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Original Article

Oridonin represses lung cancer cell proliferation and migration by modulating AFAP1-AS1/IGF2BP1

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Article Info

Abstract

Oridonin belongs to a small molecule from the Chinese herb Rabdosia rubescens with potent anticancer activity. In spite of the lncRNA AFAP1-AS1 has been proven to exert promoting function in lung cancer, its relationship with oridonin in lung cancer is obscure. Therefore, our study planned to explore the potential of oridonin in lung cancer as well as unveil the regulatory mechanism of oridonin on AFAP1-AS1 in lung cancer cells. In the present study, oridonin inhibited lung cancer cell proliferation, migration, as well as invasion, as evidenced by MTT, wound healing, as well as transwell assays. Besides, we observed that oridonin could downregulate AFAP1-AS1 expression, and overexpressed AFAP1-AS1 could reverse the repressive effects of oridonin on lung cancer cell proliferation, migration, as well as invasion. More importantly, we found that AFAP1-AS1 could bind to IGF2BP1 through starBase prediction and RIP assay. The expression level of IGF2BP1 was also reduced by oridonin treatment but reversed after AFAP1-AS1 overexpression. Additionally, we proved that overexpressed IGF2BP1 could reverse the repressive impacts of oridonin on lung cancer cell proliferation, migration, as well as invasion. Further, in vivo experiments validated the repressive role of oridonin on tumor growth of lung cancer. Together, oridonin inhibits lung cancer cell proliferation as well as migration by modulating AFAP1-AS1/IGF2BP1, and AFAP1-AS1/IGF2BP1 possesses the potential to be a promising therapy targeting for lung cancer, especially in oridonin treatment.

Keywords: Lung cancer, Oridonin, AFAP1-AS1, IGF2BP1.

1. Introduction

Lung cancer (LC) belongs to the dominating cause of malignancy-associated death all over the world, with a death rate of 18.4% [1]. LC is separated into small cell lung carcinoma as well as non-small cell lung carcinoma [2]. Surgery, chemotherapy, along with radiotherapy is the main treatment strategies for LC. Due to poor treatment and late stages of diagnosis, some patients relapse and die within a short time [3]. The molecular heterogeneity of LC is one of the reasons for these conditions. The dysregulation of the expression of cancer markers is closely related to oncogenes or anti-tumor genes involved in carcinogenesis [4]. Hence, it is essential to expand the library of potential tumor-linked molecules to promote the precise diagnosis as well as anti-tumor therapy of LC.

In recent years, natural products derived from medical plants have accepted growing attention in tumor therapy. Oridonin belongs to a kind of diterpenoid compound isolated from the Chinese herb *R. rubescens*, which has been paid more and more attention by cancer biologists because of its predominant anti-tumor activity [5]. Recently, increasing evidence has revealed that oridonin can repress tumor progression, relieve tumor burden and tumor syndrome, as well as greatly improve the survival rate of tumor patients [6]. For example, oridonin hinders breast cancer growth via blocking the Notch signaling [7]. Oridonin represses human pancreatic cancer cell migration through miR-200b-3p/ZEB1 axis. Oridonin stimulates apoptosis in esophageal cancer cells through repressing PI3K/AKT/mTOR pathways [8]. Moreover, it has been documented that oridonin can suppress LC cell migration as well as epithelial-to-mesenchymal transition [9]. However, the underlying anti-tumor mechanism of oridonin in LC is still not fully understood.

Long non-coding RNAs (LncRNAs) belong to RNA molecules that exceed 200 nucleotides long [10]. LncRNAs are able to modulate expression and/or functions of genes via serving to be microRNA (miRNA) spongers or combining with RNA binding proteins (RBPs). Dysregulation of lncRNAs is frequently detected in LC, which is involved in cancer progression [11]. LncRNAs take part in epigenetic modulation of genes required for LC growth and have also been revealed to be possible biomarkers for the diagnosis as well as prognosis of multiple tumors [12]. As a result, lncRNAs are emerging to be viable, minimally invasive diagnostic/prognostic indicators, as well as therapeutic targets [13].

Actin filament-associated protein 1 antisense RNA 1

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(AFAP1-AS1) belongs to a high-expressed lncRNA in numerous malignant tumors and is implicated in tumor development [14, 15]. In addition, it has been unveiled that silenced AFAP1-AS1 inhibits LC cell migration as well as invasion [16]. However, the relation between oridonin and AFAP1-AS1 in LC is unclear.

Our study planned to explore the underlying anti-tumor mechanism of oridonin in LC and unveil the regulatory relationship between oridonin and AFAP1-AS1 in LC.

2. Material and methods
2.1. Cell culture and treatment
Two LC cell lines (A549 and H1299) as well as human normal epithelial cell line (BEAS-2B) were acquired from Procell (Wuhan, China), and could be maintained in Ham's F-12K medium, RPMI-1640 medium, and BEGM medium, respectively. All cell mediums were added with 10% fetal bovine serum together with 1% antibiotics (penicillin/streptomycin) at 37°C with 5% CO_2.

2.2. Cell transfection
GenePharma Inc. (Shanghai, China) provided the pcDNA-3.1 and overexpression plasmids (pcDNA3.1-AFAP1-AS1 and pcDNA3.1-IGF2BP1). Cell transfection could be implemented with Lipofectamine 3000 (Thermo Fisher, USA).

2.3. RNA isolation and quantitative real-time PCR (qRT-PCR)
Isolation of total RNA from cell samples could be implemented with Trizol reagent (Invitrogen, USA). The SuperScript III first-strand synthesis system (Thermo Fisher, USA) was adopted for synthesizing cDNA. SYBR Premix Ex Taq (Takara, Japan) was implemented to perform qPCR. The $2^{-\Delta\Delta CT}$ method was implemented for calculating gene relative expression levels.

2.4. Western blot
Proteins could be extracted from cells with RIPA buffer (Beyotime, China). Proteins were isolated by SDS-PAGE after quantifying the protein concentration with a BCA assay kit (Beyotime), and then shifted onto polyvinylidene difluoride (PVDF) membranes (Solarbio, China). The membranes were incubated with specific primary antibodies against anti-IGF2BP1 (1:1000, Proteintech Group, China) and anti-GAPDH (1:1000, Proteintech Group, China) at 4°C overnight followed by sealing using 5% skim milk powder. The membranes were then treated with the appropriate secondary antibodies (1:2000, Proteintech Group, China) and visualized with enhanced chemiluminescence reagents (ECL; Beyotime).

2.5. MTT assay
1×10^4 cells were planted into 96-well plates. Followed by treating different concentrations of oridonin (1, 5, 10, 15, 20 µM) for 24 h, 20 µl of MTT dye solution could be treated into each well for another 4 h of incubation. The optical density (OD) could be examined at 490 nm wavelength with a microplate reader (Bio-Rad, USA).

2.6. Wound healing assay
The cells were cultivated in 6-well plates and grew to 80-90% confluent. The cell monolayers were then scratched with pipette tips. After washing, the cells were added

with oridonin in medium containing 1% serum for 24 h. The number of migrated cells could be measured with an inverted microscopy.

2.7. Cell invasion assay
The transwell chambers (Corning Costar, USA) were coated with Matrigel (Becton Dickinson, USA). Cells were gathered in serum-free medium, and the cell suspensions were then put in the above compartment. Culture medium including 10% fetal bovine serum was put into the bottom compartment. After 24 h of incubation, the invasive cells on the lower surface were dyed with crystal violet, manually counted, and quantified, and 5 visual fields were randomly selected for analysis in each group.

2.8. RNA binding protein immunoprecipitation (RIP)
This assay was implemented with a RIP kit (Bersin-Bio, China). The antibodies included anti-AGO2 as well as control IgG (Abcam, UK), and the coprecipitated RNAs could be implemented for cDNA synthesis and evaluated by qRT-PCR.

2.9. Xenograft tumor model
Shanghai Laboratory Animal Center (Shanghai, China) provided 10 four-week-old male BALB/C nude mice. All the animal experiments were approved by the Ethics Committee of Animal care and Use of Taizhou People's Hospital Affiliated to Nanjing Medical University. The nude mice were separated into 2 groups at random (n=5), and then subcutaneously administrated with 100 µl serum-free medium including A549 cells into right flank of mice. Followed by the tumor volumes of the xenografts reaching about 90 mm³, the oridonin group was added with 10 mg/kg oridonin. The tumor volume of nude mice was detected every 3 days. The mice could be sacrificed to harvest the tumors at day 18 and tumor weight was determined.

2.10. Statistical analysis
All experiments were performed at least 3 times. The SPSS 17.0 software (IBM, USA) was adopted for statistical analyses. The data were expressed as mean ± SD. Differences were analyzed with Student t-test or one-way analysis of variance. P<0.05 meant statistical significance.

3. Results
3.1. Oridonin inhibits LC cell proliferation, migration along with invasion
Figure 1A displays the molecular structure of oridonin. As oridonin has received widespread attention recently because of its apparent anti-cancer potential [17], we used a modified MTT assay to preliminarily detect the cytotoxic effects of oridonin on human normal epithelial cell line BEAS-2B and LC cells A549 and H1299. It was displayed that oridonin possessed no apparent cytotoxic influence on BEAS-2B cells, which was in line with previous study [18]. Besides, oridonin exhibited anti-proliferative effect on LC cells through a concentration-dependent way (Figure 1B-D). Additionally, it was observed that when the concentration of oridonin presented not higher than 10 µM, 24 h, oridonin had no obvious cytotoxicity to the two types of LC cells, and the inhibition rate was less than 10%. Hence, a concentration of oridonin was 10 µM at 24 h could be adopted in follow-up analyses, to rule out the interference of cell proliferation. To identify the role of oridnoin in LC
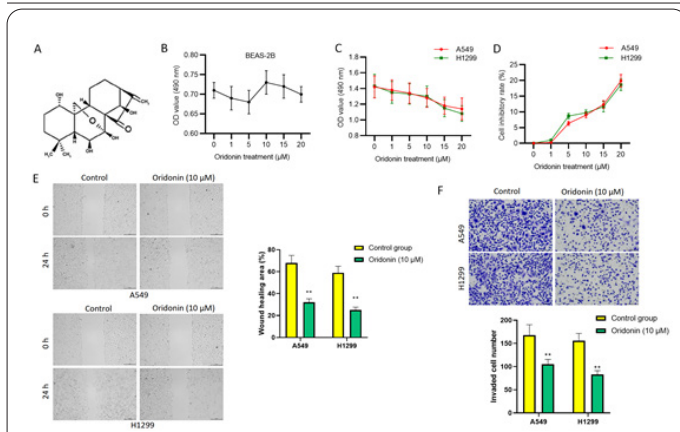
Fig. 1. Oridonin inhibits the proliferation, migration and invasion of lung cancer cells. (A) The chemical structure of oridonin. (B-D) The viability of human normal epithelial cells and lung cancer cells was assessed by MTT assay after treatment with different concentrations of oridonin for 24 h (0, 1, 5, 10, 15, 20 μM). (E) Wound healing assay detected lung cancer cell migration after oridonin treatment. (F) Transwell assay detected lung cancer cell invasion after oridonin treatment. $^{**}P<0.01$.

cells migration and invasion, wound healing and transwell assays were implemented. The outcomes revealed that the scratch width of oridonin group was wider compared to the control group in two LC cells (Figure 1E). Meanwhile, two LC cells invasion was weakened after oridonin treatment (Figure 1F).

3.2. Overexpressed AFAP1-AS1 reverses the impacts of oridonin on LC cells proliferation, migration as well as invasion

For all we know, AFAP1-AS1 has been widely reported to accelerate LC development [19]. Notably, oridonin has been documented to inhibit pancreatic cancer cell tumorigenicity via downregulating AFAP1-AS1 [20]. Thus, our study speculated that oridonin might inhibit LC development via regulating AFAP1-AS1. Based on qRT-PCR analysis, it could be observed that AFAP1-AS1 expression was reduced in LC cells after oridonin treatment (Figure 2A). Subsequently, we overexpressed AFAP1-AS1 expression in two LC cells (Figure 2B) and then performed rescue assays to validate whether overexpression of AFAP1-AS1 reversed the impacts of oridonin on LC cell proliferation, migration along with invasion. It was seen in MTT assay that the reduced proliferation in LC cells caused by oridonin treatment was offset followed by transfection of pcDNA3.1-AFAP1-AS1 (Figure 2C). Similarly, we discovered that overexpression of AFAP1-AS1 offset the inhibited LC cell migration as well as invasion mediated by oridonin treatment (Figure 2D-E).

3.3. Oridonin reduces IGF2BP1 expression by downregulating AFAP1-AS1 in LC cells

We further evaluated the downstream mechanism of AFAP1-AS1 in LC cells. An RBP IGF2BP1 was predicted to be the candidate RBP of AFAP1-AS1 using starBase database (Figure 3A). At the same time, the combination of IGF2BP1 to AFAP1-AS1 was validated by RIP assay (Figure 3B), implying a potential feedback regulatory loop of IGF2BP1 and AFAP1-AS1 in LC cells. Besides, we also detected IGF2BP1 expression in LC cells treated with oridonin, and discovered that IGF2BP1 mRNA as well as
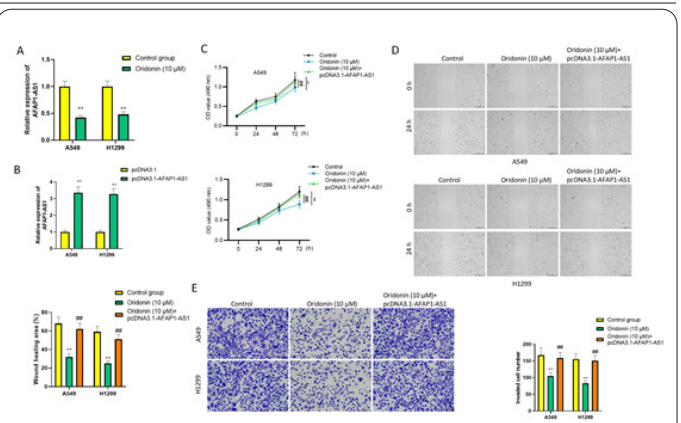

Fig. 2. Overexpression of AFAP1-AS1 reverses the impacts of oridonin on lung cancer cell proliferation, migration and invasion. (A) Relative expression of AFAP1-AS1 was assessed in lung cancer cell invasion after oridonin treatment. (B) Overexpression transfection efficiency of AFAP1-AS1 in lung cancer cells using qRT-PCR. (C) MTT assay assessed lung cancer cell viability after oridonin treatment and pcDNA3.1-AFAP1-AS1 transfection. (D) Wound healing assay assessed lung cancer cell migration after oridonin treatment and pcDNA3.1-AFAP1-AS1 transfection. (E) Transwell assay assessed lung cancer cell invasion after oridonin treatment and pcDNA3.1-AFAP1-AS1 transfection. $^{**}P<0.01$, compared with control group, $^{##}P<0.01$, compared with oridonin group.
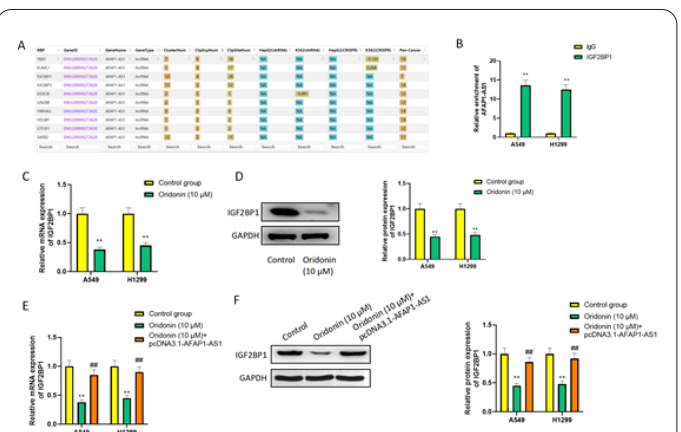

Fig. 3. Oridonin reduces IGF2BP1 expression by downregulating AFAP1-AS1 in lung cancer cells. (A) StarBase database predicted the binding of AFAP1-AS1 and IGF2BP1. (B) RIP assay detected the relationship AFAP1-AS1 and IGF2BP1. (C-D) qRT-PCR and western blot detected the mRNA and protein levels of IGF2BP1 in lung cancer cells treated with oridonin. qRT-PCR and western blot detected the mRNA and protein levels of IGF2BP1 in lung cancer cells treated with oridonin and pcDNA3.1-AFAP1-AS1 transfection. $^{**}P<0.01$, compared with control group, $^{##}P<0.01$, compared with oridonin group.

protein levels were also inhibited by oridonin treatment (Figure 3C-D). Moreover, we observed that the reduced mRNA as well as protein levels of IGF2BP1 caused by oridonin treatment could be counteracted after pcDNA3.1-AFAP1-AS1 transfection (Figure 3E-3F), suggesting the positive regulatory relationship between AFAP1-AS1 and IGF2BP1.

3.4. Overexpression of IGF2BP1 reverses the impacts of oridonin on LC cells proliferation, migration as well as invasion

We also increased IGF2BP1 expression in two l LC

cells (Figure 4A) and then performed rescue assays to validate whether overexpression of IGF2BP1 could rescue the impacts of oridonin on LC cell proliferation, migration along with invasion. We observed that the reduced proliferation in LC cells caused by oridonin treatment could be reversed after pcDNA3.1-IGF2BP1 transfection (Figure 4B). Similarly, we discovered that overexpressed IGF2BP1 offset the inhibited LC cell migration as well as invasion mediated by oridonin treatment (Figure 4C-D).

3.5. Oridonin represses the growth of xenografted A549 cells in nude mice

Next, we validated the antitumor activity of oridonin in nude mice with xenografted A549 cells. As displayed in Figure 5A-C, oridonin treatment demonstrated obvious repressive impacts on tumor volume together with tumor weight.
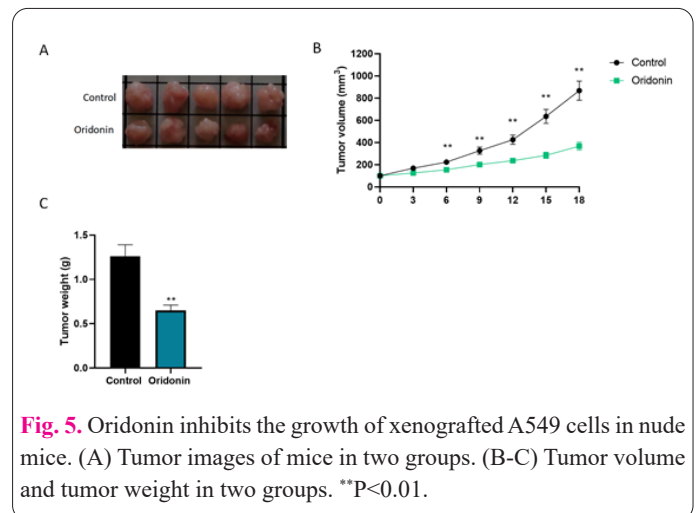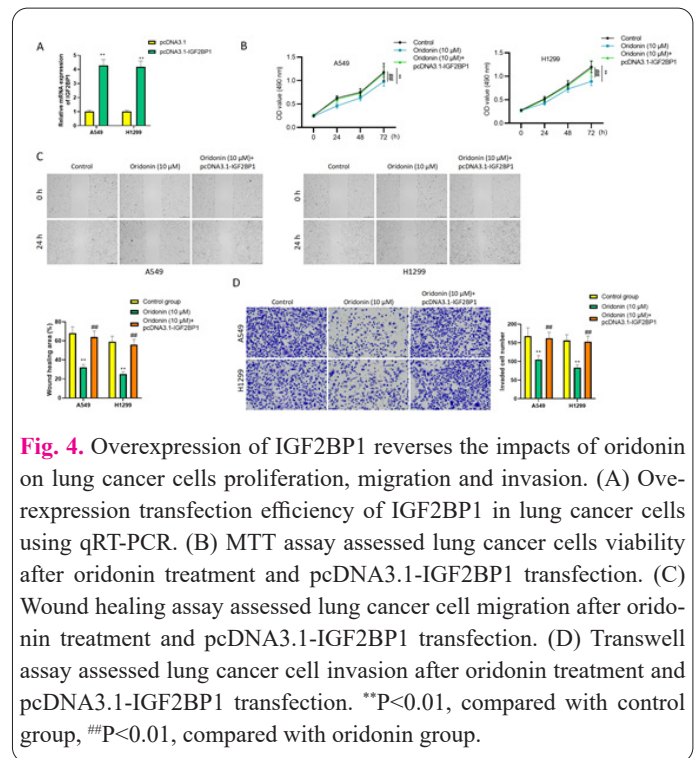
4. Discussion

Natural products have been accepting increasing attention to be cancer treatment drugs due to their less toxic effects [21]. *R. rubescens* belongs to a kind of traditional Chinese medicine, which possesses the effects of clearing heat and detoxifying, reducing inflammation and pain, and anti-tumor [22]. It has been widely used in clinical applications, especially in upper respiratory tract infections, chronic hepatitis, joint rheumatism, esophageal cancer, and other malignant tumors [23, 24]. Oridonin is the major active ingredient of *R. rubescens*, and its anti-cancer potential has attracted the attention of scholars at home and abroad in recent years [25]. Modern studies have validated that oridonin can repress the proliferation of gastric cancer, liver cancer, breast cancer and other tumor cells, and has low toxicity and no obvious damage to bone marrow, liver, kidney and other organs [26-28]. Recent literatures have suggested that oridonin also has an obvious inhibitory impact on LC cells [29], but its inhibitory effect and mechanism on the development of LC in vitro are still not clear.

In our study, we proved that oridonin could repress LC cell proliferation, migration, as well as invasion. Consistently, oridonin has been documented to inhibit the tumor growth of LC and can serve as be therapeutic agent for LC treatment. Moreover, Zheng et al. have indicated that oridonin induces G2/M arrest in A549 cells through increasing ATM activation [30].

LncRNAs have a vital function in tumor growth and can be adopted to be molecular targets for the modulation of tumor progression. More importantly, AFAP1-AS1 works as an oncogene in LC development [31]. Therefore, our study further explored the relationship between oridonin and AFAP1-AS1 in LC cells. Our study confirmed that AFAP1-AS1 was downregulated by oridonin treatment in LC cells, and further rescue assays confirmed that overexpression of AFAP1-AS1 reversed the suppressive influences of oridonin on LC cells proliferation, migration as well as invasion, which was consistent with previous study [20].

In order to further investigate the possible mechanism by which oridonin/AFAP1-AS1 influences LC development, our study proved that AFAP1-AS1 could bind to RBP IGF2BP1 in LC cells. RBPs belong to a group of proteins that combine with the relevant RNA specifically to modulate RNA function. Protein-RNA interactions are

Fig. 4. Overexpression of IGF2BP1 reverses the impacts of oridonin on lung cancer cells proliferation, migration and invasion. (A) Overexpression transfection efficiency of IGF2BP1 in lung cancer cells using qRT-PCR. (B) MTT assay assessed lung cancer cells viability after oridonin treatment and pcDNA3.1-IGF2BP1 transfection. (C) Wound healing assay assessed lung cancer cell migration after oridonin treatment and pcDNA3.1-IGF2BP1 transfection. (D) Transwell assay assessed lung cancer cell invasion after oridonin treatment and pcDNA3.1-IGF2BP1 transfection. **P<0.01, compared with control group, $^{##}$P<0.01, compared with oridonin group.

Fig. 5. Oridonin inhibits the growth of xenografted A549 cells in nude mice. (A) Tumor images of mice in two groups. (B-C) Tumor volume and tumor weight in two groups. **P<0.01.

critical parts in various cellular processes, such as splicing, stability, as well as translation, and are essential in many aspects of the gene expression cascade [32]. RBPs are abnormally expressed in cancers and are linked to patient prognosis [33]. IGF2BP1 belongs to an extensively expressed RBP that is highly expressed in a variety of cancers [34]. IGF2BP1 possesses the property to accelerate LC proliferation, migration, as well as invasion [35-37]. Our study further confirmed that IGF2BP1 was downregulated by oridonin treatment in LC cells, but reversed after transfection of pcDNA3.1-AFAP1-AS1. Besides, further rescue assays certified that overexpression of IGF2BP1 reversed the repressive influences of oridonin on LC cells proliferation, migration as well as invasion.

5. Conclusion

In conclusion, our study demonstrates that oridonin represses LC cell proliferation as well as migration by modulating AFAP1-AS1/IGF2BP1, and AFAP1-AS1/IGF2BP1 possesses the potential to be a promising therapy targeting LC, especially in oridonin treatment.

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Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Taizhou People's Hospital Affiliated to Nanjing Medical University.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

CM conducted the experiments and wrote the paper; CM and SY analyzed and organized the data; SY conceived, designed the study and revised the manuscript.

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