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The effect of thalidomide on the invasive ability of gastric cancer cells by regulating miR-524-5p/FSTL1

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ABSTRACT

This study aimed to investigate the effect of thalidomide (Thal) regulating microRNA (miR)-524-5p/follistatin-like protein 1 (FSTL1) on the invasion ability of gastric cancer cells. For this purpose, real-time fluorescent quantitative PCR (RT-qPCR) was used to detect the level of miR-524-5p in GES1, SUN-16, MGC-803, SGC-7901, MKN-28, and MKN-45 cells. Then the MGC-803 and MKN-45 cells would proceed to the next research. The MGC-803 and MKN-45 cells were cultured in vitro and added Thal to the final concentration of (6.25, 12.5, 25, 50, 100) μg/mL. The blank control group only added 0.1% dimethyl sulfoxide (DMSO) culture medium, and cultured for 48 hours. CCK8 was used to detect cell proliferation, and Transwell was used to detect cell invasion. The experiment was divided into a blank control group, Thal group (25 μg/mL Thal-treated cells), Thal+inhibitor NC group, and Thal+miR-524-5p inhibitor group (transfected with inhibitor NC and miR-524-5p inhibitor respectively on the basis of Thal group), cultivated for 48 h. The level of miR-524-5p in the cells was detected by RT-qPCR; the cell invasion was detected by Transwell; the expression of matrix metalloproteinase (MMP)-2, MMP-9, FSTL1 protein in the cells was detected by Western blot. The targeting relationship between miR-524-5p and FSTL1 was verified by dual luciferase. Results showed that compared with GES1 cells, the level of miR-524-5p in SUN-16, MGC-803, SGC-7901, MKN-28, and MKN-45 cells decreased ($P<0.05$). In MGC-803 and MKN-45 cells, compared with the blank control group, the cell proliferation rate and the number of invasions in the (50, 100) μg/mL Thal treatment groups, and the number of invasions in (6.25, 12.5, 25) μg/mL Thal treatment groups decreased ($P<0.05$). Compared with the blank control group, the level of miR-524-5p in the cells of the Thal group, Thal+inhibitor NC group, and Thal+miR-524-5p inhibitor group increased ($P<0.05$), the number of invasions, the levels of MMP-2, MMP-9 and FSTL1 proteins in cells decreased ($P<0.05$); compared with the Thal group and the Thal+inhibitor NC group, the level of miR-524-5p in the cells of the Thal+miR-524-5p inhibitor group decreased ($P<0.05$), the number of invasions, the levels of MMP-2, MMP-9, and FSTL1 proteins in the cells increased ($P<0.05$). Dual luciferase verification revealed that there was a targeting relationship between miR-524-5p and FSTL1. In conclusion, that can up-regulate the expression of miR-524-5p to reduce the expression of FSTL1 protein, inhibit the invasion of gastric cancer cells, and achieve alleviation of the disease.

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Introduction

Gastric cancer is the second leading cause of death in cancer patients, and about half of the patients come from China, which is seriously dangerous (1). Most gastric cancers have reached the advanced stage at diagnosis, and seeking safer and more effective drug treatments has always been a research hotspot. Thalidomide (Thal) has obvious anti-angiogenic effects with anti-tumor potential (2), which can inhibit the proliferation and invasion of cancer cells, promote apoptosis and thereby treat the disease (3). MicroRNAs (miRNA) are abnormally expressed during tumor occurrence and progression, which participates in the disease process. Where, miR-524-5p has reduced expression in malignant tumor cells, which can target and regulate gene expression to affect its function (4). Follistatin-like protein 1 (FSTL1), as a downstream target gene of miR-524-5p, can regulate breast cancer cell proliferation, differentiation, invasion, and apoptosis (5). This study took gastric cancer cell lines as the research object, observed cell proliferation and invasion after Thal treatment, and added miR-524-5p inhibitor on this basis to explore the mechanism by which Thal affects cell invasion.

Materials and Methods

Cell

Normal gastric epithelial mucosal cells GES1 were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences; gastric cancer cell lines SUN-16, MGC-803, SGC-7901, MKN-28, and MKN-45 were purchased from American Type Culture Collection.

Main reagents and instruments

The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.; inhibitor NC, miR-524-5p inhibitor, mimic NC, miR-524-5p mimic, and Lipofectamine 2000 were all purchased from Guangzhou RiboBioCo.,

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Ltd.; CCK8 kit, dual luciferase reporter gene detection kit, primary antibody matrix metalloproteinase (MMP)-2, MMP-9, FSTL1 (Abcam, UK, article numbers: ab228554, ab228530, ab92536, ab228402, ab223287); miRNA extraction reagent kit, miRNA cDNA first-strand synthesis kit (Thermo Fisher Technology Co., Ltd., article numbers: 740981, K1621). Real-time quantitative PCR (RT-qPCR) instrument, protein gel imager (Thermo Fisher Technology Co., Ltd., models: 7500, E-Gel Imager).

RT-qPCR detection of miR-524-5p level

GES1, SUN-16, MGC-803, SGC-7901, MKN-28, and MKN-45 cells were seeded into a 6-well plate, and cultured to logarithmic phase, with RNA extracted using miRNA extraction kit. miRNA cDNA first strand was reverse transcribed into cDNA first strand using a synthesis kit. miR-524-5p and U6 were amplified by RT-qPCR instrument. miR-524-5p forward primer (F): 5'-CTACAAAGGGAAGCACTTTTCTCAA-3', reverse primer (R): 5'-GTTGCCGTTACATCAGA-3'; U6-F: 5'-GTTGCCGTTACATCAGA-3', U6-R: 5'-TCCTAGTACCACTTGTTCTA-3'. 20 μL reaction system: 2×SYBR *Premix Ex Taq™* II Probe QPCR Mix 10 μL, ddH_2O 8.0 μL, cDNA 1 μL (50 ng/μL), F/R (10 μM) 0.5 μL each. Reaction conditions: 95°C, 5 min; 94°C, 15 s, 59°C, 30 s, 40 cycles. Relative quantitative analysis of miR-524-5p was performed by $2^{-\Delta\Delta CT}$ method.

Effect of Thal treatment at different concentrations on cell proliferation and invasion

Cell culture and drug treatment

MGC-803 and MKN-45 cells were used for the subsequent study. The cells grew to the logarithmic phase. The cells were seeded at a density of 4×10^4 cells/mL and then treated using [Thal with dimethyl sulfoxide (DMSO) as a solvent] at final concentrations of (6.25, 12.5, 25, 50, 100) μg/mL. The blank control group (0 μg/mL Thal) was only added with 0.1% DMSO medium in replace of the drug to act on the cells. The cells were cultured at 37℃, 5% CO_2 incubator for 48 h before testing.

CCK8 detection of cell proliferation

The cells were placed in a 96-well plate with 100 μL per well. After the cell treatment, add 10 μL of CCK8 and further place them in a 37℃, 5% CO_2 incubator for 2 h culture. The microplate reader was used to detect the cell optic density (optic density, OD) of each well at 490 nm. Cell proliferation rate = $OD_{\text{experimental group}}/OD_{\text{control group}} \times 100\%$.

Transwell detection of cell invasion

Process the cells according to the previous stage, and dilute the fetal-free bovine serum culture medium into a single cell suspension of 1×10^4 cells/mL. The Transwell chamber was coated with Matrigel and placed in a 24-well plate. Add 500 μL complete culture solution to the lower layer of the chamber. Add 500 μL single cell suspension to the upper layer and place it in a 37°C, 5% CO_2 incubator for 48 h culture. After staining with crystal violet, take a photo with a microscope and count. The top, bottom, left, right, and middle 5 fields of view were counted in each well. The number of cell invasion is the total number of cell penetration.

The effect of interference with miR-524-5p on cell invasion and FSTL1 protein expression

Experimental Grouping

In the experiment, MGC-803 and MKN-45 cells were divided into a blank control group, Thal group, Thal+inhibitor NC group, and Thal+miR-524-5p inhibitor group. Thal group 25 μg/mL cells were treated byThal; Thal+inhibitor NC group and Thal+miR-524-5p inhibitor group were transfected with inhibitor NC and miR-524-5p inhibitor on the basis of Thal group (Lipofectamine 2000 transfection reagent was used for transfection of inhibitor NC, miR-524-5p inhibitor for 6 h); the blank control group was only added with 0.1% DMSO culture solution in replace of drugs to act on the cells, and the cells were cultured in a 37°C, 5% CO_2 incubator for 48 h before testing.

RT-qPCR detection of miR-524-5p levels in cells

The cells in previous stage were placed in a 6-well plate and cultured for 48 h. Refer to step 1.2 to detect the miR-524-5p level in the cells.

Transwell detection of cell invasion

Treat the cells to detect cell invasion.

Western blot detection of MMP-2, MMP-9, FSTL1 protein expression in cells

The cells were placed in a 6-well plate and cultured for 48 h. Remove the culture medium, add 200 μL protein lysate to lyse for 10 min, gather the mixture into a centrifuge tube, centrifuge at 12000 r/min at 4°C for 20 min, and the supernatant is the total protein. Perform protein separation by gel electrophoresis, transfer PVDF membrane; seal in 5% skimmed milk powder at room temperature; correspondingly add primary antibodies MMP-2, MMP-9, FSTL1, and incubate overnight at 4°C; add secondary antibody and incubate at room temperature for 1 h. Avoid light in color development, and take pictures with a protein gel imager for quantitative analysis.

Dual luciferase verification of the targeting relationship between miR-524-5p and FSTL1

There are binding sites between the 3'UTR of FSTL1 mRNA and miR-524-5p. Design and synthesize FSTL1 wild-type (FSTL1 WT) and mutant type (FSTL1 MUT), which were co-transfected with miR-524-5p mimic or mimic NC into MGC-803 and MKN-45 cells. A dual luciferase reporter gene detection kit was used to detect the luciferase activity of each group.

Statistical analysis

SPSS 25.00 was used for statistical analysis. The measurement data were all expressed by the mean ± standard deviation ($\bar{x}\pm s$), comparison between multiple groups was performed by one-way analysis of variance, and pairwise comparison between groups was performed by *SNK-q* method. $P<0.05$ indicates a statistically significant difference.

Results

The expression level of miR-524-5p in different gastric cancer cell lines

Compared with GES1 cells, SUN-16, MGC-803, SGC-7901, MKN-28, and MKN-45 cells have decreased levels

of miR-524-5p (P<0.05), as shown in Table 1. MGC-803 and MKN-45 cells were later studied.

The effect of different concentrations of Thal on cell proliferation

Compared with the blank control group (0 μg/mLThal), (50, 100) μg/mLThal treatment group has a reduced cell proliferation rate (P<0.05), as shown in Table 2.

The effect of different concentrations of Thal on cell invasion

Compared with the blank control group (0 μg/mL Thal), (6.25, 12.5, 25, 50, 100) μg/mL Thal treatment groups have decreased the number of cell invasions (P<0.05), as shown in Table 3.

The effect of interference with miR-524-5p on the level of miR-524-5p in cells

In MGC-803 and MKN-45 cells, compared with the blank control group, Thal group, Thal+inhibitor NC group and Thal+miR-524-5p inhibitor group have increased miR-524-5p levels in the cells (P<0.05); Compared with the Thal group and the Thal+inhibitor NC group, Thal+miR-524-5p inhibitor group has decreased levels of miR-524-5p in the cells (P<0.05), as shown in Table 4.

Table 1. Comparison of miR-524-5p levels in 6 cell lines (n=6, $\bar{x}\pm s$).

Cell lines	miR-524-5p
GES1	1.01±0.12
SUN-16	0.62±0.07#
MGC-803	0.28±0.03#
SGC-7901	0.57±0.04#
MKN-28	0.38±0.04#
MKN-45	0.72±0.05#
F	92.998
P	0.000

Note: GES1 cell line *vs* #P<0.05.

Table 2. Comparison of the proliferation rate of MGC-803 and MKN-45 cells treated with different concentrations of Thal (n=6, $\bar{x}\pm s$ %).

Thal (μg/mL)	Cell proliferation rate	
	MGC-803	MKN-45
0	99.45±3.48	100.03±3.26
6.25	99.18±2.46	98.45±1.96
12.5	97.86±3.48	96.46±2.26
25	95.81±3.89	94.58±3.45
50	89.45±6.15#	90.15±4.16#
100	85.14±5.17#	86.34±2.46#
F	11.297	17.775
P	0.000	0.000

Note: 0 μg/mL *vs* #P<0.05.

Table 3. Comparison of the number of invasions of MGC-803 and MKN-45 cells treated with different concentrations of Thal (n=6, $\bar{x}\pm s$).

Thal (μg/mL)	Number of invasion cells	
	MGC-803	MKN-45
0	694.15±34.16	306.18±47.85
6.25	568.45±46.83#	216.45±33.42#
12.5	394.34±86.15#	145.18±27.87#
25	285.19±56.85#	103.19±42.16#
50	145.16±26.18#	35.15±6.15#
100	86.15±16.23#	9.15±4.24#
F	136.182	75.220
P	0.000	0.000

Note: 0 μg/mL *vs* #P<0.05.

Table 4. Comparison of miR-524-5p levels in cells after interference with miR-524-5p (n=6, $\bar{x}\pm s$).

Group	miR-524-5p	
	MGC-803	MKN-45
Blank control group	1.01±0.13	0.99±0.08
Thal group	1.75±0.15#	2.21±0.23#
Thal+inhibitor NC group	1.76±0.22#	2.18±0.19#
Thal+miR-524-5p inhibitor group	1.44±0.13#*&	1.58±0.16#*&
F	28.531	66.288
P	0.000	0.000

Note: Blank control group *vs* #P<0.05; Thal group *vs* *P<0.05; Thal+inhibitor NC group *vs* &P<0.05.

The effect of interference with miR-524-5p on cell invasion

In MGC-803 and MKN-45 cells, compared with the blank control group, Thal group, Thal+inhibitor NC group and Thal+miR-524-5p inhibitor group have decreased number of cell invasions (P<0.05); Compared with Thal-group and Thal+inhibitor NC group, Thal+miR-524-5p inhibitor group has increased number of cell invasions (P<0.05), as shown in Figure 1 and Table 5.

The effect of interference with miR-524-5p on MMP-2, MMP-9 and FSTL1 proteins in cells

In MGC-803 and MKN-45 cells, compared with the blank control group, the Thal group, Thal+inhibitor NC group and Thal+miR-524-5p inhibitor group have decreased MMP-2, MMP-9 and FSTL1 protein levels in the cells (P<0.05); Compared with Thal group and Thal+inhibitor NC group, Thal+miR-524-5p inhibitor

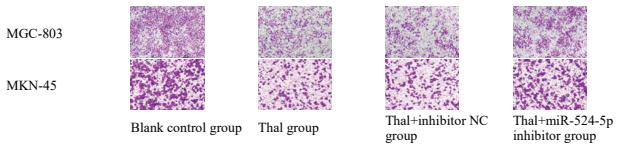

Figure 1. Cell invasion after interference with miR-524-5p (200×).

Table 5. The effect of interference with miR-524-5p on the number of cell invasion (n=6, $\bar{x}\pm s$).

Group	Number of invasion cells	
	MGC-803	MKN-45
Blank control group	648.19±41.36	245.59±31.52
Thal group	243.86±34.19#	106.16±26.15#
Thal+inhibitor NC group	273.49±48.47#	109.75±23.56#
Thal+miR-524-5p inhibitor group	364.49±45.35#*&	165.19±33.29#*&
F	112.024	30.490
P	0.000	0.000

Note: Blank control group *vs* #P<0.05; Thal group *vs* *P<0.05; Thal+inhibitor NC group *vs* &P<0.05.

Table 6. Comparison of the protein levels of MMP-2, MMP-9 and FSTL1 in cells after interference with miR-524-5p (n=6, $\bar{x}\pm s$).

Group	MGC-803			MKN-45		
	MMP-2	MMP-9	FSTL1	MMP-2	MMP-9	FSTL1
Blank control group	1.23±0.12	0.96±0.08	0.96±0.09	1.03±0.04	1.57±0.21	0.86±0.08
Thal group	0.23±0.03#	0.49±0.05#	0.13±0.02#	0.11±0.02#	0.38±0.04#	0.13±0.02#
Thal+inhibitor NC group	0.24±0.02#	0.52±0.06#	0.15±0.03#	0.12±0.02#	0.42±0.05#	0.14±0.02#
Thal+miR-524-5p inhibitor group	0.86±0.08#*&	0.72±0.08#*&	0.56±0.06#*&	0.58±0.06#*&	1.03±0.11#*&	0.61±0.05#*&
F	262.299	59.884	285.908	769.867	126.912	322.722
P	0.000	0.000	0.000	0.000	0.000	0.000

Note: Blank control group *vs* #P<0.05; Thal group *vs* *P<0.05; Thal+inhibitor NC group *vs* &P<0.05.

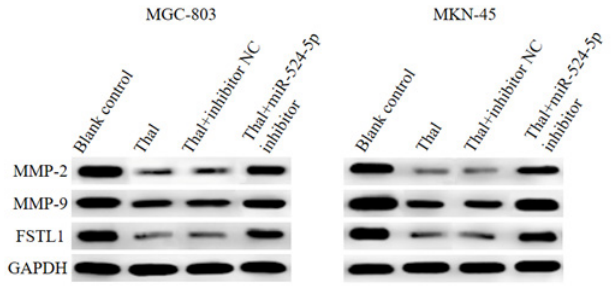

Figure 2. The effect of interference miR-524-5p on MMP-2, MMP-9 and FSTL1 proteins expression in cells.).

group has increased MMP-2, MMP-9 and FSTL1 protein levels in the cells (P<0.05), as shown in Figure 2 and Table 6.

Dual luciferase verification of the targeting relationship between miR-524-5p and FSTL1

There is a target site between miR-524-5p and FSTL1, as shown in Figure 3. The results of dual luciferase detection show that in MGC-803 and MKN-45 cells, compared with mimic NC+FSTL1 WT, miR-524-5p mimic+FSTL1 WT co-transfected cells have decreased relative luciferase activity (P<0.05), while mimic NC+FSTL1 MUT and miR-524-5p mimic+FSTL1 MUT co-transfected cells show no statistically significant difference in relative luciferase activity (P>0.05), as shown in Table 7. It proves that there is a miR-524-5p specific binding site on the FSTL1 sequence.

Discussion

Gastric cancer has rapid clinical development. Considering that Chinese patients have reached the advanced stage at diagnosis with a low 5-year survival rate, we are gradually developing targeted treatment for gastric cancer in line with the Chinese population. The new comprehensive treatment system for gastric cancer and research and development of new drugs stake a key position in this period (6). Thal was first used to treat morning sickness in pregnant women but was subsequently banned after it was found to be severely teratogenic (7). As the new use of old drugs has gradually become a hot topic in pharmaceutical research, studies have found that Thal can resist tumor angiogenesis, inhibit immunity, promote cell apoptosis, and inhibit cell invasion (8). In gastric cancer, studies have found that patients with Thal have good lymph node metastasis and tolerable toxic side effects (9). MMP-2 and MMP-9 proteins are both important regulatory factors for invasion. Cancer cell with enhanced invasion has elevated protein expression levels (10). This study found that Thal can inhibit cell invasion and protein expression in invasion proteins MMP-2 and MMP-9, and when it reaches a certain concentration, it can inhibit cell proliferation and protect against diseases, but the mechanism of action demands further study.

The abnormal expression of miRNA in tumors affects the occurrence and development of tumors. As a potential tumor suppressor, miR-524-5p is gradually regarded as a therapeutic target, which can affect the therapeutic effect and prognosis (11,12). miR-524-5p has decreased expression in gastric cancer cells. The increase of miR-524-5p reduces the cell proliferation potential, which can promote cell apoptosis and induce cell cycle arrest in G0/G1 phase (13). An increase of miR-524 -5p can inhibit the proliferation and invasion of gastric cancer cells, which may act as tumor suppressor genes in gastric cancer, and can be used as a biomarker and therapeutic target for gastric cancer (14). In this study, miR-524-5p was low expressed in gastric cancer cell lines SUN-16, MGC-803, SGC-7901, MKN-28, and MKN-45, which is similar to the findings of Zhu et al. (13), Liu et al. (14), suggesting that miR-524-5p plays a role in inhibiting tumor cell growth. After Thal treatment of MGC-803 and MKN-45 cells, the expression level of miR-524-5p in the cells increased, and following the addition of miR-524-5p inhibitor on the basis of Thal, it was found that the expression level of miR-524-5p decreased and the cell invasion ability was enhanced, sug-

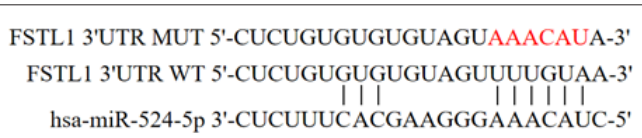

Figure 3. The targeting relationship between miR-524-5p and FSTL1.

Table 7. Dual luciferase verification of the targeting relationship between miR-524-5p and FSTL1 (n=6, $\bar{x}\pm s$).

Group	Relative luciferase activity	
	MGC-803	MKN-45
mimic NC+FSTL1 WT	1.01±0.12	0.98±0.08
miR-524-5p mimic+FSTL1 WT	0.35±0.03#	0.46±0.04#
mimic NC+FSTL1 MUT	0.99±0.07	1.00±0.08
miR-524-5p mimic+FSTL1 MUT	0.98±0.08	1.01±0.05
F	93.496	102.473
P	0.000	0.000

Note: mimic NC+FSTL1 WT group *vs* #P<0.05.

gesting that Thal can alleviate the invasion of gastric cancer cells by up-regulating the expression of miR-524-5p.

FSTL1, also known as follistatin-related protein or transforming growth factor β1 induced protein 36, is a member of the extracellular matrix protein family and is a glycoprotein with a variety of biological regulatory functions (15). By inhibiting the expression of FSTL1 in myeloma, it is possible to inhibit the proliferation, apoptosis, migration and invasion of osteosarcoma cells U2OS and Saos2 (16). As a direct target gene of miR-1252 in thyroid cancer, it can regulate the miR-1252 level and affect the expression of FSTL1, thereby affecting processes of cell viability, proliferation, apoptosis, and glucose metabolism (17). FSTL1 expression in cancer has a close relation to tumor size, pathological stage, differentiation degree, and lymph node metastasis. By inhibiting FSTL1 expression, it is possible to prevent and treat cancer, which has been used as a drug therapy for cancer metastasis and bone metabolism diseases (18). In this study, the binding site between miR-524-5p and FSTL1 was verified by dual luciferase in gastric cancer cell lines MGC-803 and MKN-45. Thal can inhibit the expression of FSTL1 and alleviate the invasion of gastric cancer cells. After the addition of miR-524-5p inhibitor, the FSTL1 protein expression level increased, suggesting that Thal up-regulates miR-524-5p to inhibit the target site FSTL1.

In summary, miR-524-5p has low expression in gastric cancer cells, and Thal can increase the expression of miR-524-5p to reduce the FSTL1 protein expression, thereby alleviating the invasion of gastric cancer cells. However, this study only initially investigates the function of Thal at the cellular level. The body's mechanism is complex, so its role still needs further verification, which is also the focus of our subsequent research.

Data availability

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declared no conflict of interest.

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