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Original Research

Effect of paeonol on proliferation, apoptosis, migration, invasion and glutamine of gastric cancer cells via circSFMBT2/miR-665 axis

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Abstract: This experiment was performed to investigate the effect of paeonol on the proliferation, apoptosis, migration, invasion and glutamine of gastric cancer HGC-27 cells and its possible mechanism. For this purpose, the MTT method was used to detect cell viability; Flow cytometry experiment was used to detect cell apoptosis; Transwell chamber experiment was used to detect cell migration and invasion; Western blotting was used to detect the expression levels of MMP2 and MMP9 protein; The decomposition of glutamine was evaluated by detecting the expression levels of glutamine, glutamic acid and α-ketoglutarate (α-KG). This study used RT-PCR to detect the expression of circSFMBT2 and miR-665. The targeting relationship between circSFMBT2 and miR-665 was verified by the dual-luciferase report experiment and RIP experiment. Results showed that different concentrations of Paeonol could significantly inhibit the proliferation, migration, invasion and glutamine decomposition of HGC-27 cells, and induce cell apoptosis in a dose-dependent manner. In gastric cancer tissues and cells, the expression of circSFMBT2 was up-regulated, and the expression of miR-665 was down-regulated. Over-expression of circSFMBT2 could partially restore the effects of paeonol on the proliferation, apoptosis, migration, invasion and glutamine of HGC-27 cells. CircSFMBT2 could target and negatively regulate the expression of miR-665. Overexpression of miR-665 could partially restore the effects of Pae and circSFMBT2 on the proliferation, apoptosis, migration, invasion and glutamine of HGC-27 cells. It was concluded that paeonol can inhibit the proliferation, migration, invasion and glutamine decomposition of gastric cancer HGC-27 cells via circSFMBT2/miR-665 axis, and also induce cell apoptosis.

Key words: Paeonol; Gastric cancer; Proliferation; Apoptosis; Migration; Invasion; Glutamine.

Introduction

Gastric cancer has high morbidity and mortality, which is a common malignant tumor of the digestive system. Due to the lack of screening methods in the early stage of gastric cancer, most patients with gastric cancer are already in the late stage. And the 5-year prognostic survival rate is lower than 30% (1, 2). Although surgery, radiotherapy and chemotherapy have achieved remarkable results, gastric cancer is still the cause of high cancer mortality in the world (3). As a small molecular phenolic compound, paeonol is the main active monomer of traditional Chinese medicine Radix Cynanchi Paniculati and Cortex Moutan, which has pharmacological activities such as anti-inflammation, anti-tumor, improving cardiovascular and cerebrovascular diseases and enhancing immunity (4). Lyu et al found that paeonol could significantly inhibit the growth, migration and invasion of gastric cancer cells by down-regulating MMP-2 and MMP-9. Circular RNA (circRNA), as a circular closed non-coding RNA molecule, is abnormally expressed in many cancers (5). For example, circSFMBT2 (Hsa_circ_0017639) is highly expressed in gastric cancer cells. CircSFMBT2 inhibits the proliferation and metastasis of gastric cancer cells by regulating miR-224-5p/USP3 (6). In recent years, miRNA has attracted much attention in the study of gastric cancer, such as the low expression of miR-665 in gastric cancer cells and gastric cancer tissues. The abnormal expression is significantly correlated with TNM stage, late metastasis and poor differentiation. Overexpression can significantly inhibit the proliferation, migration, invasion and epithelial-mesenchymal transformation of gastric cancer cells (7). However, the studies on circSFMBT2 (Hsa_circ_0017639) and paeonol have not been reported. Therefore, the purpose of this study is to explore the effects of paeonol on proliferation, apoptosis, migration, invasion and glutamine decomposition of gastric cancer via circSFMBT2/miR-665.

Materials and Methods

Cells and reagents

Normal gastric epithelial cell GES-1 and gastric cancer cell HGC-27 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences; Fetal bovine serum and RPMI 1640 culture medium were purchased from Gibco company of USA; Paeonol was purchased from Zhejiang Haizheng Company; Lipofectamine TM 2000 transfection kit was purchased from Invitrogen company of USA; Vector, circSFMBT2, miR-NC, miR-665, circSFMBT2 probe and Oligo probe were purchased from Shanghai Jima Company; MTT kit was purchased from Shanghai Tongren Institute of Chemistry. Transwell and Matrigel glue were purchased from Shanghai Yisheng Biological Company; MMP2

and MMP9 antibodies were purchased from CST Company of the United States; Glutamine Kit and glutamate Kit were purchased from Sigma-Aldrich Company of Germany; α-KG Kit was purchased from Abcam Company of the United States; TRIzol Kit and reverse transcription Kit were purchased from TaKaRa Company of Japan; RNA enzyme R was purchased from Epicenter Company of the United States; Actinomycin D was purchased from Shanghai Chunyou Biology Company. Apoptosis kit and luciferase kit were purchased from Beijing Solebo Co., Ltd.

Sample collection

Twenty-one patients with gastric cancer and paracancerous tissues who underwent gastric cancer surgery were obtained. All patients did not receive preoperative chemotherapy and radiotherapy for 3 months and were immediately transferred to-80 ℃ refrigerator storage after surgical resection. All patients signed the informed consent form before the operation, and the study was approved by the Ethics Committee.

Cell culture and grouping

Normal gastric epithelial cells GES-1 and human gastric cancer cells HGC-27 were cultured in RPMI 1640 medium containing 10% fetal bovine serum, respectively, and incubated at 37 ℃ and 5% CO2 in the incubator. HGC-27 cells in the logarithmic growth phase were treated with different concentrations of paeonol at 0.0, 0.1, 0.2, 0.4 mg/ml, which were recorded as paeonol groups. HGC-27 cells were divided into six groups. Control group: DMSO solution was added, paeonol concentration was 0 mg/ml; Pae group: paeonol concentration was 0.4 mg/ml; Pae+vector group: Vector was transfected and then treated with paeonol concentration of 0.4 mg/ml; Pae+circSFMBT2 group: CircSFMBT2 was transfected, and then HGC-27 cells were treated with paeonol 0.4 mg/ml; Pae+circSFMBT2+miR-NC group: circSFMBT2 and miR-NC were co-transfected, and then HGC-27 cells were treated with paeonol 0.4 mg/ml; Pae+circSFMBT2+miR-665 group: circSFMBT2 and miR-665 were co-transfected, and then HGC-27 cells were treated with paeonol 0.4 mg/ml. The procedure of the Lipofectamine TM 2000 transfection kit was strictly followed in cell transfection.

MTT method

HGC-27 cells were collected and inoculated into 96-well plates (3×10^3 cells / well). After 48 hours, 20 μ L MTT was added to each well for 4 h and 150 μ L DMSO was added to mix gently. The OD value was detected by enzyme labeling instrument at 490 nm, and the cell viability was calculated.

Flow cytometry

After the HGC-27 cells were digested, we mixed the cells with a 1X binding buffer. Then 5 μ L Annexin V-FITC and PI reagents were also added, followed by incubation at room temperature in the dark for 20 min. The cell apoptosis rate was detected by flow cytometry.

Transwell

Cell migration experiment: After HGC-27 cells were digested, a total of 200 μ L of cell suspension was added to the upper chamber, and 500 μ L of RPMI 1640 medium containing 10% fetal bovine serum was added to the lower chamber. After the cells were incubated for 24 h at 37 ℃, the migrated cells were fixed with 4% paraformaldehyde for 25 min and then stained with 0.1% crystal violet for 5 min at room temperature. After 5 min, 5 visual fields were randomly selected under a light microscope to calculate the number of migrating cells. As for the invasion experiment, before HGC-27 cells were inoculated, matrigel glue (1:4 dilution, Shanghai Yisheng Biological Company, China) was added to Transwell, and the other steps were consistent with the migration experiment.

Western blotting

The total proteins of HGC-27 cells in each group were extracted with RIPA buffer. The protein samples were treated with SDS-PAGE, then transferred to the membrane, and skimmed milk was closed and cultured for 1.5 h. The Primary antibody-protein MMP2 and MMP9 were added, and then continue the culture, and the second antibody was added the next day. 2 hours later, ECL photoluminescence solution was added to analyze the gray value of the protein band.

Determination of glutamine, glutamate and α-KG levels

After transfected at 48 h, the expression of glutamine, glutamate and α-KG was detected by glutamine kit, glutamate kit and α-KG kit.

RT-PCR

Total RNA was extracted from HGC-27 cells with TRIzol reagent, then cDNA was synthesized by reverse transcription using RNA as a template and then amplified by PCR. GAPDH and U6 were used as internal parameters, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of circSFMBT2 and miR-665.

RNase R + restriction endonuclease digestion, actinomycin and subcellular localization

Total RNA was extracted from HGC-27 cells with TRIzol reagent and then purified by phenol-chloroform after incubated with 3 U / μ g RNA enzyme R at room temperature for 15 min. HGC-27 cells and 2 μ g/mL actinomycin D were cultured in RPMI 1640 medium for 0, 6, 12 and 24 h, respectively. The expression of circSFMBT2 and linearSFMBT2 was detected by RT-PCR. The nuclear and cytoplasmic RNA of HGC-27 cells were extracted by TRIzol kit and fine cytoplasm was detected by RT-qPCR.

Double luciferase report

The binding site of circSFMBT2 to miR-665 was predicted by Starbase, and then the wild type luciferase vector (circSFMBT2 WT) and mutant luciferase vector (circSFMBT2 MUT) of circSFMBT2 were constructed and then cotransfected into HGC-27 cells with miR-NC or miR-665 according to the kit instructions. After transfection for 48 h, luciferase activity was detected.

RNA Immunoprecipitation

Biotinylated circSFMBT2 probe and HGC-27 cells were co-incubated for 2 h, with Oligo probe as

control, then HGC-27 cells were further incubated with Mmur280 streptavidin magnetic beads, and then the biotin-coupled RNA complex was pulled down. After 4 h, the magnetic beads were cleaned, and the binding RNA complex on the magnetic beads was extracted by TRIzol reagent and analyzed by RT-PCR.

Statistical analysis

All the experiments were statistically analyzed by SPSS 22.0 and GraphPad Prism 7.0. T-test was used for comparison between the two groups, and single-factor analysis of variance was used for comparison between multiple groups. Pearson correlation analysis was used to evaluate the correlations between circSFMBT2 and miR-665, with $P < 0.05$ indicating a statistically significant difference between the two groups.

Results

Effects of paeonol on viability, apoptosis, migration, invasion and glutamine decomposition of human gastric cancer cells

After gastric cancer cells were treated with different concentrations of paeonol (0.0, 0.1, 0.2, 0.4 mg/ml), the results showed that with the increase of paeonol concentration, the cell viability, the number of migrating cells, the number of invasive cells and the expression of MMP2 and MMP9 protein decreased gradually, while the apoptosis rate increased gradually, as shown in Figure 1A-E. The decomposition of glutamine was evaluated by observing the expression levels of glutamine, glutamate and α-KG. The results showed that the expression levels of glutamine, glutamate and α-KG decreased gradually with the increase of paeonol concentration, as shown in Figure 1F-G.

Up-regulation of circSFMBT2 in gastric cancer tissues and gastric cancer cell lines

As shown in Figure 2A, circSFMBT2 was a circular structure derived from exon Exon5, 6, 7, 8. Compared with paracancerous tissues and normal gastric epithelial cell line GES-1, the expression of circSFMBT2 in gastric cancer tissue and gastric cancer cell line HGC-27 was significantly increased (Figure 2B-C). The circular stability of circSFMBT2 was verified by RNase R + restriction enzyme digestion test and the actinomycin D experiment. The results showed that the expression of circSFMBT2 did not change significantly, but the expression of linear SFMBT2 decreased significantly after the treatment of RNase R +. After the treatment of actinomycin D, the expression of circSFMBT2 was significantly higher than that of linear SFMBT2, indicating that circSFMBT2 has good ring stability, as shown in Figure 2D-E. In addition, we also detected the location of circSFMBT2 by RT-PCR, and the results showed that circSFMBT2 mainly existed in the cytoplasm, as shown in Figure 2F.

Effect of paeonol partially restored by circSFMBT2 on the biological function of gastric cancer cells

As shown in Figure 3A, after treated with different concentrations of paeonol (0.0, 0.1, 0.2, 0.4 mg/ml), the expression of circSFMBT2 decreased gradually in gastric cancer cells. According to the experimental results of Figure 1, this study carried out the follow-up experiment by selecting the concentration of paeo-

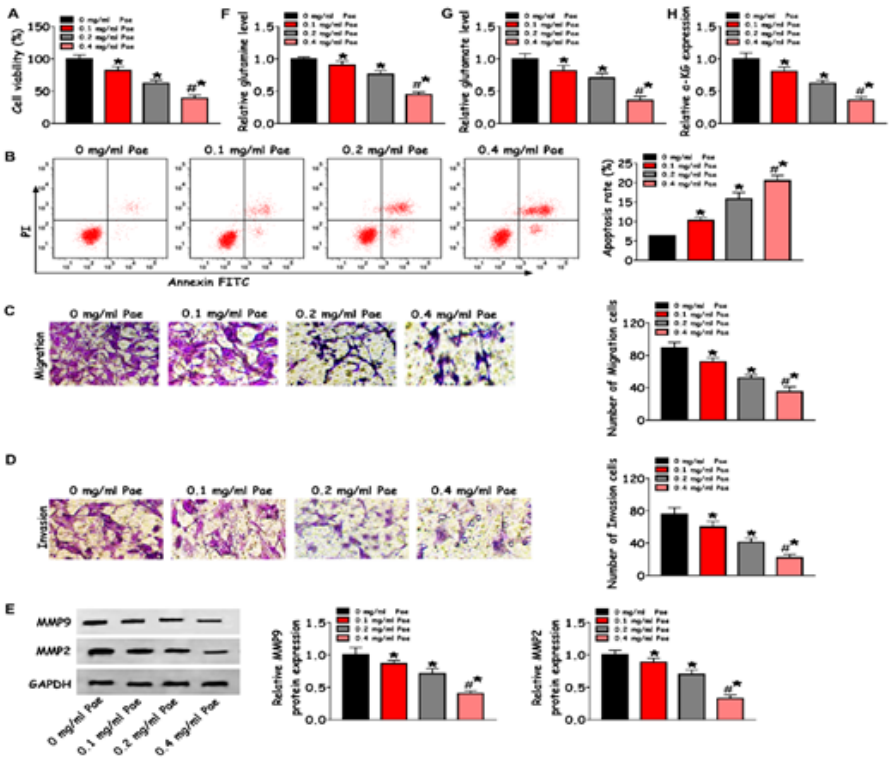

Figure 1. Effects of paeonol on viability, apoptosis, migration, invasion and glutamine decomposition of gastric cancer cells. A. Effects of different concentrations of paeonol on the viability of gastric cancer cells; B. Effects of different concentrations of paeonol on apoptosis of gastric cancer cells; C-D. Effects of different concentrations of paeonol on migration and invasion of gastric cancer cells; E. Effects of different concentrations of paeonol on the expression of MMP2 and MMP9 proteins; F-G. Effects of different concentrations of paeonol on the expression of glutamine, glutamate and α-KG.

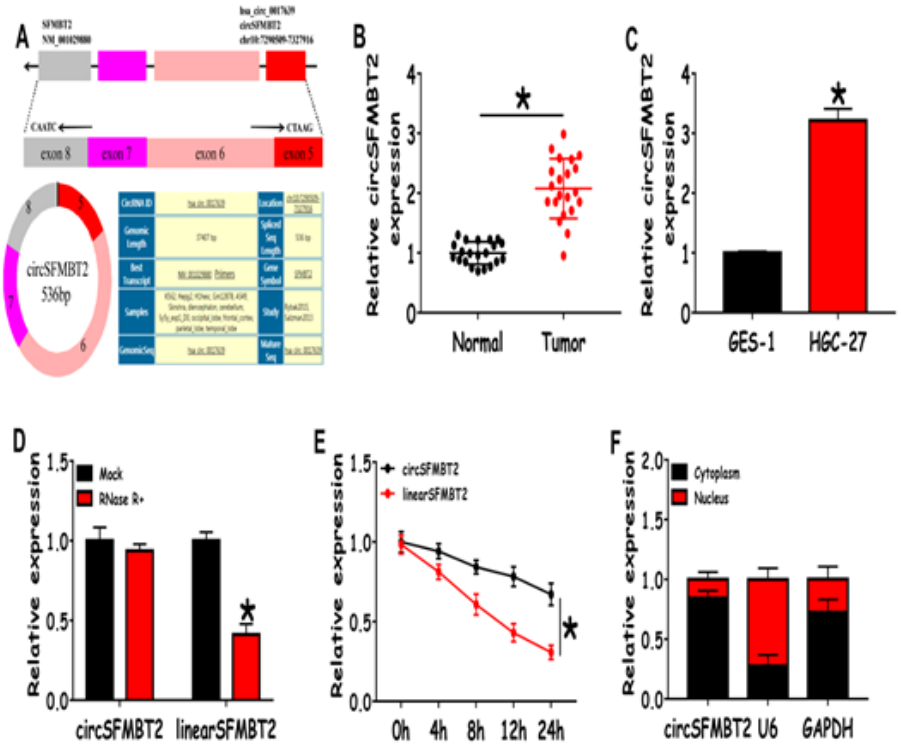

Figure 2. Up-regulation of circSFMBT2 in gastric cancer tissues and gastric cancer cell lines. A. The genomic site of circSFMBT2; B-C. The expression of circSFMBT2 in gastric cancer tissues and gastric cancer cells; D-E. RNase R + digestion and actinomycin D assay was used to detect the stability of circSFMBT2; F. circSFMBT2 was mainly located in the cytoplasm.

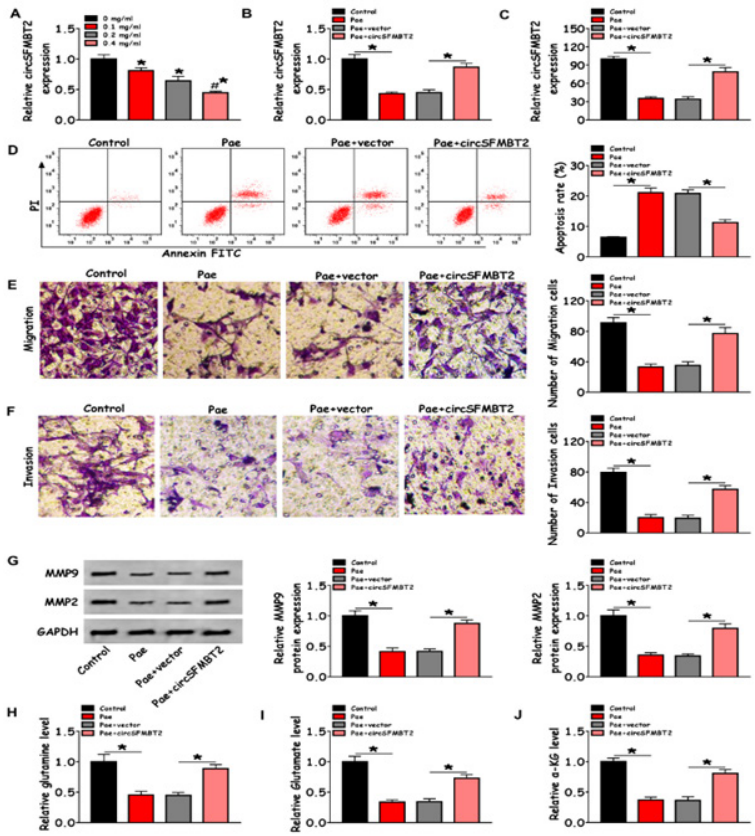

Figure 3. Effect of paeonol partially restored by circSFMBT2 on the biological function of gastric cancer cells. A. Effects of different concentrations of paeonol (0.0, 0.1, 0.2, 0.4 mg/ml) on the expression of circSFMBT2; B. Overexpression of circSFMBT2; C. circSFMBT2 could partially restore the effect of paeonol on gastric cancer cell viability; D. circSFMBT2 could partially restore the effect of paeonol on apoptosis of gastric cancer cells; E-F. CircSFMBT2 could partially restore the effect of paeonol on migration and invasion of gastric cancer cells; G. circSFMBT2 could partially restore the effects of paeonol on protein MMP9 and MMP2; H-J. CircSFMBT2 could partially restore the effects of paeonol on glutamine, glutamate and α-KG.

nol at 0.4 mg/ml. Compared with the control group, circSFMBT2 expression, cell viability, number of migrating cells, number of invasive cells, expression of MMP2 and MMP9 protein, expression of glutamine,

glutamate and α-KG decreased significantly in the Pae group, while cell apoptosis rate increased significantly in Pae+circSFMBT2 group. Compared with the Pae+vector group, circSFMBT2 expression, cell viability, the number of migrating cells, the number of invasive cells, expression of MMP2 and MMP9 protein increased significantly in the Pae+circSFMBT2 group. The expression of glutamine, glutamate and α-KG increased significantly, while the apoptosis rate decreased significantly, as shown in Figure 3B-J.

Targeted binding sites between circSFMBT2 and miR-665.

We detected the expression of circSFMBT2 after biotin transfection by RIP and RT-PCR assays. The results showed that the circSFMBT2 probe was enriched in the vector group and circSFMBT2 group, as shown in Figure 4A. In addition, as shown in the Venn diagram, there were four miRNA combined with circSFMBT2 predicted by circBank and Starbase, namely miR-107, miR-665, miR-4644 and miR-103a-3p. The expression of miRNA in si-circSFMBT2 transfected gastric cancer cell line HGC-27 was detected by RT-PCR assay. The results showed that miR-107, miR-665, miR-4644 and miR-103a-3p in the si-circSFMBT2 group were significantly higher than those in the si-NC group, and there was a significant difference in miR-665 (Figure 4B-C). The results of Figure 4D show that there were complementary nucleotide sequences between circSFMBT2 and miR-665. Compared with the miR-NC group, circSFMBT2 WT luciferase activity in the miR-665 group decreased significantly, while circSFMBT2 MUT had no significant change, as shown in Figure 4E.

Compared with paracancerous tissues and normal gastric epithelial cell line GES-1, the expression of miR-665 in gastric cancer tissue and gastric cancer cell line HGC-27 were significantly decreased, and there was a negative correlation between circSFMBT2 and miR-665, as shown in Figure 4F-H. As shown in Figure 4I, the expression of miR-665 increased gradually after treated with different concentrations of paeonol (0.0, 0.1, 0.2, 0.4 mg/ml) in gastric cancer cells.

Overexpression of miR-665 could partially restore the effects of paeonol and circSFMBT2 on the biological function of gastric cancer cells.

Compared with the control group, miR-665 and apoptosis rate were significantly increased, cell viability, the number of migrating cells, the number of invasive cells, expression of MMP2 and MMP9 protein, expression of glutamine, glutamate and α-KG were significantly decreased in the Pae group. Compared with Pae+vector, miR-665 and cell apoptosis rate decreased significantly, cell viability, the number of migrating cells, the number of invasive cells, the expression of MMP2 and MMP9 protein, and the expression of glutamine, glutamate and α-KG increased significantly in the Pae+circSFMBT2 group. Compared with Pae+circSFMBT2+miR-NC, miR-665 and apoptosis rate were significantly increased, cell viability, the number of migrating cells, the number of invasive cells, the expression of MMP2 and MMP9 protein, the expression of glutamine, glutamate and α-KG were significantly decreased in the Pae+circSFMBT2+miR-665 group (Figure 5A-J).

Discussion

Paeonol, also known as 2-hydroxy-4-methoxyacetophenone, is a class of small molecular phenolic compounds with a variety of pharmacological activities. In recent years, paeonol has achieved obvious results in anti-tumor, such as liver cancer, cervical cancer, breast cancer and gastric cancer (8). Lei et al. found that paeonol could enhance the radiosensitivity of lung adeno-

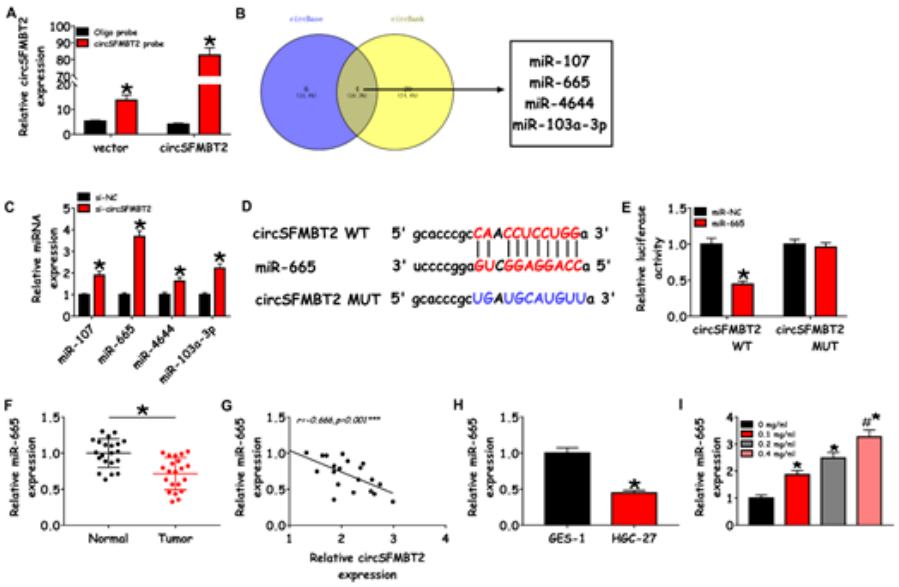

Figure 4. There were targeted binding sites between circSFMBT2 and miR-665**.** A. The expression of circSFMBT2 was detected after biotin transfection; B. Venn Diagram Wien graphic analysis software circBank and Starbase jointly predicted the expression level of miRNA in si-circSFMBT2 transfected with miRNA; C. Expression level of miRNA transfected with si-circSFMBT2; D. Starbase predicted the binding site between circSFMBT2 and miR-665; E. circSFMBT2 and miR-665 could bind to each other; F. The expression of miR-665 in gastric cancer; G. There was a negative correlation between circSFMBT2 and miR-665; H. Down-regulation of miR-665 expression in gastric cancer cell line HGC-27; I. Effects of different concentrations of paeonol (0.0, 0.1, 0.2, 0.4 mg/ml) on the expression of miR-665.

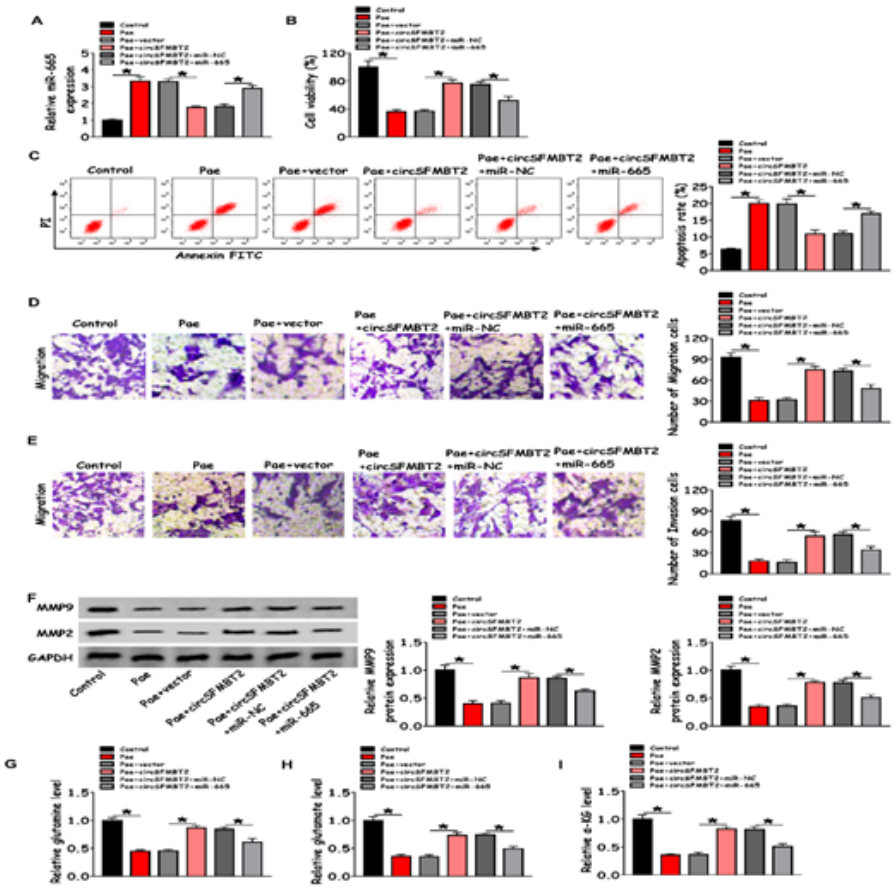

Figure 5. Overexpression of miR-665 could partially restore the effects of paeonol and circSFMBT2 on the biological function of gastric cancer cells. A. Effects of different concentrations of paeonol (0.0, 0.1, 0.2, 0.4 mg/ml) on the expression of circSFMBT2; B. Overexpression of circSFMBT2; C. circSFMBT2 could partially restore the effect of paeonol on gastric cancer cell viability; D. circSFMBT2 could partially restore the effect of paeonol on apoptosis of gastric cancer cells; E-F. circSFMBT2 could partially restore the effect of paeonol on migration and invasion of gastric cancer cells; G. circSFMBT2 could partially restore the effects of paeonol on protein MMP9 and MMP2; H-J. circSFMBT2 could partially restore the effects of paeonol on glutamine, glutamate and α-KG.

carcinoma by promoting radiation-induced apoptosis and inhibiting the PI3K/Akt pathway (9). Studies have shown that paeonol had an obvious inhibitory effect on the growth of breast cancer cells, and its mechanism may be related to its induction of apoptosis. CXCL4/CXCR3-B may promote apoptosis by regulating the expression of BACH1 and Nrf2 and down-regulating HO-1 (10). Paeonol has attracted wide attention in the treatment of gastric cancer. The results showed that paeonol could significantly inhibit the proliferation, migration and invasion of BGC823 cells, reduce the expression of MMP-2 and MMP-9 protein, and promote the apoptosis of SGC-7901 cells (11,12), which was similar to this study. The results showed that paeonol decreased the viability, invasion, migration, glutamine, glutamate and α-KG expression of HGC-27 cells in a dose-dependent manner, increased the apoptosis rate, and down-regulated the expression of MMP-2 and MMP-9 proteins, indicating that paeonol could significantly inhibit the proliferation, migration, invasion and glutamine decomposition of gastric cancer cells, and promote apoptosis.

CircRNA is a kind of non-coding RNA molecules with single-strand circular closure, which is highly conservative and stable. It can act as miRNAs sponges that regulate downstream targets, interact with genes and participate in the occurrence and development of a variety of cancers, including gastric cancer (13, 14). The results of Shen et al. (15) showed that the expression of circRNA_001569 was up-regulated and the expression of miR-145 was down-regulated in gastric cancer tissues and cells. Overexpression of circRNA_001569 could significantly inhibit the proliferation of gastric cancer cells and induce apoptosis. CircRNA_001569 plays a role in regulating the expression of miR-145 in gastric cancer, which is similar to the results of this study. The results of Liang et al. (16) showed that the expression of hsa_circ_006100 was up-regulated and the expression of miR-195 was down-regulated in gastric cancer, which was significantly related to tumor stage, cell differentiation and lymph node metastasis. Hsa_circ_006100 inhibits the proliferation, migration and invasion of extracellular cells and promotes apoptosis by regulating the miR-195/GPRC5A axis. The expression of circ_0000144 was up-regulated and the expression of miR-623 was down-regulated in gastric cancer tissues and cells. Interfering with circ_0000144 could inhibit the proliferation, migration, invasion and glutamine decomposition of gastric cancer cells by up-regulating miR-623, and promote cell apoptosis (17). MiR-665 is expressed in a variety of cancers and can play a role in cell proliferation, apoptosis, migration and invasion (18, 19). For example, miR-665 was down-regulated in gastric cancer cells, and miR-665 inhibited gastric cancer cell proliferation, invasion and epithelial-mesenchymal transformation by down-regulating PPP2R2A (20).

This study showed that the expression of circSFMBT2 was up-regulated and the expression of miR-665 was down-regulated in gastric cancer tissues and gastric cancer cells. After treated with paeonol, the expression of circSFMBT2 was down-regulated and the expression of miR-665 was up-regulated in HGC-27 cells. Overexpression of circSFMBT2 could partially restore the effects of paeonol on proliferation, apoptosis, migration, invasion and glutamine decomposition of gastric cancer cells. Further results showed that circSFMBT2 targeted negative regulation of miR-665 expression, and overexpression of miR-665 could partially restore the effects of paeonol and circSFMBT2 on gastric proliferation, apoptosis, migration, invasion and glutamine decomposition, indicating that paeonol plays a role in gastric cancer cells by regulating circSFMBT2/miR-665 axis. In general gastric cancer has many factors and components that need to be carefully evaluated (21-24).

Gastric cancer is the fourth most common cancer in the world and the second leading cause of death. Environmental factors as well as genetic factors play an important role in the development and progression of this disease. The most important of these is Helicobacter pylori, which is present in most cancerous tissues (25-30). With the advent of microRNAs in the field of genetic findings, these powerful molecules have opened their place in the field of genetic diseases. miRNAs are small, non-coding molecules involved in various cellular processes, such as cell differentiation and death (31-36).

To sum up, paeonol can inhibit the proliferation, migration, invasion and glutamine decomposition of gastric cancer, and induce apoptosis, and its mechanism may be related to the regulation of the circSFMBT2/miR-665 axis.

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