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Original Research

Guizhi Fuling pills inhibit the proliferation, migration and invasion of human cutaneous malignant melanoma cells by regulating the molecular axis of LncRNA TPT1-AS1 / miR-671-5p

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Abstract: The current research was aimed to explore the effects of Guizhi Fuling Pills on the proliferation, migration and invasion of human cutaneous malignant melanoma cells and its regulation on the molecular axis of LncRNA TPT1-AS1 / miR-671-5p. Human cutaneous malignant melanoma cells A375 were cultured in vitro and randomly divided into Con group, Guizhi Fuling pills-L group, Guizhi Fuling pills-M group, Guizhi Fuling pills-H group, Guizhi Fuling pills-H + pcDNA group, Guizhi Poria pills-H + pcDNA-TPT1-AS1 group. MTT was used to detect cell proliferation. The Transwell cell test was used to detect cell migration and invasion. qRT-PCR was used to detect the expression of TPT1-AS1 and miR-671-5p. The dual-luciferase report experiment verified the targeting relationship of TPT1-AS1, miR-671-5p. Western blot was used to detect the expression of Ki-67, PCNA, MMP-2 and MMP-9. Compared with Con group, Guizhi Fuling Pills could inhibit cell proliferation, migration and invasion ($p<0.05$), and also inhibit the expression of Ki-67, PCNA, MMP-2, MMP-9, TPT1-AS1 ($p<0.05$), promote the expression of miR-671-5p ($p<0.05$)., and the differences between the indexes of Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group and Guizhi Fuling Pill-H group were statistically significant ($p<0.05$). The dual-luciferase report experiment confirmed that TPT1-AS1 could target and bind to miR-671-5p and could regulate the expression and activity of miR-671-5p. Overexpression of TPT1-AS1 could reduce the inhibitory effect of Guizhi Fuling Pills on proliferation, migration and invasion of A375 cells. Guizhi Fuling Pill may reduce the proliferation, migration and invasion of human cutaneous malignant melanoma cells by down-regulating the expression of TPT1-AS1 and up-regulating the expression of miR-671-5p.

Key words: Guizhi Fuling Pill; LncRNA TPT1-AS1; miR-671-5p; Cutaneous malignant melanoma; Proliferation; Migration; Invasion.

Introduction

Cutaneous malignant melanoma is a clinically common malignant tumor with increasing incidence year by year. Existing studies indicate that the genesis process of cutaneous malignant melanoma is a complex biological process involving multiple factors and multiple genes (1). At present, surgery, radiotherapy or chemotherapy are the major options for its clinical treatment, but patients still have a poor prognosis. Traditional Chinese medicine plays an important role in regulating tumor cell proliferation, differentiation and apoptosis, but its regulation mechanism in the treatment of cutaneous malignant melanoma has not been fully elucidated (2,3). Guizhi Fuling Pills are mainly composed of Chinese medicine ingredients like cassia twig, Poria cocos, which have anti-cancer, anti-inflammatory, antioxidant effects (4). However, the effect of Guizhi Fuling pills on cutaneous malignant melanoma has not been elucidated. LncRNA TPT1-AS1 up-regulated in cervical cancer can promote cell growth and metastasis by acting as a sponge molecule of miR-324-5p (5). Bioinformatics analysis shows that miR-671-5p may be the target gene of TPT1-AS1. miR-671-5p has down-regulated expression in gastric cancer cells. The up-regulation of its expression can inhibit the proliferation of gastric cancer cells and promote apoptosis by targeting URGCP (6). However, it remains unknown whether Guizhi Fuling Pills can affect the biological behavior of cutaneous malignant melanoma cells by regulating the molecular axis of TPT1-AS1/miR-671-5p. Therefore, this study mainly investigates the effect of Guizhi Fuling Pills on the biological behavior of cutaneous malignant melanoma cells, and explores its regulatory effect on the molecular axis of TPT1-AS1/miR-671-5p, with a view to laying the experimental foundation for further investigation into the molecular mechanism of Guizhi Fuling Pills in fighting cancer.

Materials and Methods

Materials and reagents

Guizhi Fuling pills were purchased from Shaanxi Huaxi Pharmaceutical Co., Ltd.; human cutaneous malignant melanoma cells A375 were purchased from ATCC cell bank, USA; DMEM medium and fetal bovine serum was purchased from Gibco, USA; Trizol reagent and Lipofectamine 2000 were purchased from Invitrogen, USA; reverse transcription kit and real-time fluorescence quantitative PCR kit were purchased from TaKaRa, Japan; miR-671-5p oligonucleotide mimics (miR-671-5p mimics) and negative control mimic NC

sequence (miR-NC) were purchased from Guangzhou Ruibo Biological Technology Co., Ltd.; MTT was purchased from Wuhan Amyjet Scientific Inc.; both Transwell Chamber and Matrigel were purchased from BD, USA; rabbit anti-human Ki-67 and PCNA antibodies were purchased from CST, USA; rabbit anti-human MMP-2 and MMP-9 antibodies were purchased from Santa Cruz, USA; goat anti-rabbit IgG secondary antibody labeled with horseradish peroxidase (HRP) was purchased from Abcam, USA.

Method

Experimental processing and grouping

Preparation of Guizhi Fuling Pills-containing culture solution: 45 g Guizhi Fuling Pills were weighted, decocted after soaking, and precipitated using ethanol. After ethanol recovery, it was dried with an extraction rate of 70%, added with 100 mL of culture solution for full dissolution, centrifuged at 4℃ under 3000 r/min for 5 min. The supernatant was taken, filtered and sterilized and then packed in centrifuge tubes. The solution concentration was adjusted to 2, 4, and 6 g/L (7).

A375 cells were cultured in DMEM medium containing 10% fetal bovine serum and cultured in 37°C incubator with 5%CO_2 saturated humidity. After grown to 80% confluence, the cells were digested using 0.25% trypsin and inoculated in 96-well plates (1×10^4 /well) after cell density adjustment, added with culture solution containing different concentrations (2, 4, 6 g/L) of Guizhi Fuling Pills and cultured for 24 h, which were respectively recorded as Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group, Guizhi Fuling Pill-H group. At the same time, the normal cultured cells were taken as Con group. In the subsequent experiments, pcDNA and pcDNA-TPT1-AS1 were transfected into A375 cells, added with culture solution containing 6 g/L Guizhi Fuling Pills for 24 h culture, which was respectively recorded as Guizhi Fuling Pill-H+pcDNA group, Guizhi Fuling pill-H + pcDNA-TPT1-AS1 group.

Cell proliferation detection by MTT

A375 cells (5×10^4 cells/mL) in the logarithmic growth phase were inoculated into 96-well plates (100 μL/well), grouped according to the "1.2.1" experiment, added with 20 μL MTT solution to incubate at room temperature for 4 h. After removal of the supernatant, it was added with 150 μL DMSO, incubated at room temperature with shaking for 10 min. The absorbance value (OD value) at a wavelength of 490 nm was measured using a microplate reader, and the cell viability (%) = (experimental group OD value/control group OD value) × 100%.

Cell migration and invasion detection by Transwell assay

Cell migration experiment: for each group, A375 cells (5×10^4 cells/mL) in the logarithmic growth phase were inoculated into the upper Transwell chamber (200 μL/well). Culture solution (600 μL)containing 10% fetal bovine serum was added to the lower Transwell chamber, placed in 37℃ and 5% CO_2 incubator for 24 h, washed with PBS, followed by paraformaldehyde fixation (20 min) and crystal violet dyeing (10 min) to observe the number of migrated cells. Cell invasion experiment: Matrigel (9: 1) was diluted with a pre-cooled medium and added to the upper Transwell chamber (40 μL/well), incubated for 5 h in the incubator. Subsequent experimental steps were carried out as in cell migration experiments to observe the number of invasive cells.

Detection of TPT1-AS1, miR-671-5p expression level in cells by real-time fluorescence quantitative polymerase chain reaction (Quantitative Real-time PCR, qRT-PCR)

A375 cells in the logarithmic growth phase were collected from each group, with total RNA extracted by the Trizol method. cDNA was synthesized with reference to the reverse transcription kit. QRT-PCR reaction was carried out using cDNA as a template. For the reaction system: 10×PCR Buffer 2.5 μL, $MgSO_4$ 2.5 μL, dNTPs 2.5 μL, forward and reverse primers 0.5 μL each, cDNA 2 μL, RNase-Free ddH_2O supplemented to 25 μL; For reaction conditions: 95 ℃ 60 s, 95 ℃ 60 s, 58 ℃ 30 s, 72 ℃ 30 s, a total of 36 cycles. TPT1-AS1 took GAPDH as the internal reference, while miR-671-5p took U6 as the internal reference. Relative expression of TPT1-AS1 and miR-671-5p was calculated by $2^{-\Delta\Delta Ct}$.

Detection of TPT1-AS1 target genes by dual-luciferase reporter gene

starBase prediction suggests that miR-671-5p might be the target gene of TPT1-AS1. Wild-type vector WT-TPT1-AS1 and mutant vector MUT-TPT1-AS1 were built, respectively. miR-NC, miR-671-5p mimics and WT-TPT1-AS1, MUT-TPT1-AS1 were co-transfected into A375 cells. Cells were collected 24 h after transfection and luciferase activity of each group was detected. PcDNA and pcDNA-TPT1-AS1 were transfected into A375 cells respectively, and the expression level of miR-671-5p in each group of cells was detected by qRT-PCR.

Western blot detection of Ki-67, PCNA, MMP-2, MMP-9 protein expression

A375 cells in the logarithmic growth phase were collected from each group, with total protein extracted. Protein concentration and protein denaturation were detected, and protein was isolated by SDS-PAGE, followed by membrane transfer, blocking. Ki-67, PCNA, MMP-2, MMP-9 and primary antibody dilution buffer (1: 1000) against internal reference protein were added successively, incubated at 4℃ for 24 h, washed with TBST, added with secondary antibody dilution buffer (1: 5000), incubated at room temperature for 1 h, followed by gray value analysis of each band using ImageJ software.

Statistical processing

Data were analyzed using SPSS21.0 statistical software. The measurement data were expressed as ($\bar{x}\pm s$) and all met normal distribution. An independent sample *t*-test was used for comparison between two groups, and one-way analysis of variance was used for comparison between multiple groups. $P<0.05$ indicates a statistically significant difference.

Results

Effect of Guizhi Fuling Pills on the proliferation of human cutaneous malignant melanoma cells A375

Compared with the Con group, Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group, Guizhi Fuling Pill-H group have significantly reduced cell viability (p<0.05), and significantly decreased Ki-67 and PCNA protein levels (p<0.05). The differences in cell viability and Ki-67 and PCNA protein levels are statistically significant between the Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group and Guizhi Fuling Pill-H group (p<0.05), as shown in Figure 1, Table 1.

Effect of Guizhi Fuling Pills on migration and invasion of human cutaneous malignant melanoma cells A375

Compared with the Con group, Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group, Guizhi Fuling Pill-H group have significantly reduced the number of migrating cells and invasive cells (p<0.05), and significantly decreased MMP-2, MMP-9 protein levels(p<0.05). The differences in migrating, invasive cell numbers and MMP-2 and MMP-9 protein levels are statistically significant between the Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group, Guizhi Fuling Pill-H group (p<0.05), as shown in Figure 2, Table 2.

Effect of Guizhi Fuling Pills on the expression of TPT1-AS1, miR-671-5p in human cutaneous malignant melanoma cells A375

Compared with the Con group, Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group, and Guizhi Fuling Pill-H group have significantly reduced TPT1-AS1expression levels (p<0.05), and significantly increased miR-671-5p expression levels(p<0.05). The differences in TPT1-AS1 and miR-671-5p expression levels are statistically significant between Guizhi Fuling Pill-L group, Guizhi Fuling Pill-M group and Guizhi Fuling Pill-H group(p<0.05), as shown in Table 3.

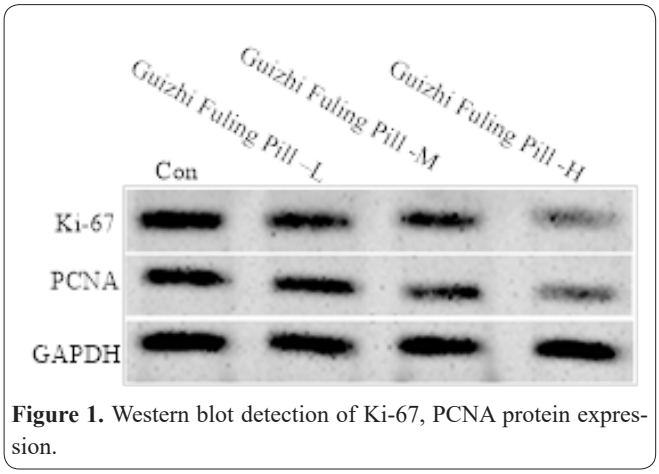

Figure 1. Western blot detection of Ki-67, PCNA protein expression.

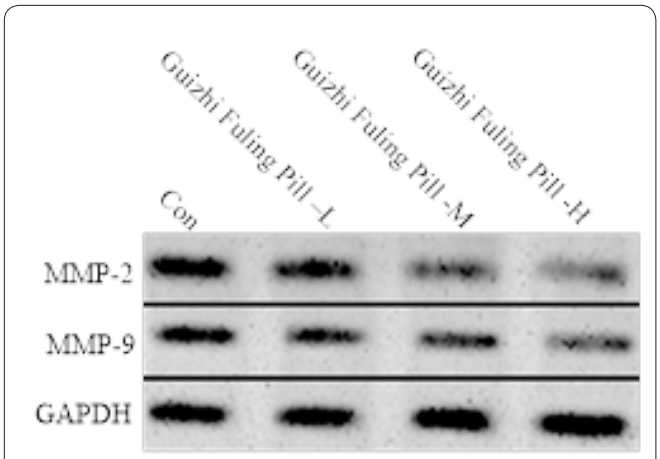

Figure 2. Western blot detection of MMP-2, MMP-9 protein expression.

Table 1. Effect of Guizhi Fuling Pills on the proliferation of human cutaneous malignant melanoma cells A375($\bar{x}$ ±s, n=9).

Group	Cell viability (%)	Ki-67	PCNA
Con	100.03±1.32	0.96±0.10	0.93±0.15
Guizhi Fuling Pill -L	86.71±5.28*	0.78±0.12*	0.71±0.07*
Guizhi Fuling Pill -M	67.10±3.88*#	0.51±0.05*#	0.50±0.09*#
Guizhi Fuling Pill -H	42.41±3.51*#&	0.28±0.05*#&	0.31±0.04*#&
F	396.796	109.500	69.372
p	0.000	0.000	0.000

Note: Compared with the Con group, *p<0.05; compared with Guizhi Fuling Pill-L group, #p<0.05; compared with Guizhi Fuling Pill-M group, &p<0.05.

Table 2. Effect of Guizhi Fuling Pills on migration and invasion of human cutaneous malignant melanoma cells A375($\bar{x}$ ±s, n=9).

Group	Number of migrating cells	Number of invasive cells	MMP-2	MMP-9
Con	126.89±16.31	90.78±10.69	0.93±0.13	0.94±0.09
Guizhi Fuling Pill-L	78.33±7.92*	66.67±9.82*	0.67±0.05*	0.69±0.09*
Guizhi Fuling Pill-M	60.22±8.38*#	50.78±4.58*#	0.44±0.09*#	0.45±0.13*#
Guizhi Fuling Pill-H	41.56±3.71*#&	36.67±3.32*#&	0.31±0.04*#&	0.32±0.05*#&
F	117.115	79.859	91.907	75.708
p	0.000	0.000	0.000	0.000

Note: Compared with the Con group, *p<0.05; compared with Guizhi Fuling Pill-L group, #p<0.05; compared with Guizhi Fuling Pill-M group,&p<0.05.

Table 3. Effect of Guizhi Fuling Pills on the expression of TPT1-AS1, miR-671-5p in human cutaneous malignant melanoma cells A375($\bar{x}$ ±*s*, n=9).

Group	TPT1-AS1	miR-671-5p
Con	1.00±0.07	1.01±0.12
Guizhi Fuling Pill-L	0.70±0.04*	1.59±0.19*
Guizhi Fuling Pill-M	0.51±0.09*#	2.09±0.19*#
Guizhi Fuling Pill-H	0.32±0.04*#&	3.23±0.29*#&
F	186.870	187.529
p	0.000	0.000

Note: Compared with the Con group, *p<0.05; compared with Guizhi Fuling Pill-L group, #p<0.05; compared with Guizhi Fuling Pill-M group, &p<0.05.

Targeted regulation of miR-671-5p by TPT1-AS1

starBase prediction indicates that the TPT1-AS1 sequence contains a nucleotide sequence complementary to miR-671-5p, as shown in Figure 3. The results of dual-luciferase reporter assay showed that in the cell experiment involving co-transfection of wild-type vector WT-TPT1-AS1, compared with the miR-NC group, the miR-671-5p group has significantly reduced luciferase activity (p<0.05). In the cell experiment involving co-transfection of mutant vector MUT-TPT1-AS1, luciferase activity of the miR-671-5p group is not significantly different from that of the miR-NC group (p>0.05), as shown in Table 4. Compared with the pcDNA group, the pcDNA-TPT1-AS1 group has significantly reduced miR-671-5p expression level (p<0.05), as shown in Table 5.

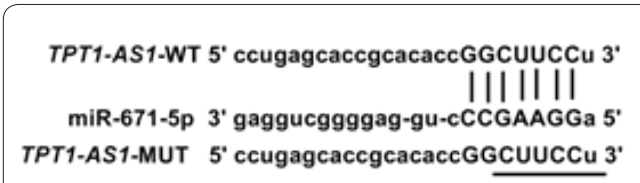

Figure 3. TPT1-AS1sequence contains a nucleotide sequence complementary to miR-671-5p.

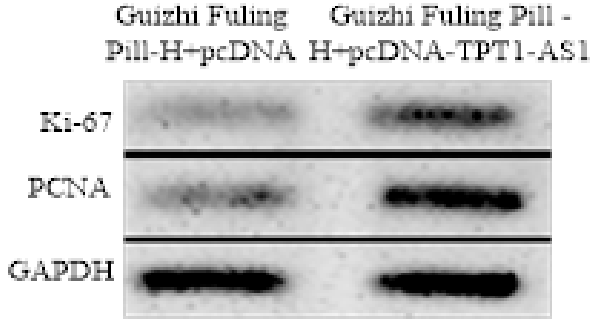

Figure 4. Western blot detection of Ki-67, PCNA protein expression.

Table 4. Dual-luciferase reporter assay ($\bar{x}$ ±*s*,n=9).

Group	WT-TPT1-AS1	MUT-TPT1-AS1
miR-NC	1.00±0.07	1.00±0.05
miR-671-5p	0.59±0.10*	1.01±0.08
t	10.077	0.318
p	0.000	0.755

Note: Compared with the miR-NC group, *p<0.05.

Table 5. Targeted regulation of miR-671-5p expression by TPT1-AS1($\bar{x}$ ±*s*, n=9).

Group	miR-671-5p
pcDNA	1.01±0.08
pcDNA-TPT1-AS1	0.34±0.05*
t	21.306
p	0.000

Note: Compared with the pcDNA group, *p<0.05.

Overexpression of TPT1-AS1 can reduce the inhibitory effect of Guizhi Fuling Pills on the proliferation of human cutaneous malignant melanoma cells

Compared with the Guizhi Fuling Pill-H+pcDNA group, Guizhi Fuling Pill-H + pcDNA-TPT1-AS1 group has significantly increased cell viability (p<0.05), and significantly increased Ki-67, PCNA protein levels(p<0.05), as shown in Figure 4, Table 6.

Overexpression of TPT1-AS1 can reduce the inhibitory effect of Guizhi Fuling Pills on migration and invasion of human cutaneous malignant melanoma cells

Compared with the Guizhi Fuling Pill-H+pcDNA group, the Guizhi Fuling Pill-H+pcDNA-TPT1-AS1 group has a significantly increased number of migra-

Table 6. Overexpression of TPT1-AS1 can reduce the inhibitory effect of Guizhi Fuling Pills on the proliferation of human cutaneous malignant melanoma cells($\bar{x}$ ±*s*, n=9).

Group	TPT1-AS1	miR-671-5p	Cell viability (%)	Ki-67	PCNA
Guizhi Fuling Pill-H+pcDNA	0.33±0.04	3.16±0.32	40.81±4.16	0.26±0.03	0.31±0.05
Guizhi Fuling Pill-H+pcDNA-TPT1-AS1	0.85±0.11*	1.20±0.26*	61.23±2.51*	0.69±0.18*	0.75±0.08*
t	13.328	14.261	12.609	7.069	13.992
p	0.000	0.000	0.000	0.000	0.000

Note: Compared with Guizhi Fuling Pill--H+pcDNA group, *p<0.05.

Table 7. Overexpression of TPT1-AS1 can reduce the inhibitory effect of Guizhi Fuling Pills on migration and invasion of human cutaneous malignant melanoma cells($\bar{x}$ ±*s*, n=9).

Group	Number of migrating cells	Number of invasive cells	MMP-2	MMP-9
Guizhi Fuling Pill-H+pcDNA	42.33±3.91	38.11±3.89	0.35±0.05	0.33±0.09
Guizhi Fuling Pill-H+pcDNA-TPT1-AS1	86.67±6.86*	75.22±8.07*	0.83±0.18*	0.75±0.11*
t	16.846	12.427	7.708	8.865
p	0.000	0.000	0.000	0.000

Note: Compared with the Guizhi Fuling Pill-H+pcDNA group, *p<0.05.

ting cells and invasive cells(p<.05), and significantly increased MMP-2, MMP-9 protein levels(p<0.05), as shown in Figure 5, Table 7.

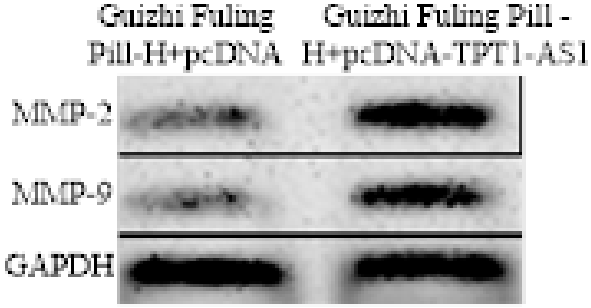

Figure 5. Western blot detection of MMP-2, MMP-9 protein expression.

Discussion

Cutaneous malignant melanoma is prone to early metastasis, making most patients less sensitive to radiotherapy or chemotherapy drugs. Cutaneous malignant melanoma is concealed, so patients are already in the progressive or even late stage when the diagnosis is made. Radices trichosanthis can induce apoptosis of cutaneous malignant melanoma cells (8). LncRNA with abnormal expression in cutaneous malignant melanoma cells can regulate cell proliferation, migration and invasion (9). However, it remains unclear whether Guizhi Fuling pills can regulate the biological behavior of cutaneous malignant melanoma cells by regulating the expression of LncRNA.

Guizhi Fuling pills can achieve therapeutic purposes by inhibiting the proliferation of tumor cells like breast cancer (10,11). This study showed that after adding different concentrations of Guizhi Fuling Pills, A375 cells have significantly decreased viability with the increase of the dosage, suggesting that Guizhi Fuling Pills can inhibit the proliferation of cutaneous malignant melanoma cells in a dose-dependent manner. Studies have shown that Ki-67 and PCNA up-regulated in tumors can promote cell proliferation (12). This study indicated that Guizhi Fuling pills can inhibit Ki-67 and PCNA expression, making it significantly down-regulated as the dosage increases, suggesting that Guizhi Fuling pills may inhibit proliferation of cutaneous malignant melanoma cells by regulating the expression of Ki-67 and PCNA. Studies have shown that matrix metalloproteinases have close relation with tumor cell metastasis. MMP-2 and MMP-9 are matrix metalloproteinases, whose increased expression levels in tumors can promote tumor cell migration and invasion (13). This study indicated that Guizhi Fuling pills can inhibit the migration and invasion of A375 cells, and significantly reduce the expression levels of MMP-2 and MMP-9, suggesting that Guizhi Fuling pills may inhibit migration and invasion of cutaneous malignant melanoma cells by regulating the expression of MMP-2 and MMP-9.

TPT1-AS1 can promote angiogenesis and metastasis of colorectal cancer (14). Up-regulation of TPT1-AS1 expression can promote tumorigenesis and metastasis of epithelial ovarian cancer (15). CircPIP5K1A can act as a sponge molecule for miR-671-5p to promote the development of gastric cancer (16). miR-671-5p inhibits osteosarcoma cell proliferation by blocking the cell cycle (17). By dual-luciferase reporter assay and qRT-PCR experiment, this study confirmed that TPT1-AS1 can target and bind to miR-671-5p, and negatively regulate its expression. Further study showed that after adding Guizhi Fuling pills, the expression level of TPT1-AS1 was significantly reduced, while the expression level of miR-671-5p was significantly increased. It is, therefore, speculated that Guizhi Fuling Pill may inhibit proliferation, migration and invasion of malignant cutaneous melanoma cells by inhibiting the expression of TPT1-AS1 and promoting the expression of miR-671-5p. This study showed significantly enhanced cell proliferation, migration and invasion capabilities, and significantly increased expression levels of Ki-67, PCNA, MMP-2, and MMP-9, indicating that overexpression of TPT1-AS1 can reduce the inhibitory effect of Guizhi Fuling pills on proliferation, migration and invasion of human cutaneous malignant melanoma cell. It is suggested that Guizhi Fuling Pills may affect the proliferation, migration and invasion of cutaneous malignant melanoma cells by regulating the expression of TPT1-AS1/miR-671-5p.

The associations of non-coding RNAs with genomic lesions, colorectal primary lesions, in cancers, and even in the rate of fetal growth such as intrauterine growth restriction (IUGR), highlight the importance of miRNAs (34-36).

Gene expression is the process by which information is used within a gene to produce a functional product. Gene regulation allows the cell to control its structure and function, and this is the basis for cellular differences (differentiation), transformation (evolution), and the ability of organisms to adapt to new conditions. Genetic modification is the key word in the medical world in the near future. A technology called "CRISPR" technology with the ability to cut, move and regulate the genes of the human body in order to treat diseases and increase longevity and perhaps even the immortality of mankind (18-33). In current research, the expression of miR-671-5p was studied.

To conclude, Guizhi Fuling Pills can inhibit the pro-

liferation, migration and invasion of cutaneous malignant melanoma cells. The mechanism of action may be related to the inhibition of TPT1-AS1 expression and the promotion of miR-671-5p expression, which may provide a scientific basis for further revealing the molecular mechanism of Guizhi Fuling Pills in inhibiting cutaneous malignant melanoma, and also provide a new direction for the treatment of cutaneous malignant melanoma.

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