'
(start with 596 base site), and 5'-AACTGCAGCAG-GAATCCAGGT-3'(start with 1660 base site). A pair of oligonucleotide sequences were then synthesized for the two respective sequences. The constructed oligonucleotide sequences are described below:
1) 596 top strand: 5'-TGCTg**AATGGATTCAC-TGTATGGGTT**gttttggccactgactgacAACCCATAG-TGAATCCATT-3';
596 bottom strand:5'-cctgAATGGATTCACTATG-GGTTGTCAGTCAGTGGCCAAAACAACCCA-TACAGTGAATCCATTC-3'.
2) 1660 top strand:
5'-TGCTg **ACCTGGATTCCTGCTGCAGTT**gttttg-
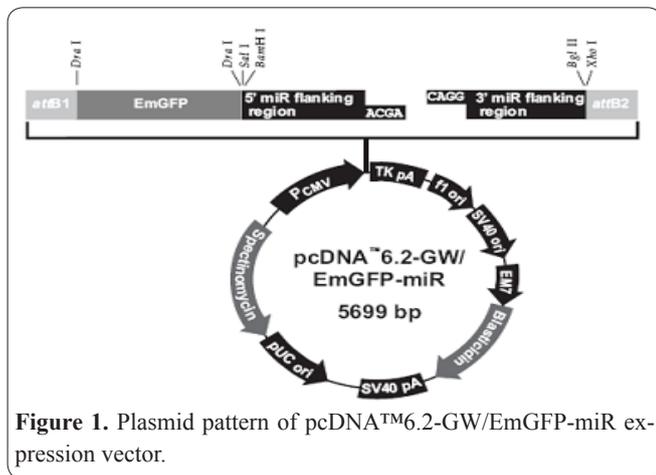
Figure 1. Plasmid pattern of pcDNA™6.2-GW/EmGFP-miR expression vector.

gccactgactgacAACTGCAGGGAATCCAGGT-3';
1660 bottom strand: 5'-cctgACCTGGATTCCCTG-CAGTTGTCAGTCAGTGGCCAAAACAACTG-CAGCAGGAATCCAGGTC-3'.

The annealed oligonucleotides were then cloned into the pcDNA™6.2-GW/EmGFP-miR expression vector (Invitrogen), as shown in Figure 1, according to manufacturer recommendations. After the FUT3-miRNA recombinant plasmid was successfully constructed, DNA sequencing was performed to verify the correct insertion of the target miRNA oligonucleotides into the vector without base mutations.

Cell culture and transient transfection of plasmids

KATO-III cells were obtained from ATCC. One day prior to transfection, 500,000 cells per well were plated in a 6-well plate with 80% confluence at the time of transfection. Cells were divided into three groups: the blank control (group 1), the negative control transfected with empty miRNA plasmids (group 2),and the FUT3-miRNA group transfected with FUT3-miRNA plasmids (group 3).

Cells were transfected with plasmids according to the FuGENE (Roche) HD transfection reagent instructions. Transfection was optimized by adjusting the ratio of liposome to plasmid vector , 4 μL:2μg, 6μL:2μg, 8 μL:2μg ,and 10 μL:2μg. Forty-eight hours after transfection, transfection efficiency was estimated by evaluating green fluorescent protein expression by flow cytometry.

RNA extraction and RT-PCR to detect relative expression level of FUT3-mRNA

Total RNA was isolated from cells 48 hours after tranfection using TRIzol (Invitrogen). RNA was reverse transcribed into cDNA using an RNA PCR Kit (AMV) Ver.3.0 (Takara) according to manufacturer instructions. Human FUT3 PCR primers are 5'-GCAAGGCT-TAGACCAGTTCG-3' and 5'-AAAGGCCATGTC-CATAGCAG-3'; human GAPDH primers are 5'-ATT-CAACGGCACAGTCAAGG-3' and 5'-CACCAGTG-GATGCAGGGAT-3'. Reactions were conducted on an ABI PRISM® 5700 sequence detection system (Applied Biosystem). The expression level of FUT3 mRNA was standardized by the reference gene GAPDH.

Western blot analysis for detection of sLeA protein expression in KATO-III cells

Forty-eight hours after transfection, cells were col-lected and lysed in 50 mM Tris ,1mM EDTA and 1% Triton X-100 buffer. sLeA was detected by running protein extracts (30μg/well) on 8% SDS-polyacryla-mide gels. Proteins were then blotted onto polyviny-lidene fluoride (PVDF) membranes, blocked with 5% skim milk at room temperature for two hours, and then incubated overnight with 1:500 mouse monoclonal anti-sLeA (KM231) (Chemicon) at 4°C. Proteins were then washed with TBST and incubated with anti-mouse-HRP antibodies, and membranes were developed with ECL Western Blotting Substrate. Results were analyzed and calculated by gray scanner. The relative expression of sLeA protein was represented by the ratio between the gray value of sLeA and that of GAPDH. Experiments were conducted in replicates of three for each group, and the average and mean values of each group were calculated.

Detection of KATO-III cell proliferation by CCK-8

According to the CCK-8 Kit (Beyotime) instruc-tions, cell suspensions (100μL/well) were incubated in a 96-well plate, with each well containing serum-free medium 100 μL, and cultured for 24 hours. After 24 hours, the cells were divided into their three respective groups, as described above, and transfected accordingly. Cell activity was measured at 24, 48, 72, 96, and 120 hours, respectively. Results at each time point were verified with three duplicated wells. Then, 10μL CCK-8 solution was added to each well. After incubation at 37°C for 2 hours, the optical density (D) of each well was measured at 450 nm to determine the cell growth curve. Each experiment was repeated three times.

Transwell detection of tumor cell invasion and migration

Forty-eight hours after transfection, transwell cham-bers (Millipore) were placed into a 24-pore culture plate, and a micropore layer of 8 μm diameter was placed in the bottom of the chamber. After a 1:4 dilution with pre-cooled RPMI-1640 culture solution, 100μL of diluted Matrigel gel was added to the upper Transwell cham-ber and then incubated for 2 hours at 37°C to induce gel formation. Three groups of cells in the logarithmic growth phase were digested by trypsin and re-sus-pended in serum-free culture medium with a density of 5×10^5 /mL. Then, 200 μL of cell suspension was added to the chamber of the basement membrane, while 600 μL RPMI 1640 containing 10% FBS was added into the lower chamber as a chemotactic factor; each condition was replicated three times.

After being cultured at 37°C under 5% CO_2 for 24 hours, the chamber was removed, the liquid was separa-ted, and cells were gently wiped off the basement mem-brane of the small chamber with cotton swabs dipped in PBS. The cells then were fixed in 4% formaldehyde for 20 minutes, and stained for 15 minutes with crystal violet. Under SEM (×200), five fields of view were ran-domly selected to conduct the cell count, and averages were calculated. Each experiment was replicated three times.

The migration experimental method was identical to that of the invasion test, with the exception of the Matrigel on the inner surface of the bottom membrane of the Transwell chamber. Cells were cultured at 37°C

under 5% CO_2 for 24 hours, and the migration ability of the tumor cells was then determined by counting the number of cells present in the membrane.

Statistical analysis

Statistical software SPSS was employed for statistical analysis. Experimental data is expressed by mean±SD, and comparisons between groups are made according to one factor analysis of variance; a p-value of $p<0.05$ indicates statistical significance.

Results

DNA sequencing of recombinant FUT3-miRNA

Sequencing results demonstrated that the target miRNA oligonucleotides were correctly inserted into the vector, without insertion, deletion or mutation, thus proving the successful construction of the FUT3-miRNA expression plasmid as shown in Figure 2.

Transfection efficiency determined by flow cytometry

Cells were collected 48 hours after transfection in order to determine transfection efficiency by flow cytometry. Results indicate high transfection efficiency and low liposome toxicity when liposomes and plasmid are expressed in a ratio of 8μL:2μg. Transfection efficiency was 53.83% (Figure 3B), while that of the negative control was equal to 0.34% (Figure 3A). When the amount of liposomes exceeded the optimal ratio, the rate of transfection did not significantly increase.

Effect of miRNA recombinant plasmid on FUT3 gene expression in mRNA level

RT-PCR was performed 48 hours after tranfection to evaluate mRNA levels representing FUT3 gene expression in the three target groups. Results were analyzed using relative expression levels of FUT3 mRNA as compared to GAPDH. Results indicate that FUT3 gene expression in FUT3-miRNA group was reduced (0.31 ± 0.04, p<0.05) to a greater extent than that determined in the blank control group (0.92 ± 0.03) and the negative control group (0.99 ± 0.04). There was no statistical significance observed between the FUT3 gene expression in the negative control and blank control groups (p>0.05), as shown in Figure 4.
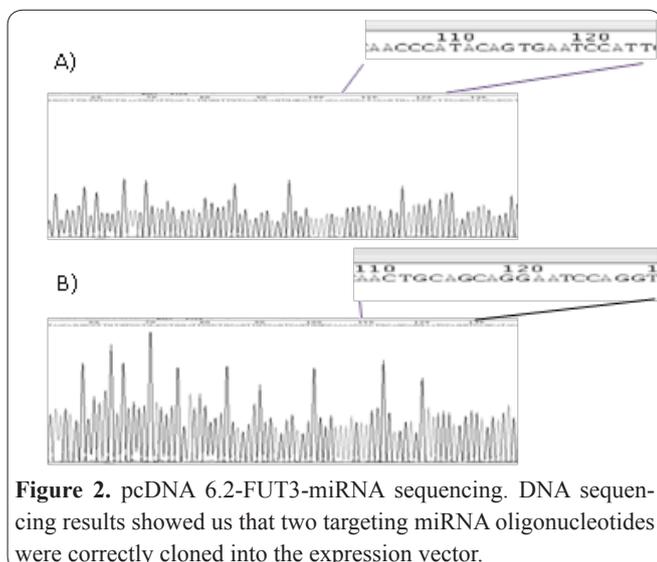
Figure 2. pcDNA 6.2-FUT3-miRNA sequencing. DNA sequencing results showed us that two targeting miRNA oligonucleotides were correctly cloned into the expression vector.
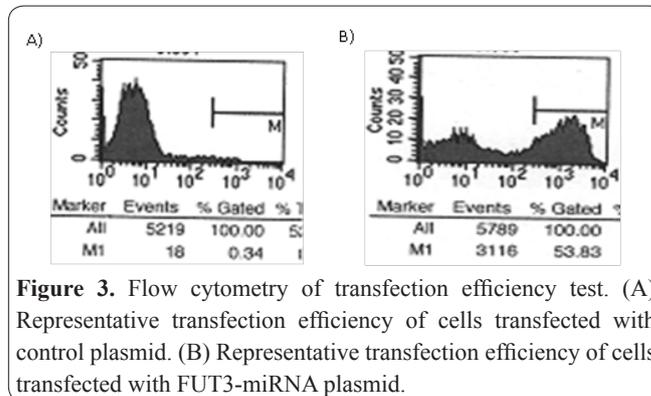
Figure 3. Flow cytometry of transfection efficiency test. (A) Representative transfection efficiency of cells transfected with control plasmid. (B) Representative transfection efficiency of cells transfected with FUT3-miRNA plasmid.

Effect of miRNA recombinant plasmid on sLeA expression of KATO-III cells

Western blot detected the expression of sLeA antigens on the surface of KATO-III cells after transfection. Western blot results demonstrated that the relative expression of sLeA in the FUT3-miRNA group was significantly lower (0.36 ± 0.03) than that of the other two groups. The difference was statistically significant (p<0.05) as compared to the blank control group (0.71 ± 0.01) and negative control group (0.79 ± 0.05), while no significant difference was observed between the negative control and blank control groups (p>0.05), as shown in Figure 5. The results confirmed that FUT3 gene silencing can reduce sLeA synthesis on KATO-III cells.

Inhibition of FUT3-miRNA on KATO-III cell proliferation after transfection

Determination of CCK-8 levels indicated that the
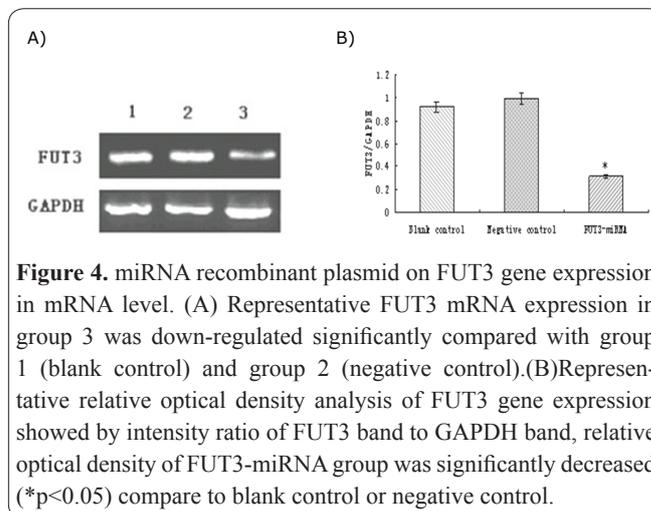
Figure 4. miRNA recombinant plasmid on FUT3 gene expression in mRNA level. (A) Representative FUT3 mRNA expression in group 3 was down-regulated significantly compared with group 1 (blank control) and group 2 (negative control).(B)Representative relative optical density analysis of FUT3 gene expression showed by intensity ratio of FUT3 band to GAPDH band, relative optical density of FUT3-miRNA group was significantly decreased (*p<0.05) compare to blank control or negative control.
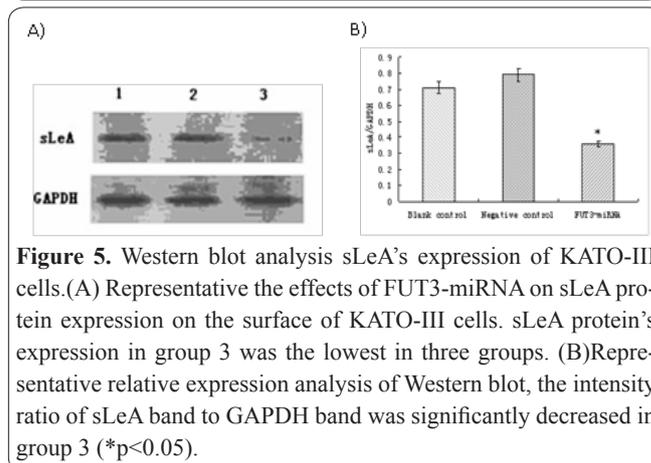
Figure 5. Western blot analysis sLeA's expression of KATO-III cells.(A) Representative the effects of FUT3-miRNA on sLeA protein expression on the surface of KATO-III cells. sLeA protein's expression in group 3 was the lowest in three groups. (B)Representative relative expression analysis of Western blot, the intensity ratio of sLeA band to GAPDH band was significantly decreased in group 3 (*p<0.05).

FUT3-miRNA transfection group demonstrated significant inhibition of cell proliferation at four time points: 0.44 ± 0.02 at 48 hours, 0.55 ± 0.01 at 72 hours, 0.70 ± 0.06 at 96 hours, and 0.89 ± 0.03 at 120 hours ($p<0.05$). KATO-III cell proliferation demonstrated no significant differences at various time points in the blank or negative control groups ($p>0.05$). Results indicate that the down-regulation of FUT3 can effectively inhibit proliferation of KATO-III cells, as shown in Figure 6.

Inhibition of FUT3-miRNA in the invasion and migration of KATO-III cells

Transwell cell invasion tests indicated that the wear membrane cell number in the FUT3-miRNA transfection group (34.79 ± 3.20) was dramatically lower than that of the blank control (71.41 ± 4.10) and negative control (67.85 ± 3.12) groups 48 hours after transfection. The difference was statistically significant ($p<0.05$), whereas no statistical difference was observed between the blank and negative control groups ($p>0.05$), as shown in Figure 7A. Results indicate that FUT3-miRNA recombinant plasmid can effectively inhibit the invasion of KATO-III cells.

Cell migration assay demonstrated that the number of trans-membrane cells observed in the FUT3-miRNA transfection group was 63.12 ± 1.27, which was significantly less than that of the blank control (101.10 ± 2.2) and negative control (100.30 ± 1.9) groups ($p<0.05$), as shown in Figure 7B. Results indicate that the FUT3-miRNA recombinant plasmid can effectively inhibit the migration of KATO-III cells *in vitro*.

Discussion

This study investigated the effect of FUT3 on sLeA expression in gastric cancer cells through their inhibition by miRNA. With the development of molecular biology, molecular genetics, molecular immunology and transgenic technique disciplines, many genes have been determined to be closely related to tumorigenesis, as well as the development and prognosis of tumors. Of these, the fucosylation transfer enzyme gene and its synthetic product has received increasing research attention in
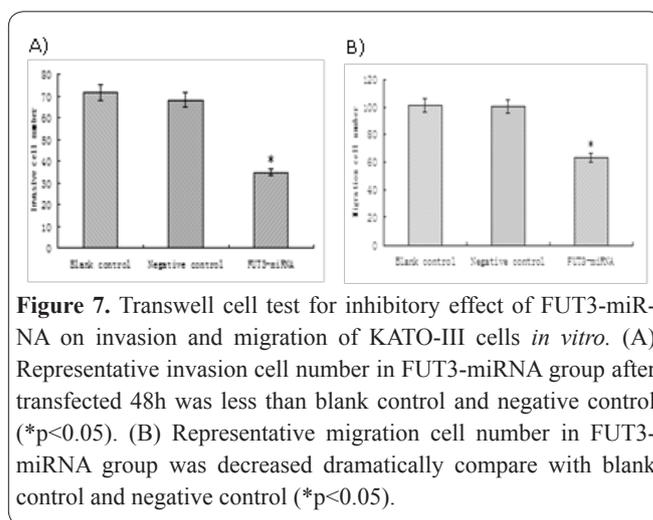
Figure 7. Transwell cell test for inhibitory effect of FUT3-miRNA on invasion and migration of KATO-III cells *in vitro*. (A) Representative invasion cell number in FUT3-miRNA group after transfected 48h was less than blank control and negative control (*$p<0.05$). (B) Representative migration cell number in FUT3-miRNA group was decreased dramatically compare with blank control and negative control (*$p<0.05$).

recent years. Previous research has identified sLeA as a tumor-associated antigen which is highly expressed in many malignant tumor cells and has an important effect on the tumor cell adhesion and recognition processes; it has also been correlated to metastasis and poor survival of cancer patients (8-11). Other research has proved greater frequencies of sLeA expression on the surface of gastric cancer cells in patients with lymphatic invasion and lymph node metastasis (12). Additionally, increased expression of sLeA carbohydrates have been correlated to clinically advanced stages and metastasis of gastric and colon cancers (13). Recent studies have provided further evidence that sLeA is frequently implicated in lymphohematogenous metastasis, while its decreasing expression could reduce cancer cells spreading to blood vessels, thus inhibiting metastasis (14, 15) .

Gene expression of the fucosylation transfer enzyme (FUTs) is key to the regulation of fucosylated oligosaccharide synthesis.Deta representing in several gastrointestinal cell lines that FUT3 and FUT5 play essential role in the synthesis and regulation of sialyl Lewis antigens (16, 17). Glycosyltransferase expression analyses have identified FUT3 as the key enzyme on sLeA biosynthesis by transfecting sLeA-deficient intestinal epithelial cells (IECs) with FUT3 (18). Based on FUT3 gene regulation in the biosynthesis of Lewis blood group antigens, this may prove a beneficial molecular target due to its creation of a base for the design of cell-specific therapies. Since increased levels of FUT3 have been widely detected in gastric tumors, researchers have employed antisense nucleic acid targeting of the FUT3 gene to block expression of the $\alpha1,3/4$-fucosyltransferase gene, which in turn down-regulates sLeA expression and could thus affect the proliferation, adhesion, and migration of tumor cells (19, 20). There have also been numerous reports indicating that FUT3 knockdown using RNA interference (RNAi) technology,such as shRNA and siRNA, could induce a decrease in sialyl Lewis antigen in tumor cells and thus prevent cell adhesion to E-selection (21,22). It is proposed that FUT3 could be used as a key gene for reducing the migration and metastatic potential of tumor cells.

Artificial miRNA was constructed by using natural miRNA as a basic skeleton; its pre-miRNA fragment was synthesized *in vitro* and contained a specific stem loop structure. When transfected into the cell, it could

Figure 6. CCK-8 test for inhibitory effect of FUT3-miRNA on cell proliferation. Representative KATO-III cells proliferation was detected at 24, 48, 72, 96, and 120 hours after transfection with recombinant plasmid. Cell proliferation curve showed that FUT3-miRNA group had significant decreased compare to the other two groups in 48,72,96 and 120hours after transfection.

then imitate the formation of endogenous miRNA and its effect on the degradation of target gene processes. The effective amount of miRNA with the silencing sequence was far less than that of traditional siRNA while its silence effects seem exacerbated. Based on these advantages, miRNA has become an important research method and experimental tool in the RNA interference field (23).

Combined with the miRNA research platform previously developed in the authors' laboratory, FUT3-miRNA is a promising conjunctive therapy aimed at the interruption of tumor cell proliferation, invasion, and migration, among other abilities. This study analyzed the implications of FUT3 in the synthesis of sLeA antigens in KATO-III cells by the down-regulation of sLeA achieved by miRNA recombinant plasmid. The target FUT3-miRNA plasmid was transfected into human gastric cancer KATO-III cells. Results demonstrate that the FUT3-miRNA plasmid is able to down-regulate expression of the fucosylation transfer enzyme FUT3, and thus can significantly reduce sLeA synthesis on the surface of KATO-III cells. Experimental results also indicate that FUT3-miRNA could significantly inhibit the growth and proliferation of KATO-III gastric cancer cells, and effectively inhibit their invasion and migration abilities. Therefore, FUT3-miRNA could be used as a new gene therapy for gastric cancer primarily due to its effect on the biosynthesis of sLeA. Further experiments are necessary to prove and further discuss the related mechanism.

Acknowledgements

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