

Effect of aromatase inhibitor letrozole on the proliferation of spermatogonia by regulating the MAPK pathway

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Abstract. The molecular mechanism of the aromatase inhibitor letrozole was investigated. It promotes the proliferation of spermatogonia by regulating the mitogen-activated protein kinase (MAPK) pathway. Six different concentrations were selected for letrozole in order to incubate mouse spermatogonia [GC-1 spermatogonia (spg)] for 24, 48 and 72 h, respectively. Cell Counting Kit-8 (CCK-8) was used to observe the effect of letrozole on the proliferation of GC-1 spg cells, and the effect was further verified by cell plate clone formation assay. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis were used to detect the effects of letrozole on MAPK signaling pathways [Ras/extracellular signal-regulated kinase 1 (ERK1)/c-Myc], proliferation indexes [Ki-67 and proliferating cell nuclear antigen (PCNA)]. Bromodeoxyuridine (BrdU) staining was used to study the effects of letrozole and MAPK signaling pathways on cell proliferation. The results of CCK-8 showed that the proliferation rate of GC-1 spg cells was improved. Study results also revealed a significant increase in letrozole concentration along with the time of action. The results of plate clone formation assay further indicated that letrozole could significantly promote the proliferation capacity of GC-1 spg cells ($p < 0.05$). The results of RT-PCR and western blot analysis confirmed letrozole significantly activated the expression of Ras/ERK1/c-Myc in the classical MAPK pathway. A significant increase was noted in the protein levels of Ki-67 and PCNA ($p < 0.05$). By contrast, inhibition of the MAPK pathway resulted in a significant decrease in the levels of the above indexes ($p < 0.05$). The number of BrdU cells in the letrozole group was also higher than that of the control group, while the number of BrdU-stained cells in the letrozole + MAPK inhibition group showed a significant decrease in comparison to the letrozole group. In conclusion, letrozole activated the MAPK signaling pathway and promoted

the proliferation of mouse spermatogonia GC-1 spg cells. The present study provides a theoretical basis for the clinical application of letrozole.

Introduction

Infertility is an important global health problem that causes great distress to 15% of couples (1). Findings have shown that 50-60% of the cases are due to male factors. Semen abnormalities including oligozoospermia and asthenozoospermia or azoospermia have been reported to be the main causes of infertility in men (2). Although the current test-tube baby technique solves the fertility problems of some patients, the technique has many shortcomings such as low success rate, high cost and the proneness of fetus to disease (3).

At present, the important treatment methods for oligozoospermia, asthenozoospermia and other symptoms caused by different pathological conditions in males include endocrine therapy, anti-inflammatory therapy, supplementation of trace elements and arginine (4,5). However, there is still a lack of clinically effective drug treatments for the above diseases. Letrozole is a new generation of highly selective aromatase inhibitor. It is a synthetic benzotriazole derivative that is used mainly in the radiotherapy and chemotherapy for breast cancer (6,7). A recent report also suggested the use of the aromatase inhibitor letrozole for the successful treatment of male infertility and human non-obstructive azoospermia (8). However, its molecular mechanism is not yet clear. Thus, the relationship between letrozole and mitogen-activated protein kinase (MAPK) signaling pathways and their respective effects on the proliferation of mouse spermatogonia were focus areas of the present study.

The primary aim of the present study was to explore mechanism of action of letrozole that has been shown to promote the MAPK pathway including RAS. APS-2-79 is an inhibitor, which was utilized in the present study to confirm the stimulatory actions of letrozole.

Materials and methods

Reagents. MAPK pathway inhibitor, APS-2-79, was procured from Selleck Chemicals (cat. no. S8355; Houston, TX, USA). Letrozole in the test was procured from MCE Corp. (cat. no. CGS-20267; Shanghai, China).

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Cell culture. Spermatogonia of GC-1 spg mice were purchased from the Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum as well as 100 U/ml penicillin and streptomycin. Cells in the cell culture flask were incubated in the environment containing 5% CO₂ at 37°C. The Ethics Committee of Chongqing Three Gorges Central Hospital approved the study.

Cell Counting Kit-8 (CCK-8) detection. GC-1 spg cells were inoculated in three 96-well plates at a density of 1x10⁴/ml. The cells were completely adherent to the wall 24 h later. Letrozole (100 µl) at a final concentration of 0.1, 1, 10, 100, 1,000 nm was added and used for incubation for 24, 48 and 72 h, respectively. Then, 10 µl CCK-8 reagent (Biotool, Shanghai, China) was added to each well and incubated for 1 h in an incubator at 37°C. The optical density (OD) of each well was measured at the wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell plate clone. GC-1 spg cells in the logarithmic growth phase were selected, and digested with 0.25% trypsin into single cells. Cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum, and the cell concentration was adjusted to 1x10³/ml. Then, 1 ml cell suspension and 3 ml complete culture solution were placed in 6-well plates and gently rotated to disperse the cells evenly. The 6-well plates were incubated in a cell incubator with 5% CO₂ at 37°C for 2-3 weeks. When a clone visible to the naked eye appeared in the 6-well plates, the culture was terminated. The supernatants were then discarded. This was followed by two washings with phosphate-buffered saline (PBS) 5 ml of 4% paraformaldehyde was utilized for fixing cells for 15 min. An appropriate amount of crystal violet staining solution was used for staining for 30 min, and then washed with double distilled water. Images were taken of the colonies with cell count more than 50.

Reverse transcription-polymerase chain reaction (RT-PCR). The total ribonucleic acid (RNA) was extracted from GC-1 spg cells in each group using the PureLink[®] RNA Silica Column Extraction kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized with 1.0 µg total messenger RNA (mRNA) using a reverse transcription kit (SuperScript[®] VILO cDNA Synthesis kit and Master Mix; Invitrogen; Thermo Fisher Scientific, Inc.). The annealing was performed at 55°C while extension was completed at 72°C. The expression of each index was detected by the gene reactivation kit (Guangzhou, China) and quantitative PCR (Thermo Fisher Scientific, Inc.). The calculation formula of the expression level of mRNA as per kit: $2^{-\Delta Cq}$ [$\Delta Cq = Cq$ (target gene) - Cq (glyceraldehyde 3-phosphate dehydrogenase)]. The corresponding primer sequences are shown in Table I.

Western blot analysis. Cells in each treatment group were inoculated in 6-well plates, and an appropriate amount of protease inhibitor and protein lysate were added. The cell

Table I. Primer sequences of RT-PCR.

| Gene names | Primer sequences |
|--------------|--|
| <i>Ras</i> | 5'-3' ACTGAATATAAACTTGTGGTAGTTGGACCT 3'-5' TCAAAGAATGGTCCTGGACC |
| <i>ERK1</i> | 5'-3' CTACACGCAGTTGCAGTACAT 3'-5' CAGCAGGATCTGGATCTCCC |
| <i>c-Myc</i> | 5'-3' GGACTGCGCAGGGAGACCTACAGGGG 3'-5' GAGGGAGCCGGCTGAGAGAAGTTGGG |
| <i>GAPDH</i> | 5'-3' AGGTTCGGTGTGAACGGATTG 3'-5' TGTAGACCATGTAGTTGAGGTCA |

ERK1, extracellular signal-regulated kinase 1.

lysate was aspirated and centrifuged at 12,000 x g at 4°C for 30 min. Total protein (40 µg) was electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gel, followed by transfer to the polyvinylidene difluoride (PVDF) membrane. According to the instructions of the marker, the bands containing the target proteins were cut and incubated overnight with Ras (cat. no. ab52939), ERK1 (cat. no. ab17942), c-Myc (cat. no. ab32072), Ki-67 (cat. no. ab156956), proliferating cell nuclear antigen (PCNA) (cat. no. ab29) and GAPDH (cat. no. ab8245) antibodies. All the antibodies were procured from Abcam (Cambridge, UK). Dilutions were made with the blocking buffer (1:500 for all antibodies) and the incubation time was 12 h at 4°C. The membranes were visualized with an enhanced chemiluminescence (ECL) detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and gray scale analysis was performed using a gel analyzer. The bands were quantified by using ImageJ software.

Detection of cell proliferation by bromodeoxyuridine (BrdU) staining. Cells were inoculated on a chamber slide (NEST Science Co. Ltd., Shanghai, China) at a density of 10-20%. The cells were incubated for 48 h according to the test requirements by addition of letrozole and MAPK inhibitors. Before the termination of cell culture, BrdU (final concentration: 30 µg/l; MCE Corp.) was added, and the cells were further incubated at 37°C for 40 min. The culture medium was then discarded, and the slide was washed 3 times with PBS. Methanol was used for fixation for 10 min, after which the slide was dried with air. Subsequently, 0.3% H₂O₂-methanol was used for inactivating endogenous oxidases for 30 min. Bovine serum albumin (BSA) (5%) was used for sealing cells at room temperature for 1 h. Formamide was used for denaturation of nucleic acid at 100°C for 5 min. After cooling in the ice bath, PBS washing was performed, and the anti-mouse BrdU monoclonal antibody (working concentration = 1:50) was added. 4',6-Diamidino-2-phenylindole (DAPI) was used for staining nuclei for 2 min. Ten high-power fields were randomly chosen to calculate the total number of cells in each field of vision and the number of BrdU-positive cells.

Statistical analysis. The results were analyzed using GraphPad Prism software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). The t-test was used to compare the

differences in samples between the two groups. One-way analysis of variance (ANOVA) was used to compare the differences in the mean among multiple groups and Tukey test was used as post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. The LSD post hoc test was also performed for comparisons among groups.

Results

Detection of the effect of letrozole on the proliferation of spermatogonia by CCK-8. Six gradient concentrations were set for letrozole to treat mouse spermatogonia (GC-1 spg) for 24, 48 and 72 h, respectively. CCK-8 assay showed that letrozole could promote the proliferation of GC-1 spg cells and exhibited time and dose effects (Fig. 1). In the follow-up experiment, 100 nm letrozole was selected to treat cells for 72 h, and then the detection was performed.

Detection of the effect of letrozole on the proliferation of spermatogonia by the plate clone formation assay. The plate clone formation assay was used to further verify the effect of letrozole on the proliferation of GC-1 spg cells. As shown in Fig. 2, the number of cell colonies in the letrozole treatment was significantly higher than the control group ($p < 0.05$).

Detection of the effects of letrozole on the MAPK pathway by RT-PCR. As MAPK is an important signaling pathway for cell proliferation, letrozole and letrozole + MAPK inhibitors were set to explore the relationship between letrozole and MAPK pathways. RT-PCR results showed that, letrozole significantly increased the mRNA levels of Ras/ERK1/c-Myc in the classical MAPK pathway ($p < 0.05$) as compared to those of control group. Further, letrozole + MAPK inhibition significantly decreased the mRNA levels of Ras/ERK/c-Myc ($p < 0.05$) (Fig. 3).

Detection of effects of letrozole on the MAPK pathway by western blot analysis. The results of western blot analysis were consistent with those of RT-PCR, which further verified that compared with those in the control group, letrozole significantly increased the protein levels of Ras/ERK1/c-Myc in the classical MAPK pathway ($p < 0.05$). On the other hand, letrozole + MAPK inhibition significantly decreased the protein levels of Ras/ERK/c-Myc ($p < 0.05$) (Fig. 4).

Detection of the expression of proliferation indexes. The protein expression levels of Ki-67 and PCNA (two classical proliferation indexes), were examined to further investigate the effects of letrozole on the proliferation of GC-1 spg cells. Letrozole significantly increased the protein levels of Ki-67 and PCNA ($p < 0.05$) (Fig. 5), in comparison to the control group. The protein levels of Ki-67 and PCNA in the letrozole + MAPK inhibition group showed a significant declining trend as compared to the letrozole group ($p < 0.05$).

Detection of effects of letrozole on cell proliferation by BrdU. The BrdU assay was used to detect the effects of letrozole and the MAPK pathway on the proliferation of GC-1 spg cells. The results (Fig. 6) indicated that the number of BrdU-stained cells in the letrozole group was higher than

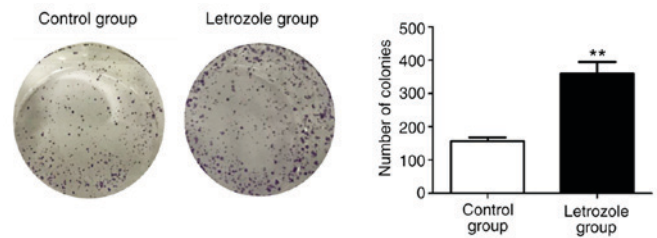


Figure 1. Detection of the effect of letrozole on the proliferation of mouse spermatogonia by CCK-8. CCK-8, Cell Counting Kit-8. ** $P < 0.05$.

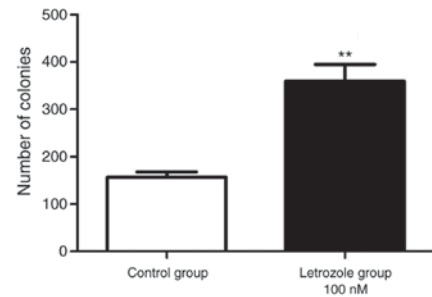


Figure 2. Detection of the effect of letrozole on the proliferation of mouse spermatogonia by the plate clone formation assay. Compared with the control group, ** $p < 0.01$.

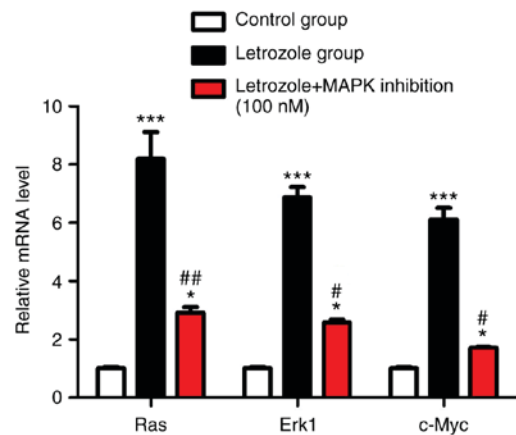


Figure 3. Detection of the effect of letrozole on the MAPK pathway by RT-PCR. Compared with the control group, * $p < 0.05$, *** $p < 0.001$; compared with the letrozole group, # $p < 0.05$, ** $p < 0.01$.

that in the control group, while that in the letrozole + MAPK inhibition group was significantly less in comparison to letrozole group.

Discussion

Oligozoospermia is a complex disease, which is caused by the combination of genetic factors and acquired conditions (9). The occurrence of spermatozoa is also a complex cell process, which is divided into the division stage (proliferation and differentiation of spermatogonia), the meiosis stage (meiosis of spermatogonia) and the haploid stage (from sperm cells to sperm differentiation) (10). In this process, the number of spermatogonia directly affects the production of sperm.

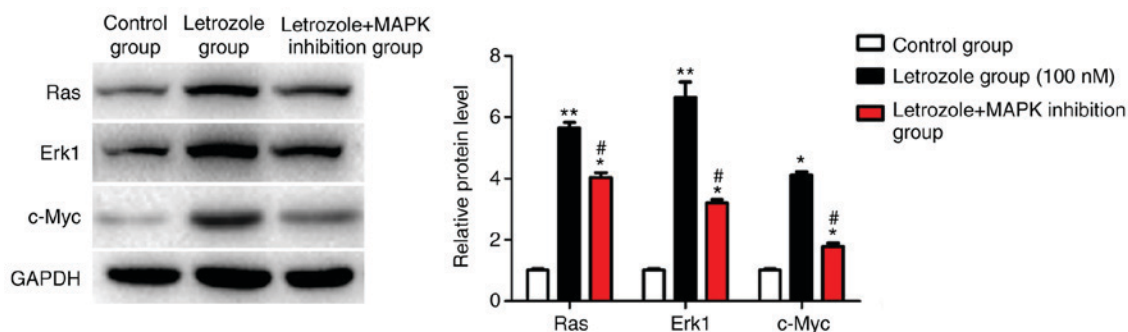


Figure 4. Detection of the effect of letrozole on the MAPK pathway by western blotting. Compared with the control group, * $p < 0.05$, ** $p < 0.01$; compared with the letrozole group, # $p < 0.05$. MAPK, mitogen-activated protein kinase.

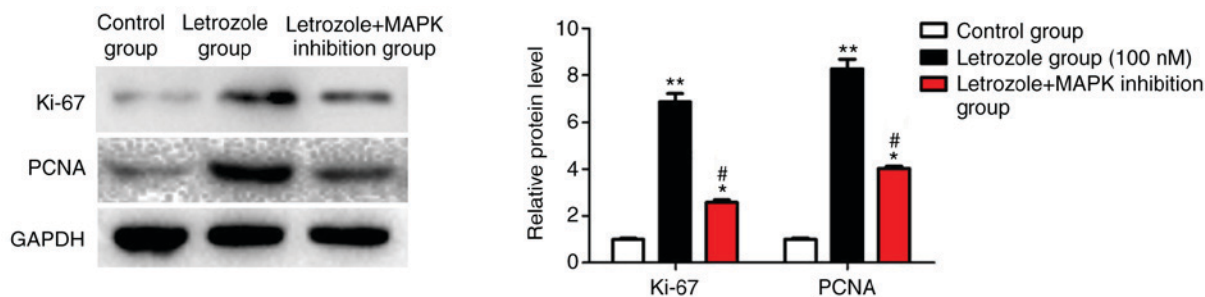


Figure 5. Detection of the expression of proliferation indexes. Compared with the control group, * $p < 0.05$, ** $p < 0.01$; compared with the letrozole group, # $p < 0.05$.

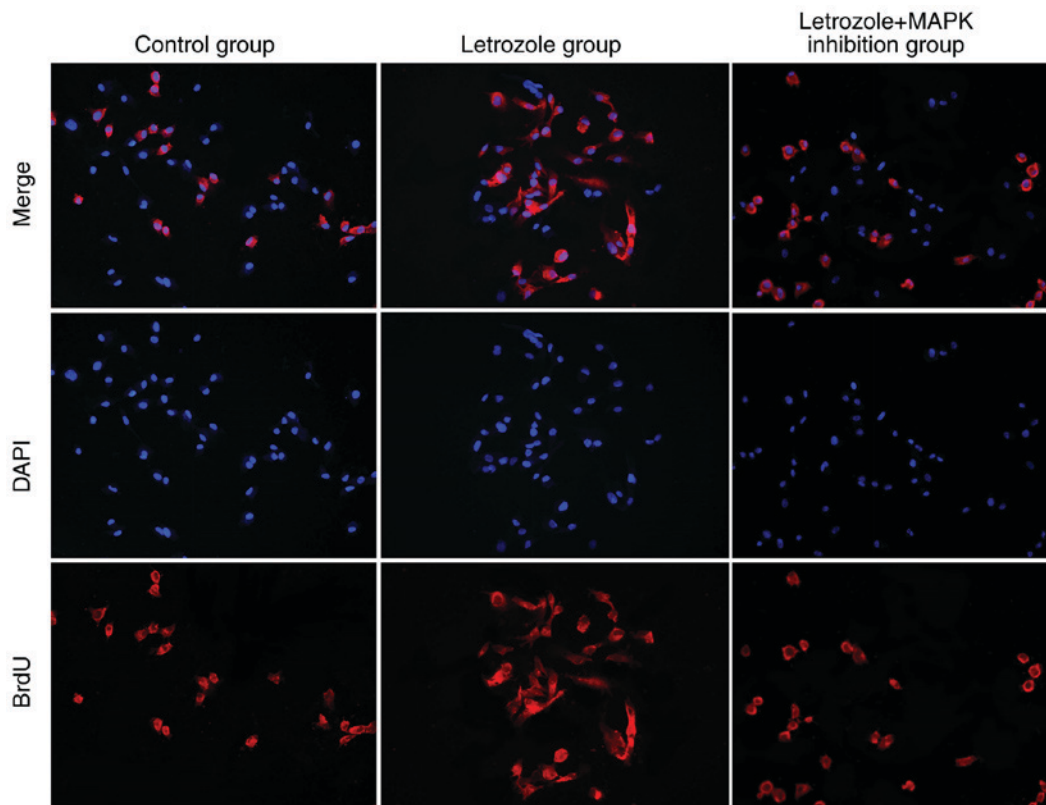


Figure 6. Detection of the effects of letrozole and the MAPK pathway on the proliferation of GC-1 spg cells by BrdU. MAPK, mitogen-activated protein kinase; spg, spermatogonia; BrdU, bromodeoxyuridine.

Abnormalities in spermatogonia often lead to oligozoospermia, asthenozoospermia and sperm abnormalities (11).

Therefore, improvement in the number of spermatogonia is crucial during male oligozoospermia.

Letrozole is considered as a safe as well as an effective aromatase inhibitor that could be orally taken. It has the ability to act as an aromatase inhibitor to decrease the estrogen level. In this way, it eliminates the stimulation of estrogen on tumor growth. Besides, no potential toxicity to the normal systemic system has been reported. Moreover, target organs showed good tolerance and strong pharmacological effects (12,13). The recent 3-phase clinical trials utilized letrozole as a chemotherapy drug for postmenopausal metastatic breast cancer (14). In addition, the literature also suggested that the main source of estrogen in men is the process that showed aromatase-catalyzed testosterone into estradiol. So, it has a strong negative feedback on the hypothalamic-pituitary axis so as to reduce the production and the release of follicle stimulating hormone (FSH). Aromatase inhibitors could inhibit the conversion of testosterone into estradiol; thereby reduced the concentration of serum estradiol. Thus, the negative feedback on the hypothalamic-pituitary axis increased the levels of serum FSH (15). FSH regulates the development, growth, adolescent sexual maturity and a series of physiological processes related to reproduction, which acts on seminiferous tubules of the testis and promotes sperm formation. This, in turn, might exert positive effects on sperms (16). However, concerning the molecular mechanism, the main role of letrozole in the signaling pathway has not yet been reported.

MAPK pathway plays important roles in the differentiation, proliferation, survival and migration of mammalian cells (17). The four major MAPK cascade reactions [ERK1/2, c-Jun N-terminal kinase 1/3 (JNK1/3), p38 and ERK5] in mammalian cells have been well studied. The action mechanism of cascade reactions includes activation of the Ras protein-coupled receptor of the Ras family [such as Ras, cell division control protein 42 homolog (Cdc42) and Rac], followed by the activation of mitogen activated protein kinase kinases (MAPKKs) and MAPKs (18). A study of Jaldety and Breitbart showed that ERK1 and 2 were expressed at all stages of mouse germ cells, but the activation of ERK1/2 peaks in mouse spermatogonia (19). In addition, ERK1/2 actively participates in the process of mouse spermatogonial stem cell factor (SCF) accelerating the cell cycle progression by inducing the transient activation of MAPK cascades (20). Besides, c-Myc (as the target gene of the MAPK pathway), is involved in the process of cells from G0 to S phases, and its increased expression positively regulates cell proliferation (21).

In the present study, *in vitro* cell experiments showed that letrozole significantly increased the proliferation of mouse GC-1 spg cells accompanied by the activation of the MAPK signaling pathway. However, the exploration of RAF is also crucial for concrete conclusion. So, it could be one of the prime limitations of the study but we will include this in our future studies. We did not include APS-2-79 group in the present study, which is the second limitation of the study. Experiments using MAPK inhibitors found that MAPK signaling pathway played an important role in promoting the proliferation ability of spermatogonia. Based on the above experimental results, we speculated that letrozole might have a good clinical effects on infertility caused by male oligozoospermia and asthenozoospermia. Thus, letrozole has strong potential for its clinical application against oligozoospermia. However, further studies are essential for concrete conclusions.

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Availability of data and materials

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

Authors' contributions

ShundeW wrote the manuscript and was responsible for cell culture. ShuhongW and HL performed CCK-8 assay, XL was devoted to PCR. MX and JW helped with western blot analysis, ML and TL contributed to BrdU staining. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of Chongqing Three Gorges Central Hospital (Chongqing, China) approved the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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