Original Article
Oxymatrine inhibits proliferation and apoptosis of human breast cancer cells through the regulation of miRNA-140-5P

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Abstract: Objective: Oxymatrine has shown strong anti-cancer ability, but its mechanism is not well-studied. Methods: The inhibitory rates of oxymatrine with various concentrations (0, 1, 2, 4, 6, 8, 16, 32 mg/ml) on MCF-7 cells were detected by CCK-8. The effects of oxymatrine on the expression of miRNA-140-5P in MCF-7 cells were detected by real-time fluorescent quantitative PCR (RT-PCR). miRNA-140-5P mimics or NC mimics were transfected into cells using Lipofectamine 2000. Eventually, the cells were divided into control-group, drug-group, miRNA-140-5P mimics group, NC mimics group, and miRNA-140-5P mimics + drug group. Cell viability was detected by CCK-8 assay and apoptosis rate of each group were measured by using Flow cytometry. Western blot was carried out to detect the protein expression of TGFBR1 and FGF9. Results: Oxymatrine at various concentrations had conspicuous inhibitory effect on the proliferation of MCF-7 cells ($P<0.05$), and the inhibitory effect of oxymatrine on MCF-7 cells showed both dose- and time-dependent manners. The relative expression of miRNA-140-5P in MCF cells was remarkably lower than that in MCF-10A. Oxymatrine could effectively promote the expression of miRNA-140-5P in MCF-7 cells, and the relative expression of miRNA-140-5P increased significantly with the increased dose of oxymatrine ($P<0.05$). Both transfection of miRNA-140-5P mimics and oxymatrine treatment could reduce the proliferation of MCF-7 cells ($P<0.05$), and the proliferation of cells in miRNA-140-5P mimics + drug-group was significantly lower than that of other groups ($P<0.05$). Compared with the control-group, the protein expressions of TGFbR1 and FGF9 in low-dose, medium-dose and high-dose groups were dramatically decreased ($P<0.05$), in a dose-dependent manner ($P<0.05$). Conclusion: Oxymatrine inhibits proliferation and promotes cell apoptosis of breast cancer MCF-7 cells. The mechanism may contribute to the regulation of miRNA-140-5p and its target genes.

Keywords: Oxymatrine, miRNA-140-5P, breast cancer, cell proliferation, apoptosis

Introduction

Breast cancer is one of the most common female malignant tumors worldwide and is also the major cause of cancer-related deaths, which poses a serious threat to women's health and life safety [1]. Although the screening and therapeutic methods of breast cancer have been continuously improved over the years, the pathogenesis of breast cancer has not been fully clarified yet. Thus, breast cancer is still a major public health challenge [2]. The treatment of breast cancer is basically dependent on surgery supplemented by radiotherapy

and/or chemotherapy. However, the side effects, drug resistance, and high cost of radiotherapy and chemotherapy have become the main obstacles to cure breast cancer. Therefore, it is of great urgency to explore new method with good curative effect and little side effects [3, 4]. Oxymatrine, the main active components in Chinese herbal medicine Sophora flavescens, exerts strong potentials in anti-inflammatory, anti-arrhythmic and anti-tumor effects [5]. Studies have revealed that oxymatrine has remarkable inhibitory effects on a variety of tumors such as cervical cancer, lung cancer, liver cancer, etc. However, mechanism

of how oxymatrine affects the tumor cell growth remains unclear [6]. In clinical work, there are currently a small number of reports showing that the use of oxymatrine injection (oxymatrine) can help improve the efficacy of chemotherapy in breast cancer patients. Oxymatrine injection also improves the level of white blood cells in breast cancer patients [7]. miRNA is a non-coding small RNA that widely exists in eukaryotes and regulates the level of genes after transcription. miRNA-140-5P is a popular miRNA in recent years which is considered to exert regulatory effects on tumor immune micro-environment [8]. It has been found that miRNA-140-5P expression level is increased in many types of tumors including non-small cell lung cancer, breast cancer, osteosarcoma and so on [9, 10]. This study aimed to find out whether the anti-tumor effects of oxymatrine on breast cancer was through regulation of micRNA-140-5P.

Material and methods

Cells and reagents

Breast tumor MCF-7 cells and healthy breast MCF-10A cells were procured from American Type Culture Collection (ATCC). Oxymatrine was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. The purchase information of the remaining test reagents and materials are as follows: DMEM media and fetal bovine serum (Gbico, USA); trypsin (Hyclone, Inc.); Trziol reagent (Invitrogen, USA); M-MLV reverse transcription kit and SYBR Green PCR kit (Takara, Japan); Annexin V FITC/PI Apoptosis Kit (Jiangsu Kaiji Biotechnology Co.); CCK-8 reagent (Wuhan Boster Bioengineering Co., Ltd.); TGFBR1 antibody (Thermo Fisher, Inc., USA) and FGF9 Antibody (Abcam, USA).

Culture and grouping of cells

Both MCF-7 and MCF-10A were cultured in high-glucose DMEM medium that contains 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were cultured in an incubator under 37°C with 5% of CO_2, and the culture medium was replaced on alternate days. 0.25% trypsin was used for digestion and subculture when cell confluence reached 85%, and the cells in logarithmic-growth-phase were chosen for experimental detection.

Detection of inhibitory effect of oxymatrine on proliferation of MCF-7 cells by CCK-8

The cell concentration was adjusted to 5×10^4 cells/ml, and the cells were seeded in a 96-well plate with 200 μl per well. After the cells adhered to the wall, different concentrations of oxymatrine were added (adjusted final concentration was 0, 1, 2, 4, 6, 8, 16, and 32 mg/mL), and 5 replicas were set for each concentration. After culturing for 24 h, 48 h, and 72 h, 10 μl/well of CCK-8 reagent was added to the cells. The cells were placed in an incubator for further culture for 2 h, and the absorbance value (A) at 450 nm was detected with a microplate analyzer.

Detection of the effect of oxymatrine on miRNA-140-5P expression in MCF-7 cells by real-time fluorescence quantitative PCR (RT-PCR)

2 ml MCF-7 cells in logarithmic-growth-phase at the concentration of 2.5×10^5 cells/ml were inoculated into a 6-well plate and cultured at 37°C and 5% CO_2. After the cell adherence, 0 mg/mL oxymatrine (control group), 4 mg/mL oxymatrine (low-dose group), 8 mg/mL oxymatrine (medium-dose group) and 16 mg/mL oxymatrine (high-dose group) were added respectively and collected after continuous culture for 48 h. The total RNA of each group of cells was extracted with Trizol reagent, and the purity was detected with ultraviolet spectrophotometer. RNA was reverse-transcribed into cDNA by reverse transcription kit and RT-PCR was performed with 2×SYBR Premix Ex Taq II. With GAPDH as the internal reference gene, the relative expression level of miRNA-140-5P was calculated by $2^{-\Delta\Delta CT}$. The primer sequences were as follows: The forward primer of miRNA-140-5P was 5'-ACACTCCCCAGAGCCTGTCCTG-AGCTGGGTC-3', and the reverse primer was 5'-TGGTTGGAG TGTCGCG-3'; The forward primer of GAPDH was 5'-GCTTCGCACATGGCAATA-CT-3', and the reverse primer was 5'-AACG-AATTTCTTCA CGGCGT-3'.

Cell transfection

2 ml MCF-7 cells in logarithmic-growth-phase at the concentration of 2.5×10^5 cells/ml were inoculated into a 6-well plate. When the cells

were fused to 40-50%, Lipofectamine 2000 transfection reagent was used to transfect miRNA-140-5P mimics and NC mimics into the cells. The cells were divided into control-groups (normal culture without any treatment), drug-group (treated with 8 mg/ml oxymatrine), miRNA-140-5P mimics group (transfected with miRNA-140-5P mimics), NC mimics group (transfected with NC mimics), and miRNA-140-5P mimics + drug-group (transfected with miRNA-140-5P mimics combined with 8 mg/mL oxymatrine treatment). The treatment method of oxymatrine was the same as above.

miRNA-140-5P-mimics transfection combined with oxymatrine treatment in MCF-7 cells

Cells in each group were treated according to the cell treatment and grouping method as described in the section above. The absorbance value of each group at 450 nm was detected by CCK-8 method.

Detection of apoptosis by flow cytometry

2 ml MCF-7 cells in logarithmic-growth-phase at the concentration of $2.5×10^5$ cells/ml were inoculated into a 6-well plate and cultured at 37°C and 5% CO_2. The cells were treated according to the cell grouping and cultured for 48 h, and then collected in the tube. According to the instructions of Annexin V-FITC kit, 150 μL Annexin V-FITC binding solution, 3 μL Annexin V-FITC and 2 μL PI dye were added into each tube to resuspend the cells successively, and then incubated at room temperature for 15 min. Each sample tube was added with PBS to 500 μl, and the undigested cell clusters were removed by using a 300-mesh sieve. The sample was submitted to flow cytometer within 30 min. Cell Quest software was used to analyze the apoptosis of cells in each group.

Luciferase reporter assays

The putative complementary sequences of miR-140-5p were identified in the 3'UTR of TGFBR1 and FGR9 mRNAs. We inserted the two different parts of 3'UTR of aforementioned mRNAs into the vector, constructing two different luciferase plasmids p-TGFBR1 and P-FGR9, respectively. The MCF-7 cells were seeded in a 24-well plate, and co-transfected with TGFBR1 and FGR9. After 48 h incubation, relative lucif-

erase activities were accessed by using a Dual Luciferase Reporter Assay Kit (Promega, WI).

Detection of oxymatrine on TGFBR1 and FGF9 proteins expression in each group of cells by Western blot

MCF-7 cells at the concentration of $1×10^6$ cells/ml were inoculated into a 6-well plate. The cells were treated according to the cell grouping and cultured for another 48 h. After treatment, the cells were collected by centrifugation for protein isolation. The cell pellets were mixed with protein lysate buffer, and incubated on ice for 10 min. The cell lysate was centrifuged at 12000×g at 4°C for 10 min. The supernatant was taken as the protein lysate and the protein concentration was determined by BCA assay. The electrophoresis samples, SDS-PAGE separation gel and concentrate gel were prepared, 20 μL samples with same total protein amounts were loaded in each well, and the transfer to membrane was conducted after gel electrophoresis. The PVDF membrane was blocked with 5% milk for 60 min under room temperature. The PVDF membrane was cleaned with TBST for 3 times with 10 min for each time. After blocking, the PVDF membrane was incubated with TGFBR1 (Abcam, USA. Ab31013. diluted at 1:1500) and FGF9 antibodies (Abcam, USA. Ab206408. diluted at 1:1500) at 4°C overnight, respectively. Subsequently, the sample was rinsed again with TBST for 3 times, 10 min each time, and incubated with secondary antibody (Abcam, USA. Ab205718. diluted at 1:5000) under room temperature for 1 h. After the secondary antibody incubation, the PVDF membrane was washed 3 time with TBST. To show the bands, the PVDF membrane was added with ECL luminescent droplets, and placed into the Chemiscope gel imaging system for image.

Statistical analysis

SPSS 22.0 software was used for data analysis in this study. The measurement data was expressed by mean ± standard deviation ($\bar{x}$ ±sd), and compared by analysis of variance, and the paired comparison between groups was done by two independent sample t test. The difference was considered statistically significant if $P<0.05$.

Table 1. The inhibitory effect of oxymatrine on MCF-7 cells (n=5, %, $\overline{x}\pm$sd)

Concentration of oxymatrine (mg/ml)	24 h	48 h	72 h
0	0.00±0.000	0.00±0.000	0.00±0.000
1	2.36±0.72*	6.95±1.47*,#	11.98±2.11*,#,Δ
2	6.28±1.63*	13.82±2.78*,#	20.03±2.19*,#,Δ
4	13.46±3.56*	18.35±5.21*,#	27.93±3.75*,#,Δ
6	19.80±5.38*	26.36±7.52*,#	42.64±11.06*,#,Δ
8	25.62±8.33*	39.37±5.77*,#	49.57±10.62*,#,Δ
16	37.45±7.61*	51.75±8.26*,#	61.37±7.31*,#,Δ
32	50.69±6.82*	63.47±7.93*,#	65.32±6.93*,#,Δ

Note: Compared with 0 mg/ml at the same time, *$P<0.05$; Compared with 24 h at the same concentration, #$P<0.05$; and compared with 48 h at the same concentration, Δ$P<0.05$.
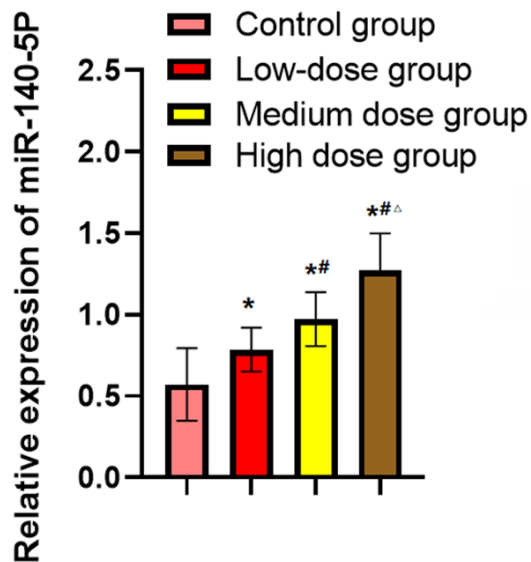
Figure 1. Effects of oxymatrine on miRNA-140-5P expression in MCF-7 cells. Note: Compared with control-group, *$P<0.05$; Compared with low-dose group, #$P<0.05$; Compared with medium-dose group, Δ$P<0.05$.

Results

Effects of oxymatrine on proliferation of MCF-7 cells

Oxymatrine at various concentrations had conspicuous inhibitory effect on the proliferation of MCF-7 cells ($P<0.05$), and the inhibitory effect of oxymatrine on MCF-7 cells showed a dose- and time-dependent manner (**Table 1**). For example, the IC$_{50}$ of oxymatrine in 24 h incubation group was around 32 mg/mL, while the IC$_{50}$ in 48 h treatment group was less than

16 mg/mL. The highest inhibitory effect of oxymatrine was found in the 72 h incubation group.

Effects of oxymatrine on miRNA-140-5P expression in MCF-7

Oxymatrine could effectively promote the expression of miRNA-140-5P in MCF-7 cells in a dose-dependent manner ($P<0.05$) (**Figure 1**).

Effects of transfection of miRNA-140-5P mimics combined with oxymatrine on proliferation of MCF-7 cells

Both transfection of miRNA-140-5P mimics and oxymatrine treatment could reduce the proliferation of MCF-7 cells ($P<0.05$), and the cell proliferation in miRNA-140-5P mimics + drug-group was notably lower than that of the other groups (all $P<0.05$) (**Figure 2**).

Effects of transfection of miRNA-140-5P-mimics combined with oxymatrine on apoptosis of MCF-7 cells

Both miRNA-140-5P mimics transfection and oxymatrine treatment could increase the apoptosis of MCF-7 cells ($P<0.05$), and the apoptosis in miRNA-140-5P mimics + drug group was obviously higher than that of other groups (all $P<0.05$) (**Figure 3**).

miR-140-5P targets TGFBR1 and FGR9

Bioinformatics software, including TargetScan, PicTar and miRanda, was used for a comprehensive analysis. It was found that TGFBR1 and FGR9 may be potential downstream target genes of miR-140-5P. The binding sites of miR-140-5P with 3'UTR of TGFBR1 and FGR9 are shown in **Figure 4A**.

Luciferase report assay showed that the fluorescence intensity in the wild-type (WT) groups, TGFBR1-WT and FGR9-WT, decreased significantly (P<0.05) than that in MCF-7 cells with miR-140-5P overexpression. However, no significant difference in fluorescence intensity was found in TGFBR1 and FGR9 mutant (Mut) group (P>0.05).

Those results suggest that miR-140-5P could bind to the 3'UTR region of TGFBR1 and FGR9
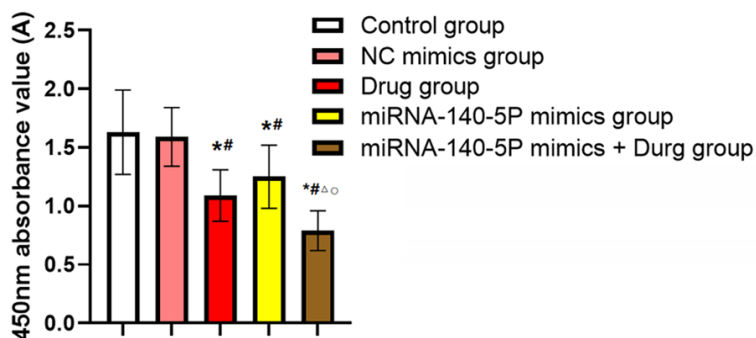
Figure 2. Absorbance values of each group at 450 nm. Note: Compared with the control-group, *$P<0.05$; Compared with NC-mimics group, #$P<0.05$; Compared with miRNA-140-5P mimics group, △$P<0.05$, and compared with drug-group, ○$P<0.05$.

directly to inhibit the expression of TGFBR1 and FGF9 (**Figure 4B**).

Expressions of TGFbR1 and FGF9 in each group

Compared with the control-group, the protein expressions of TGFbR1 and FGF9 in low-dose, medium-dose and high-dose groups decreased dramatically ($P<0.05$), and showed a significant decrease with the increased oxymatrine concentration in a dose-dependent manner ($P<0.05$) (**Figure 5**).

Discussion

According to the National Cancer Center (NCC) of China, the fatality rate due to breast tumor in China has been ascending year by year, and the age of breast cancer patients is gradually becoming younger. The NCC has predicted that by 2030, the mortality rate of breast cancer will be up to 47.94% [11]. The current therapeutics for breast cancer mainly includes surgery, chemotherapy, radiotherapy, immunotherapy, etc. Studies of ingredients from natural products, which show strong anti-tumor activity and low toxicity, are also a trend in current anti-tumor research [12, 13]. Oxymatrine, which is extracted from conventional Chinese medicine Sophora flavescens, is an alkaloid with a variety of pharmacological effects. Studies have shown that oxymatrine has anti-inflammatory, anti-bacterial and anti-oxidant effects [14, 15]. In addition, it has also revealed that oxymatrine can restrain the proliferation and induce apoptosis of various tumor cells, reverse multidrug resistance, and inhibit tumor angiogenesis, invasion and metastasis [16, 17]. Although there are many researches on the antitumor effects of oxymatrine, its molecular mechanism has not been fully understood.

CCK-8 showed that with the increase of oxymatrine concentration, the absorbance value at 450 nm decreased remarkably in a dose-dependent way. This result elaborated that oxymatrine can effectively restrain the proliferation of MCF-7, and its inhibitory effect increases obviously with the increase of drug concentration [18, 19]. Apoptosis is the autonomous and orderly death of cells controlled by genes in order to maintain a stable environment. At present, it is known that the development of cancer is highly associated with the absence of apoapsis induction in the tumor cells. Therefore, the selective induction of apoptosis in tumor cells has become one of the promising strategies for developing anti-cancer therapeutics [20]. The results of this study illustrated that as oxymatrine concentration increased, the apoptotic rate of cells also increased markedly. This suggests that oxymatrine can not only inhibit the proliferation of MCF-7, but also effectively promote cell apoptosis, thereby exerting a distinctive tumor suppressor effect.

miRNAs are a class of endogenous small RNAs with around 20-24 nucleotides and have been identified as the regulator in various biological process [21]. One miRNA may have multiple target genes, and several miRNAs can regulate the same gene. Previous studies have revealed that the miRNA-140-5P has abnormal expression in many types of tumors, and the differential RNA expression profile is tightly related to the tumorigenesis and progression of many tumor cells [22]. Studies [23] have shown that miRNA-140-5P is involved in invasion and migration of NSCLC. Decreased expression level of miRNA-140-5P reduces the cells' adhesion to outer stromal cells. Overexpression of miRNA-140-5P in NSCLC cells showed a clear inhibition on cancer cell growth. In this study, the expression level of miRNA-140-5p in normal breast MCF-10A cells and tumor MCF-7 cells was compared. The results showed that the
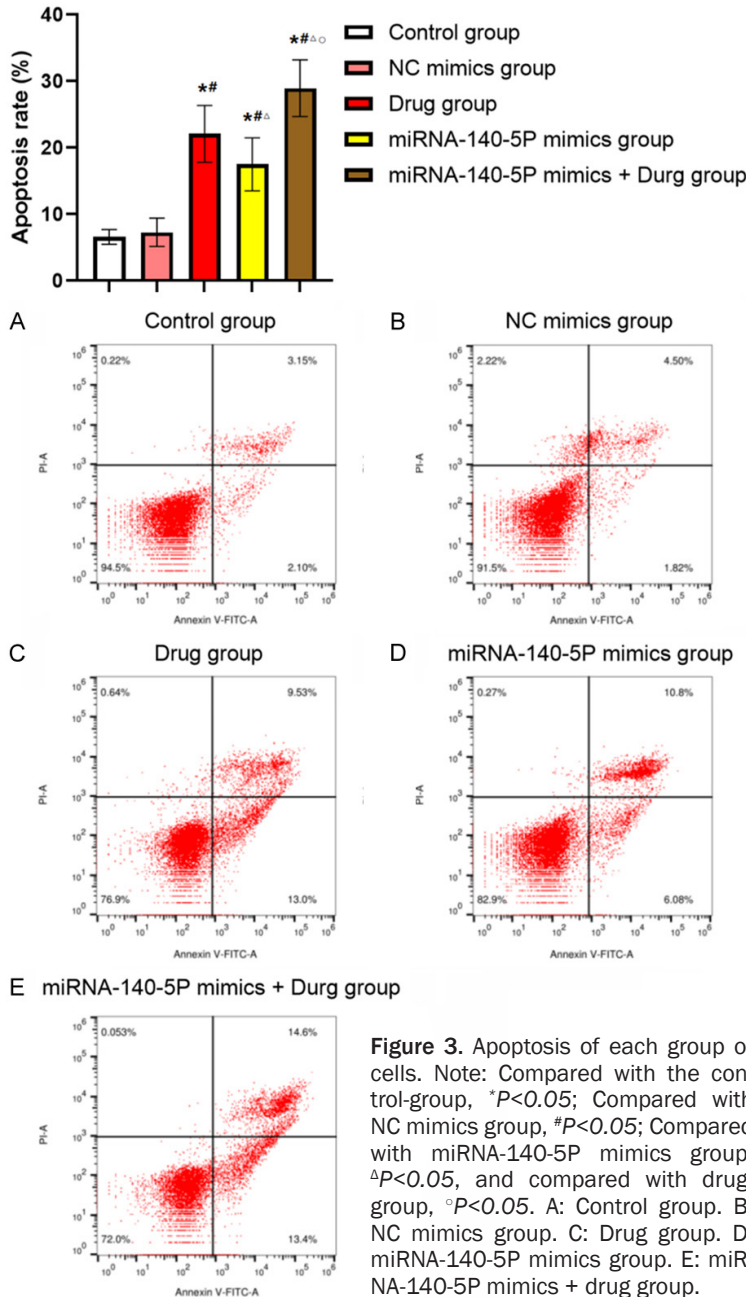
Figure 3. Apoptosis of each group of cells. Note: Compared with the control-group, $^*P<0.05$; Compared with NC mimics group, $^\#P<0.05$; Compared with miRNA-140-5P mimics group, $^\Delta P<0.05$, and compared with drug-group, $^\circ P<0.05$. A: Control group. B: NC mimics group. C: Drug group. D: miRNA-140-5P mimics group. E: miRNA-140-5P mimics + drug group.

proliferative restrictions and apoptotic promotion effect on cells.

In addition, several studies [26-28] have identified the TGFbR1 and FGF9, two key proteins in tumor-related TGF-β signaling pathways, as the down-stream target genes of miR-140-5P. TGFbR1 and FGF9 play an important role in activation of TGF-β signaling pathway, which promotes tumor cell growth and enhances the ability to environment stress. It has been elegantly proven in the liver cancer cells that miR-140-5P mainly targeted TGFbR1 and FGF9. Thus, it is rational to expect that the oxymatrine can modulate the cell activity via the miR-140-5P/TGFbR1 axis in breast cancer cells. In this study, Western blot was applied to detect the protein expression of miR-140-5p target genes TGFBR1 and FGF9. TGFbR1 and FGF9 expressions in low-dose, medium-dose and high-dose groups decreased dramatically as compared with control-group. And the inhibitory rates of TGFbR1 and FGF9 protein expression were proportional to the oxymatrine concentration, suggesting that oxymatrine can inhibit the proliferation of breast cancer cells by the regulation of miRNA-140-5P.
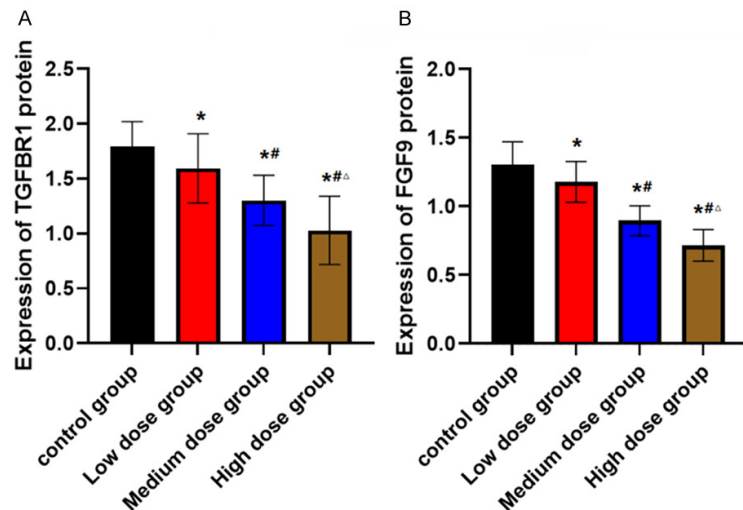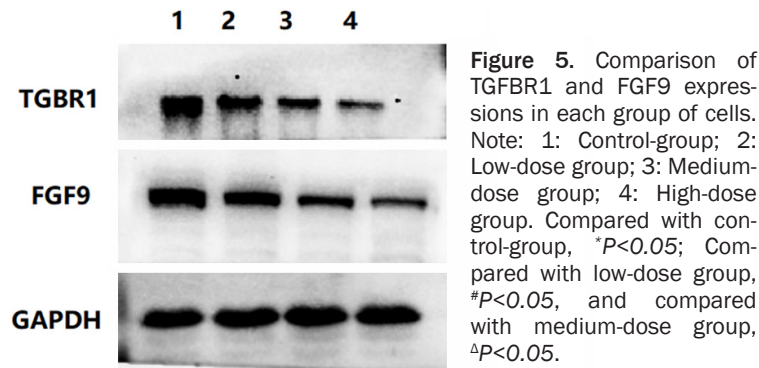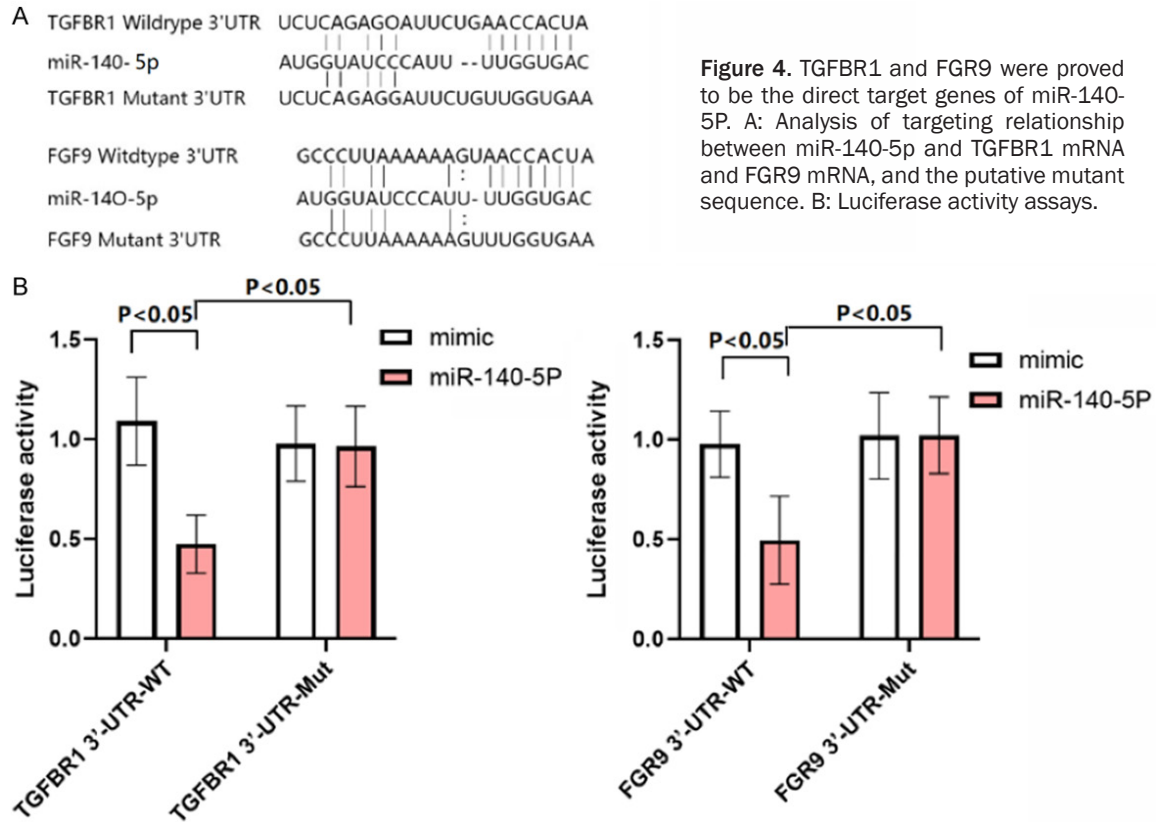
relative miRNA-140-5p expressions in MCF-7 were remarkably lower than that in MCF-10A cells. In fact, the inhibition of miRNA-140-5p expression has been reported in many types of cancer cells [24, 25]. After treated with oxymatrine, the relative expression of miRNA-140-5p in the cells of each group was significantly higher than that of the control-group, which was in a dose-dependent manner. This indicates that oxymatrine can promote miRNA-140-5P expression in MCF-7 cells, and this may be the mechanism of oxymatrine exerting

We further analyzed the effects of miRNA-140-5P and oxymatrine on MCF-7 cells, and the results illustrated that the transfection of miRNA-140-5P mimics and the treatment of oxymatrine could both reduce the proliferation activity of MCF-7 cells. And the cell proliferation activity of miRNA-140-5P mimics + drug group was substantially lower than that of other groups. Both miRNA-140-5P mimics transfection and oxymatrine treatment could increase the apoptosis of MCF-7 cells, and the apoptosis of miRNA-140-5P mimics + drug group was

A

TGFBR1 Wildrype 3'UTR	UCUCAGAGOAUUCUGAACCACUA
	‖ ‖ ‖ ‖ ‖ ‖ ‖ ‖ ‖ ‖
miR-140- 5p	AUGGUAUCCCAUU --UUGGUGAC
TGFBR1 Mutant 3'UTR	UCUCAGAGGAUUCUGUUGGUGAA

FGF9 Witdtype 3'UTR	GCCCUUAAAAAAGUAACCACUA
	‖ ‖ ‖ : ‖ ‖ ‖ ‖ ‖
miR-14O-5p	AUGGUAUCCCAUU- UUGGUGAC
FGF9 Mutant 3'UTR	GCCCUUAAAAAAGUUUGGUGAA

Figure 4. TGFBR1 and FGR9 were proved to be the direct target genes of miR-140-5P. A: Analysis of targeting relationship between miR-140-5p and TGFBR1 mRNA and FGR9 mRNA, and the putative mutant sequence. B: Luciferase activity assays.

B

Figure 5. Comparison of TGFBR1 and FGF9 expressions in each group of cells. Note: 1: Control-group; 2: Low-dose group; 3: Medium-dose group; 4: High-dose group. Compared with control-group, *P<0.05*; Compared with low-dose group, #P<0.05, and compared with medium-dose group, △P<0.05.

remarkably higher than that of other groups. This suggests that miRNA-140-5P mimics and oxymatrine treatment can effectively inhibit the proliferation of MCF-7 cells and promote cell apoptosis. Moreover, the combined application of miRNA-140-5P mimics and oxymatrine has better anti-tumor effect. The major limitation of this work is that we didn't use the animal model to verify the mechanism of oxymatrine *in vivo*.

In summary, oxymatrine can inhibit the *in vitro* proliferation activity of MCF-7 cells and promote the cell apoptosis, possibly through the up-regulation of miRNA-140-5p and its target genes.

Acknowledgements

Disclosure of conflict of interest

None.

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