

Mechanism of miR-548b-5p down-regulating FZD7 to impede the migratory and invasive behavior of gastric carcinoma cells

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ABSTRACT

This study aimed to investigate the possible mechanism of Micro RNA-548b-5p (miR-548b-5p) down-regulating frizzled (FZD) 7 to suppress the migration and invasion of gastric carcinoma cells. For this purpose, HGCC (Human gastric carcinoma cell) lines were selected (Hs-746T, NCI-N87, SGC-7901, MKN-45, SNU-1), and human normal gastric mucosa cells GES-1. QRT PCR was adopted to reveal and screen the cell line with low expression of miR-548b-5p (hs-746t) for research; the Hs-746T cells were randomly assigned into control group, miR-548b-5p NC group, miR-548b-5p mimic group, miR-548b-5p mimic+pc-FZD7 group. The CCK-8 assay was utilized to measure Hs-746T cell viability, while flow cytometry, Trans well chamber, and scratch test were utilized to examine the apoptotic, invasive, and migratory properties of the cells, respectively. WB was used to detect the SATB1, as well as the expression levels of proteins involved in apoptosis, including Caspase-3, Bax, and Bcl-2, as well as Matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) in SW620 cells. The binding of miR-548b-5p to FZD7 was evaluated through the dual-luciferase reporter assay. The results indicate that MiR-548b-5p showed low expression in HGCCs; in contrast to the control group ($P>0.05$), the Hs-746T cell viability, invasion, migration ability, MMP-2, MMP-9 protein significantly downregulated in miR-548b-5p mimic group ($P<0.05$), the apoptosis rate, Caspase-3, Bax protein expression were upregulated markedly, and Bcl-2 protein expression was downregulated significantly ($P<0.05$); in contrast to miR-548b-5p mimic group, the Hs-746T cell viability, invasion, migration ability, MMP-2, MMP-9 protein significantly were upregulated in miR-548b-5p mimic+pc-FZD7 group ($P<0.05$), the apoptosis rate, Caspase-3, Bax protein expression were significantly, and the level of Bcl-2 was down-regulated significantly ($P<0.05$); Double Luciferase Report shows that miR-548b-5p can target and regulate *fzd7*. It was concluded that MiR-548b-5p can suppress cell growth and migration of HGCC Hs-746T, which may be achieved by targeted down-regulation of FZD7.

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Introduction

Gastric cancer is a common malignancy with high morbidity and mortality rates. Patients with early gastric cancer show no obvious symptoms. With the continuous progress of the disease, some adverse manifestations such as weight loss, dysphagia, anemia and cachexia gradually appear (1,2). Biological activities of tumor cells, such as abnormal proliferation, malignant invasion, and migration have an adverse effect on the progress of tumor diseases. Research on the possible etiological and developmental mechanisms of gastric carcinoma carries great significance for clinical treatment and alleviation of the disease. miRNA is an endogenous non-coding RNA, which has become a recent research hotspot due to its important role in biological activities of cellular growth, infiltration, motility, and other related processes. A variety of miRNAs (such as miR-155-5p and miR-27A) are related to the onset and progression of gastric carcinoma as well as the duplication and migration of gastric carcinoma cells (3,4). miR-548b-5p, belonging to the miRNA family, is downregulated in several types of tumors. According to Shi et al. (5) overexpression of miR-548 can suppress breast cancer

cell growth and enhance apoptosis. Jin et al. (6) observed that decreased miR-548 expression may facilitate the proliferation and invasion of bladder cancer cells, but the influence of miR-548b-5p on gastric carcinoma cell invasion and migration is poorly characterized. In primary gastric cancer tissues and gastric carcinoma cell lines, Frizzled 7 (FZD7), belonging to the FZD family, is upregulated and strongly correlated with tumor invasion, metastasis, advanced TNM stage, poor survival, and acceleration of tumor growth (7,8). Therefore, this paper studies the possible mechanism by which miR-548b-5p regulates FZD7 and affects the invasive and migratory behaviors of gastric carcinoma cells, thereby providing a theoretical basis for treatment-induced remission and control of gastric carcinoma.

Materials and Methods

Cells

HGCCs (Hs-746T, NCI-N87, SGC-7901, MKN-45, SNU-1, (article numbers: CL-0115, CL-0169, CL-0206, CL-0292, CL-0474); Human gastric mucosa cells (GES-1, article number: CL-0563) were provided by Wuhan Pu-

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nuosai Life Science and Technology Co., Ltd.

Main reagents and instruments

Fetal Bovine Serum (FBS) and RPMI1640 medium (article No.: 164210, PM150110A) were provided by Wuhan Procell Life Science & Technology Co., Ltd.; Entranster™-R4000 (RNA Transfection Reagent, Article No.: 4000-3) was bought from Engreen Biosystem (Beijing) Co, Ltd; RNA extraction kits (article number: R1200) were procured from Solaibao (Beijing) Technology Co., Ltd.; reverse transcription kits (article number: 11141ES10) were obtained from Yisheng Biotechnology Co., Ltd.; primer sequences were synthesized by Beijing Liuhe BGL.; rabbit source FZD7, MMP-2, MMP-9, Caspase-3, Bax, Bcl-2, GAPDH, goat anti-rabbit IgG secondary antibody labeled with horseradish peroxidase (article No.: ab64636, ab181286, ab76003, ab184787, ab194583, ab32503, ab9485, ab150077) were procured from Abcam Company; cell incubator (model: MCO-15AC, Japan SANYO Company); cell counter (model: NC-100, Denmark ChemimeteC Company); inverted microscope (model: DMi8, Leica, Germany); microplate reader (model: XELx800, Perkin Elmer Company, USA); flow cytometer (model: Accuri C6, BD Company, USA); gel imaging system (model: GIS-500, Miulab Company).

Cell culture

Cell lines including Hs-746T, NCI-N87, SGC-7901, MKN-45, SNU-1 and GES-1 were cultured in an incubator (37°C, 5% CO₂). PMI1640 (10% FBS) medium was selected, and the growth medium was renewed every two days. After observing that the cell fusion rate was more than 80%, subculture was carried out, and then 0.25% trypsin was applied to the cells. After digestion, a fresh medium was introduced to stop digestion, and the cells were continuously grown for subsequent experiments.

qRT-PCR method to determine miR-548b-5p expression in each cell line

When each cell line grows to the logarithmic growth phase, collect them and extract the total RNA in the above cell lines by RNA extraction kit, and determine the purity of RNA. Perform reverse transcription to acquire cDNA with the aid of a kit for reverse transcription. The reaction mixture mainly consisted of SYBR Mix (2.5 μL), upstream primer (0.1 μL), downstream primer (0.1 μL), cDNA (1 μL), deoxyribonuclease water (1.3 μL), etc. miR-548b-5p PCR primers: upstream 5'-AGACCCTGGTCTGCACTC-TAT-3', downstream 5'-GCGAGCACAGAATTAATACGA-3'; U6 PCR primers: upstream 5'-ATTGGAACGATACAGAGAAGATT-3', downstream 5'-GGAACGCTT-CACGAATTTG-3'. The experiment was conducted in triplicate, and the normalized expression of miR-548b-5p mRNA in gastric carcinoma cells and gastric mucosa was analyzed according to 2^{-ΔΔCT} algorithm.

Grouping transfection of gastric carcinoma cells and detection of miR-548b-5p expression

Collect Hs-746T HGCCs in the logarithmic growth phase and randomly divide them into control group (no transfection), miR-548b-5p NC group, miR-548b-5p mimic group, miR-548b-5p mimic+pc-FZD7 group. Dilute miR-548b-5p NC, miR-548b-5p mimic, and pc-FZD7 with serum-free RPMI1640 medium to RNA solution with a

concentration of 26.80 ng/μL. Add 1 μL Entranster™-R4000 to dilute 24 μL serum-free RPMI1640 medium to obtain Entranster™-R4000 diluent. Uniformly mix 25 μL RNA solution and 25 μL Entranster™-R4000 diluent to obtain a composite solution. According to the transfection requirements of each group, the composite solution was taken for transfection operation. The level of miR-548b-5p was assessed by the method in 2.2.

The determination and calculation method of miR-548b-5p expression in Hs-746T gastric carcinoma cells in each group is the same as 2.2.

CCK-8 determination

Collect the Hs-746T cells during their logarithmic growth stage and culture them in a 96-well plate at a density of approximately 1500 cells per well. The cells were cultured in a 37°C, 5% CO₂ incubator and taken out after 24 h. CCK-8 reagent was added for 4h further incubation in the incubator. Measure the absorbance with a microplate photometer, and set the control well without cells. The calculation formula: cell viability (%) = $(A_{\text{determination}} - A_{\text{control}}) / (A_{\text{Control}}) \times 100\%$.

Assess apoptotic cell death of gastric carcinoma cells using flow cytometry

Collect Hs-746T in the logarithmic growth phase, set the cell density to 1×10⁶ cells per milliliter, and prepare cell suspension. Add 5 μL Annexin V/Alexa Fluor 647 and 10 μL PI working solution (20 μg/mL) to 100 μL cell suspension, mix well, and incubate in the dark at normal temperature. After 15 minutes, add PBS buffer and place it in flow cytometry to assess apoptotic cell death of gastric carcinoma cells.

Scratch test, transwell chamber test

The scratch assay (9) was performed to assess the migratory ability of gastric carcinoma cells Hs-746T. Gastric carcinoma cells Hs-746T in the logarithmic growth phase were obtained, inoculated in a 6-well plate, and grown until the cell fusion efficiency reached 90%. Make a scratch on the bottom of the 6-well plate with spearhead, continue to culture for 24 h, observe and analyze it under a microscope. Use the software (Image J) to calculate the cell scratch healing rate.

Transwell migration assay (10) was carried out to evaluate the invasive ability of Hs-746T gastric carcinoma cells. Gastric carcinoma cells Hs-746T in each group were collected, with cell concentration adjusted to 1×10⁵ cells/mL. The Transwell chamber was pre-laid with Matrigel glue and placed in 24-well plate. Introduce 200 μL of cell solution into the upper well, add an appropriate amount of RPMI1640 culture medium (20% FBS) into the lower well, culture in an incubator for 24 hours. Wash out the upper chamber cell with PBS buffer, and fix the chamber in 4% paraformaldehyde for 20min. Stain with crystal violet, observe and record the number of invaded cells in a microscope after drying.

WB detects the expression of FZD7, apoptotic and invasive proteins in gastric carcinoma cells

Collect the Hs-746T gastric carcinoma cells of the logarithmic growth phase in each group, wash them with PBS buffer for 3 times, add high-efficiency RIPA lysis solution to extract the protein, conduct centrifugal treatment

for the lysed sample to collect the supernatant, extract the protein concentration. Adjust the protein concentration of each group to the same as the protein loading buffer, perform SDS polyacrylamide gel electrophoresis after heat denaturation, and transfer the isolated protein to nitrocellulose (PVDF) membrane by wet electrotransfer. Put the PVDF membrane in a petri dish, add 5% nonfat dry milk powder for 1-h blocking, dilute the antibody according to the instructions, and add rabbit-derived FZD7, MMP-2, MMP-9, Bcl-2, Caspase-3, Bax, Bcl-2, MMP-2, MMP-9, GAPDH primary antibodies, incubate at 4°C for the entire night Wash with TBST buffer, add horseradish peroxidase-labeled and diluted secondary antibody, and incubate at normal temperature for 1 h at normal temperature on a shaker. Remove the secondary antibody, wash with TBST buffer, and make analysis after color development, exposure, and fixation.

Luciferase experiment to identify the interaction between miR-548b-5p and FZD7

Targets can database analyzes the potential binding domains of miR-548b-5p and FZD7. Take gastric carcinoma cell Hs-746T to construct FZD7 3'-UTR luciferase expression vector of mutant-type (FZD7-MUT) and wild-type (FZD7-WT) gene targets. FZD7-MUT and FZD7-WT were respectively transfected with miR-548b-5p mimic NC and miR-548b-5p mimic into Hs-746T cells. After culture in an incubator for 24 h, each group was measured for cell luciferase activity. The test was repeated in triplicate to verify the targeting relationship between them.

Statistical evaluation

Data were processed using GraphPad Prism 8, and the results were expressed as mean±SD ($\bar{x}\pm s$). P<0.05 was considered statistically significant.

Results

Comparison of miR-548b-5p expression level in HGCCs

In contrast to human gastric mucosa cells GES-1, HGCCs Hs-746T, NCI-N87, SGC-7901, MKN-45, and SNU-1 have significantly reduced expression levels of miR-548b-5p (P<0.05). Where Hs-746T cells have the lowest expression of miR-548b-5p, so Hs-746T cells were selected for follow-up experimental cells (Table 1).

Comparison of miR-548b-5p and FZD7 expression levels in Hs-746T cells of each group

In contrast to the control group, the miR-548b-5p mimic group has significantly up-regulated expression of miR-548b-5p in Hs-746T cells (P<0.05) and significant-

Table 1. Cellular expression of miR-548b-5p ($\bar{x}\pm s$, n=3).

Cells	miR-548b-5p/U6
GES-1	1.01±0.20
Hs-746T	0.21±0.07 ^a
NCI-N87	0.39±0.09 ^a
SGC-7901	0.33±0.08 ^a
MKN-45	0.37±0.03 ^a
SNU-1	0.40±0.07 ^a

Note: In contrast to human normal gastric mucosa cells GES-1, ^aP<0.05

ly reduced expression of FZD7 protein (P<0.05); compared with miR-548b-5p mimic group, the miR-548b-5p mimic+pc-FZD7 group has significantly up-regulated expression of FZD7 protein in Hs-746T cells (P<0.05). The control group did not differ significantly from miR-548b-5p NC group (P>0.05) (Figure 1 and Table 2).

Comparison of cell viability of Hs-746T cells

In contrast to the control group, miR-548b-5p mimic group has obviously lower viability of Hs-746T cells (P<0.05); compared with miR-548b-5p mimic group, miR-548b-5p mimic+pc-FZD7 group has significantly higher Hs-746T cell viability (P<0.05); the control group did not differ from the miR-548b-5p NC group in cell viability of Hs-746T (p>0.05) (Figure 2).

Apoptosis of Hs-746T cells

In contrast to the control group, the miR-548b-5p mi-

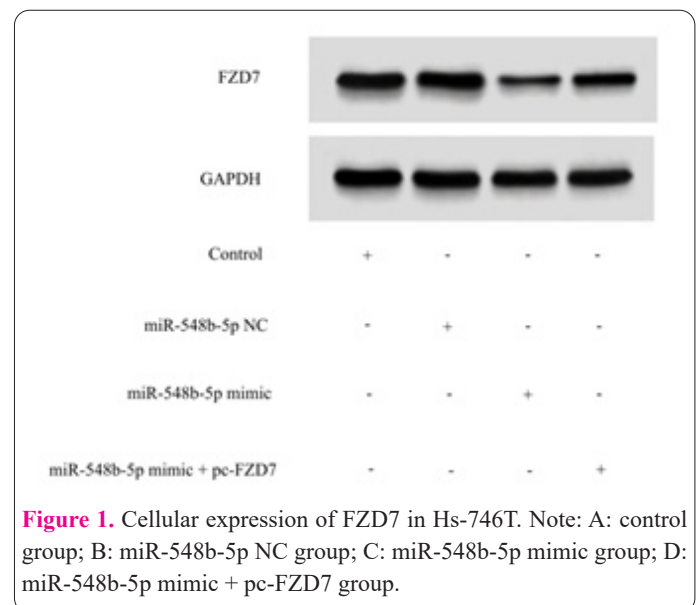


Figure 1. Cellular expression of FZD7 in Hs-746T. Note: A: control group; B: miR-548b-5p NC group; C: miR-548b-5p mimic group; D: miR-548b-5p mimic + pc-FZD7 group.

Table 2. Cellular expressions of miR-548b-5p and FZD7 in Hs-746T ($\bar{x}\pm s$, n=3).

Groups	miR-548b-5p/U6	FZD7/GAPDH
Control group	1.02±0.13	1.04±0.12
miR-548b-5p NC group	1.01±0.17	1.02±0.15
miR-548b-5p mimic group	1.71±0.22 ^{ab}	0.31±0.08 ^{ab}
miR-548b-5p mimic+pc-FZD7 group	1.68±0.24 ^{ab}	0.67±0.12 ^{abc}

Note: In contrast to the control group, ^aP<0.05; In contrast to miR-548b-5p NC group, ^bP<0.05; In contrast to miR-548b-5p mimic group, ^cP<0.05.

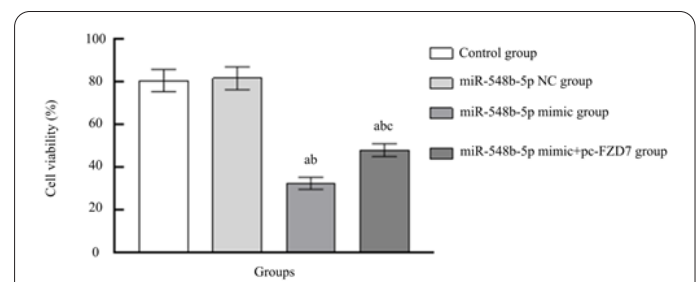


Figure 2. Cell viability of Hs-746T cells ($\bar{x}\pm s$, n=3).

Table 3. Apoptosis of Hs-746T cells ($\bar{x}\pm s$, n=3).

Groups	Apoptosis rates(%)	Apoptosis protein expression		
		Caspase-3/GAPDH	Bax/GAPDH	Bcl-2/GAPDH
Control group	13.97±2.06	0.82±0.11	0.83±0.12	1.02±0.15
miR-548b-5p NCgroup	14.06±2.17	0.85±0.14	0.79±0.10	1.04±0.14
miR-548b-5p mimicgroup	35.58±5.26 ^{ab}	1.65±0.15 ^{ab}	1.61±0.16 ^{ab}	0.23±0.05 ^{ab}
miR-548b-5p mimic+pc-FZD7group	23.39±4.17 ^{abc}	1.17±0.11 ^{abc}	1.19±0.10 ^{abc}	0.60±0.12 ^{abc}

Table 4. Invasive and migratory capacities of Hs-746T cells ($\bar{x}\pm s$, n=3).

Groups	Number of invasive cells	Wound healing rate (%)	Expression of invasive proteins	
			MMP-2/GAPDH	MMP-9/GAPDH
Control group	149.43±12.76	78.39±2.19	1.02±0.19	1.01±0.18
miR-548b-5p NCgroup	148.38±12.39	77.04±1.30	1.01±0.18	1.02±0.17
miR-548b-5p mimicgroup	82.39±7.64 ^{ab}	45.25±4.70 ^{ab}	0.28±0.05 ^{ab}	0.25±0.03 ^{ab}
miR-548b-5p mimic+pc-FZD7group	115.26±11.05 ^{abc}	59.76±2.32 ^{abc}	0.66±0.11 ^{abc}	0.61±0.10 ^{abc}

mic group has a significantly increased rate of programmed Hs-746T cell death, expression of Caspase-3 and Bax protein (P<0.05) and significantly reduced Bcl-2 protein expression (P<0.05). In contrast to the miR-548b-5p mimic group, the miR-548b-5p mimic+pc-FZD7 group has a significantly reduced rate of programmed Hs-746T cell death, expression of Caspase-3 and Bax protein (P<0.05), and significantly up-regulated level of Bcl-2 (P<0.05); there was no statistical difference between miR-548b-5p NC group and control (p>0.05) (Table 3 and Figures 3 and 4).

Invasive and migratory capacities of Hs-746T cells

In contrast to the control group, mir-548b-5p mimetic group Hs-746t cells' scratch healing rate, invasion cell number, and MMP-2 and MMP-9 protein expressions were significantly reduced (p<0.05); in contrast to the miR-548b-5p mimic group, the Hs-746t cells of mir-548b-5p mimetic+PC-fzd7group had significantly higher scratch healing rate, invasion cell number, MMP-2 and MMP-9 expressions (P<0.05); the control group did not differ from the miR-548b-5p NC group in the indexes of Hs-746T cells (p>0.05) (Table 4 and Figures 5-7).

Targeting interaction between miR-548b-5p and FZD7

TargetScan database predicts that miR-548b-5p has a corresponding binding domain in the 3'-UTR region of FZD7; the dual-luciferase experiment indicates that in contrast to FZD7 WT+miR-548b-5p NC group, FZD7 WT+miR-548b-5p mimic group has significantly reduced luciferase activity in Hs-746T cell (P<0.05), FZD7 MUT+miR-548b-5p NC group and FZD7 MUT+miR-548b-5p mimic group show no statistical difference (P<0.05) (Figures 8 and 9).

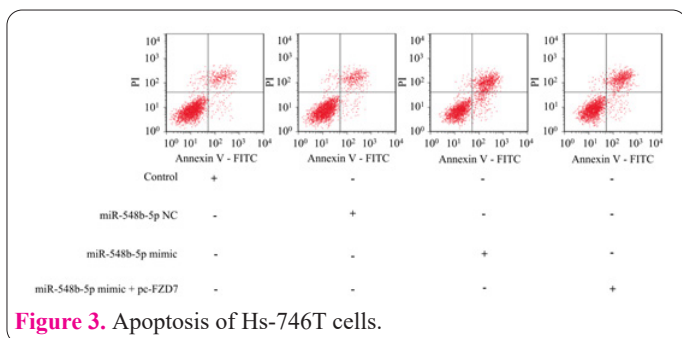


Figure 3. Apoptosis of Hs-746T cells.

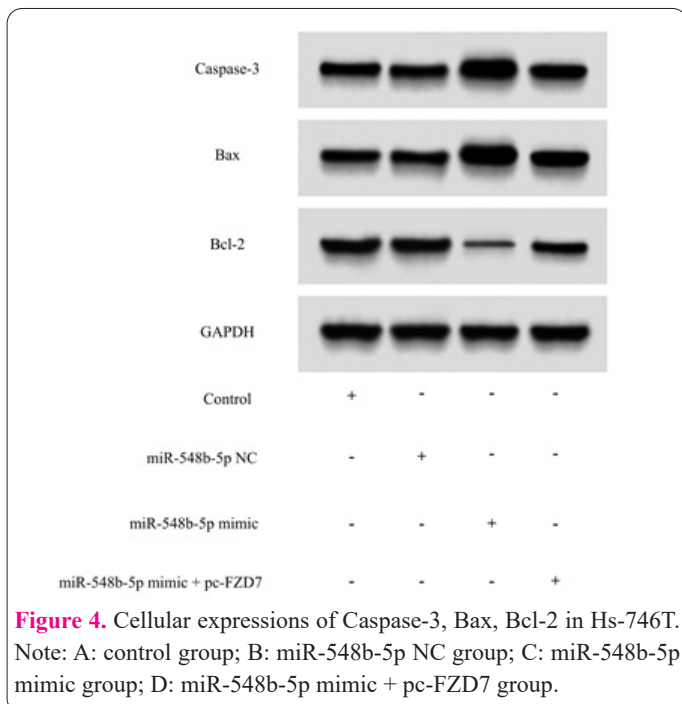


Figure 4. Cellular expressions of Caspase-3, Bax, Bcl-2 in Hs-746T. Note: A: control group; B: miR-548b-5p NC group; C: miR-548b-5p mimic group; D: miR-548b-5p mimic + pc-FZD7 group.

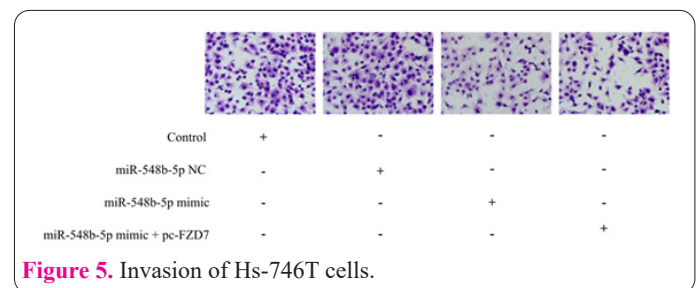


Figure 5. Invasion of Hs-746T cells.

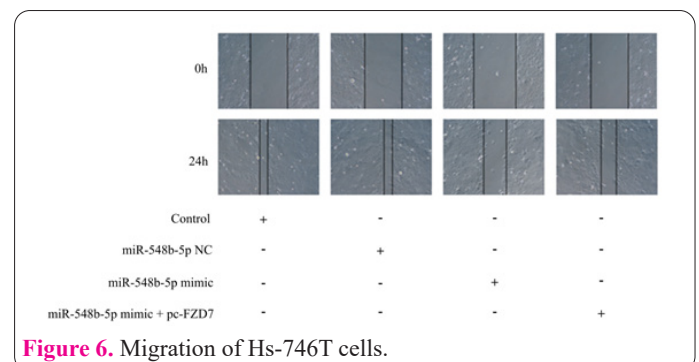


Figure 6. Migration of Hs-746T cells.

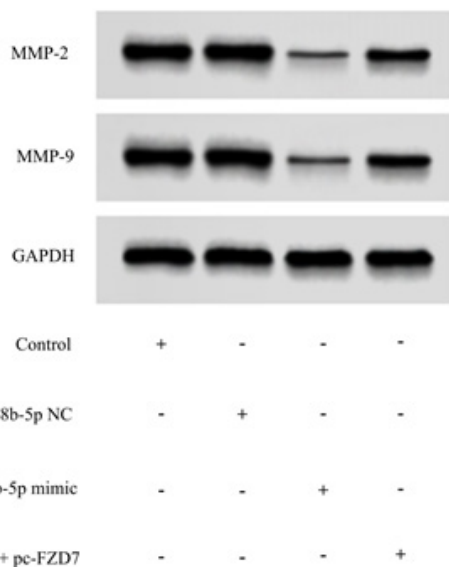


Figure 7. Cellular expressions of MMP-2, MMP-9 in Hs-746T. Note: A: control group; B: miR-548b-5p NC group; C: miR-548b-5p mimic group; D: miR-548b-5p mimic + pc-FZD7 group.

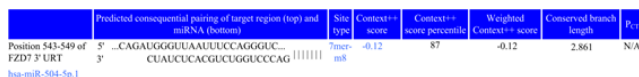


Figure 8. Binding of miR-548b-5p to FZD7 3'UTR.

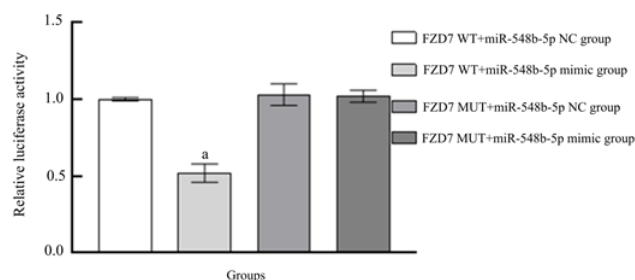


Figure 9. Luciferase Reporting Test Results ($\bar{x} \pm s$, $n=3$). Note: In contrast to FZD7 WT + miR-548b-5p NC group, ^a $P < 0.05$.

Discussion

Gastric cancer is related to poor eating habits, *Helicobacter pylori* infection, genetic factors, etc. Gastric cancer features multiple influencing factors, gradual development, and long duration. At the time of diagnosis, most patients have advanced to the stage of gastric cancer. At this time, gastric cancer has significantly enhanced invasion and migration capabilities, posing a greater threat to the patient's life. The current treatments for gastric cancer are mainly radiotherapy and chemotherapy, surgical resection and drug therapy, which can reduce the patient's pain, and appropriately improve the patients' quality of life, but the survival rate is still low. Therefore, further exploration into the possible development mechanism of gastric cancer is of great importance for improving the survival rate of gastric cancer (11,12). miRNA is a non-coding endogenous micromolecule RNA discovered in 1993. Often composed of 20~25 nucleotides, it participates in the onset and progression of diverse diseases, which has a close relation to the proliferative, invasive and migratory capacities of tumor cells and occupies an important position in the invasion and metastasis of tumor cells such as gastric cancer

(13). miR-548 has a down-regulated expression in multiple cancers: liver cancer (14), breast cancer (15), bladder cancer (16), etc. Zhu et al. (17) found that miR-548 had obvious down-regulation in pancreatic carcinoma tissues. By up-regulating the expression of miR-548, it is possible to significantly inhibit the proliferative and invasive capabilities of pancreatic carcinoma cells. The results of this study showed that HGCCs Hs-746T, NCI-N87, SGC-7901, MKN-45, and SNU-1 had significantly lower miR-548b-5p expression levels than GES-1, suggesting that miR-548b-5p could play a role in the emergence and advancement of gastric cancer. This study selected Hs-746T cells with relatively low miR-548b-5p expression levels for subsequent experiments.

Invasion and migration of tumor cells refer to the activities in which malignant cells dissociate from the original site, invade adjacent normal tissues, and then transfer to distant sites, which promotes the emergence and advancement of tumor diseases. As the degree of tumor malignancy increases, tumor cells have gradually enhanced invasion and migration capabilities (18), so exploring the effect of miR-548b-5p on the proliferative, invasive and migratory behaviors of gastric carcinoma cells and its possible mechanisms means great significance for alleviating gastric cancer. Bcl-2 is an important antiapoptosis protein that can prevent abnormal cell apoptosis. Caspase-3 and Bax are important pro-apoptotic factors. Bax can play a role by enhancing Caspase-3 level, which can reflect cell apoptosis capability (19). MMP-2 and MMP-9 can stimulate the invasion and dissemination of cancer cells, mainly by degrading the extracellular matrix (20). This study reveals that up-regulation of miR-548b-5p expression can significantly reduce the viability, invasive and migratory capabilities of Hs-746T cells and the expression of MMP-2, MMP-9 and Bcl-2, up-regulate the apoptosis capability of Hs-746T cells as well as the expression levels of Caspase-3 and Bax, suggesting that overexpression of miR-548b-5p can suppress the malignant growth, infiltration and motility of gastric carcinoma cell Hs-746T, and induce its apoptosis, possibly by regulating the expression of apoptosis-related factors and invasion-related factors in cells.

The FZD family plays a role in the management of biological activities such as cell growth, programmed cell death, cell maturation, and cell motility, which is associated with the onset and advancement of tumors. FZD7 is up-regulated in gastric cancer. By silencing FZD7 expression, it is possible to significantly inhibit the invasion and migration of gastric carcinoma cells (11-24). Data show that miRNA can pair with the 3'-UTR region of the target gene to form a silent complex, thereby promoting or suppressing cancer (25-28). Geng et al. (29) found that miRNA (such as miR-27b) can negatively regulate FZD7, thereby affecting *Helicobacter pylori*-induced gastric tumorigenesis and inhibiting the proliferation of gastric tumor cells. However, the interaction between miR-548b-5p and FZD7 has not been extensively studied. According to TargetScan database prediction, miR-548b-5p and FZD7 have corresponding binding domains in the 3'-UTR region. The luciferase experiment results demonstrated that upregulation of miR-548b-5p could markedly decrease the luciferase expression driven by the wild-type FZD7-3'UTR. It suggests that miR-548b-5p may impede the invasive and migratory capacity of gastric carcinoma cell Hs-746T pos-

sibly by down-regulating FZD7.

In summary, elevated expression of miR-548b-5p can suppress cell growth, invasion and migration of gastric carcinoma cell Hs-746T, possibly by targeted down-regulation of FZD7. However, the malignant biological behavior of tumor cells has a complicated development mechanism. In this study, there were no verification tests on multiple tumor cells, so in-depth research is still needed.

To sum up, the overexpression of miR-548b-5p is likely to suppress the cell growth, and invasive and migratory behaviors of gastric carcinoma cell Hs-746T through targeted down-regulation of FZD7. However, the development mechanism of the malignant biological behavior of tumor cells is complex, and further verification tests are needed to explore whether other pathways are indirectly involved in regulation.

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Consent for publication

All authors give consent for publication.

Declaration of Conflicting Interests

None.

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Data Availability Statement

The [DATA TYPE] data used to support the findings of this study are included within the article.

Research involving human participants and/or animal

Not applicable.

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