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ORIGINAL ARTICLE

Silencing of RNFT2 suppresses cell proliferation and migration through mTORC1 signaling pathway in gastric cancer

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Abstract

Excellent biomarkers for predicting survival or therapeutic targets are still lacking in gastric cancer (GC), which is one of the most common causes of cancer-related death worldwide. Ring finger protein, transmembrane 2 (RNFT2), which has been reported to be involved in proteolytic process, but how it functions in tumors is rarely investigated. In the present study, we explored the biological property of RNFT2 in GC, we found that RNFT2 was significantly upregulated in GC, and could serve as a tumor marker to predict prognosis. A series of in vitro cell function experiments were performed, we found that knockdown of RNFT2 expression in GC cells could inhibit cell invasion, migration and proliferation. Besides, in vivo experiments also showed that silencing RNFT2 expression in gastric cancer cells significantly reduced tumor size. Furthermore, through gene set enrichment analysis (GSEA) and immunoblotting studies, we observed that RNFT2 might influence the proliferation, invasion and migration of GC cells through the mTORC1 signaling pathway. In summary, our results clarified the carcinogenic role of RNFT2 in GC progression, provided inspiration to further understand the molecular mechanism of GC and made RNFT2 as a potential target for GC diagnosis and therapy.

Keywords Gastric cancer · RNFT2 · mTORC1 signaling · Malignant phenotypes

Introduction

Gastric cancer patients worldwide are mainly distributed in Asia according to the latest research data(Sung et al. 2021). In China, due to the fact that more than half of GC patients are often diagnosed at the local late stage of the tumor, the inevitable mortality rate is very high, seriously affecting the health of the people(Chen et al. 2016; Sung et al. 2021). The development of gastric cancer is a multi-stage process characterized by slow progression and multifactorial pathological changes, among which the risk factors for GC include nitrate and *Helicobacter pylori* infection(Oliveira et al. 2015). In addition, molecular level risk factors such as genomic variations and epigenetic changes are also involved in the carcinogenesis of GC(Nagini 2012). For examples, RAD50, SMYD5 and KLHL21 have recently been reported to influence the development of gastric cancer(Kwak et al. 2024; Park et al. 2024); Huang et al. 2024). In order to better figure out the molecular mechanism of gastric cancer and determine more effective biomarkers for predicting metastasis, recurrence and diagnosis, extensive and in-depth studies on various aspects of gastric cancer were needed(Kanda et al. 2020; Wadhwa et al. 2013).

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Ring finger protein, transmembrane 2 (RNFT2), also known as TMEM118, was firstly reported to act as a downstream target gene for miR-627-5p in a study on the effects of smoking on airway epithelial changes(Huang et al. 2019). In addition, RNFT2 was rarely reported to be involved in other biological behavioral processes. RNFT2 could play a significant role in the innate immune response chain of lung disease via influencing ubiquitin-mediated degradation of IL-3Ra (IL-3 cytokine receptor)(Tong et al. 2020). Overexpression of RNFT2 could reduce pneumonia and injury, while knockdown of RNFT2 could exacerbate the inflammatory response of mouse lung injury(Tong et al. 2020). In a study on asthma, RNFT2 was reported to influence the pharmacological effects of long-acting beta-agonists and inhaled corticosteroids(Ortega et al. 2021). In bladder cancer, RNFT2 was reported to be upregulated in tumor tissues and cell lines, overexpression of RNFT2 suggested a poor prognosis. Besides, functional experiments showed that RNFT2 facilitated bladder cancer cell migration and proliferation(Lv et al. 2022). In hepatocellular carcinoma, RNFT2 was reported to predict prognostic condition(Yuemaierabola et al. 2023). In gastric cancer, higher RNFT2 expression was obviously related with a worse postoperative overall survival and a significantly higher peritoneal recurrence(Sasahara et al. 2021). However, the effects of RNFT2 on the phenotype of GC cells and the associated mechanisms remain unknown and have not been reported.

In the present study, we demonstrated that RNFT2 was remarkably upregulated in GC tissues and cells, and could serve as a tumor marker to predict prognosis. Notably, the biological behavior of RNFT2 in GC cells has not been investigated until now. Through a series of in vitro and in vivo cell function assays, we discovered that knockdown of RNFT2 expression in GC cells could inhibit cell proliferation, invasion and migration. Furthermore, through mechanism exploration, we observed that RNFT2 could affect the malignant progression of GC cells through the mTORC1 signaling pathway. Taken together, our results clarified the carcinogenic role of RNFT2 in GC progression and made RNFT2 as a potential target for GC diagnosis and therapy.

Materials and methods

Patient specimen collection

All GC tissues and matched normal tissues (the distance from the tumor tissue margin was greater than 5 cm) were rapidly frozen in liquid nitrogen and stored for protein and mRNA extraction, once obtained from patients undergoing gastrectomy at the Department of General Surgery, the Affiliated Cancer Hospital of Nanjing Medical University. This study also obtained informed consent from all GC participants and was approved by the Ethics Committee of the mentioned hospital.

Cell culture

All GC cells including AGS and HGC27, which cultured with F12K medium (Gibco, USA), supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS, Gibco, USA), GC cells MKN45 and the human normal gastric epithelial cell line GES-1, which cultured with RPMI-1640 medium (Gibco, USA), supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS, Gibco, USA), were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in a humidified incubator under 5% CO^2 at 37 °C.

Cell transfection

The full-length RNFT2 or shRNA sequences that targeting RNFT2 were designed and synthesized into lentiviral expression vectors (GenePharma, China) to construct stable overexpression and knockdown GC cell lines according to the provided protocol. We added puromycin (MedChemExpress, China) into mediums to single out stably transfected cells (which lasted one month) and the transfection efficiency was validated by RT-qPCR and western blot. The specific sequences of shRNA could be found in supplementary Table 1.

Western blot analysis

Total protein in GC tissues and cells were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) adding with protease and phosphatase inhibitor cocktail (Beyotime, China) as described in a previous study(Lin et al. 2019), separated using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (Millipore, USA) membranes. Then, the membranes were incubated with primary antibodies (p-S6K, S6K (Abcam, USA), RNFT2 (MyBioSource, USA), Actin, p-S6 and S6 (CST, USA) and secondary antibodies, respectively. The enhanced chemiluminescence (ECL) detection kit (Beyotime, China) was employed to detect chemical signals.

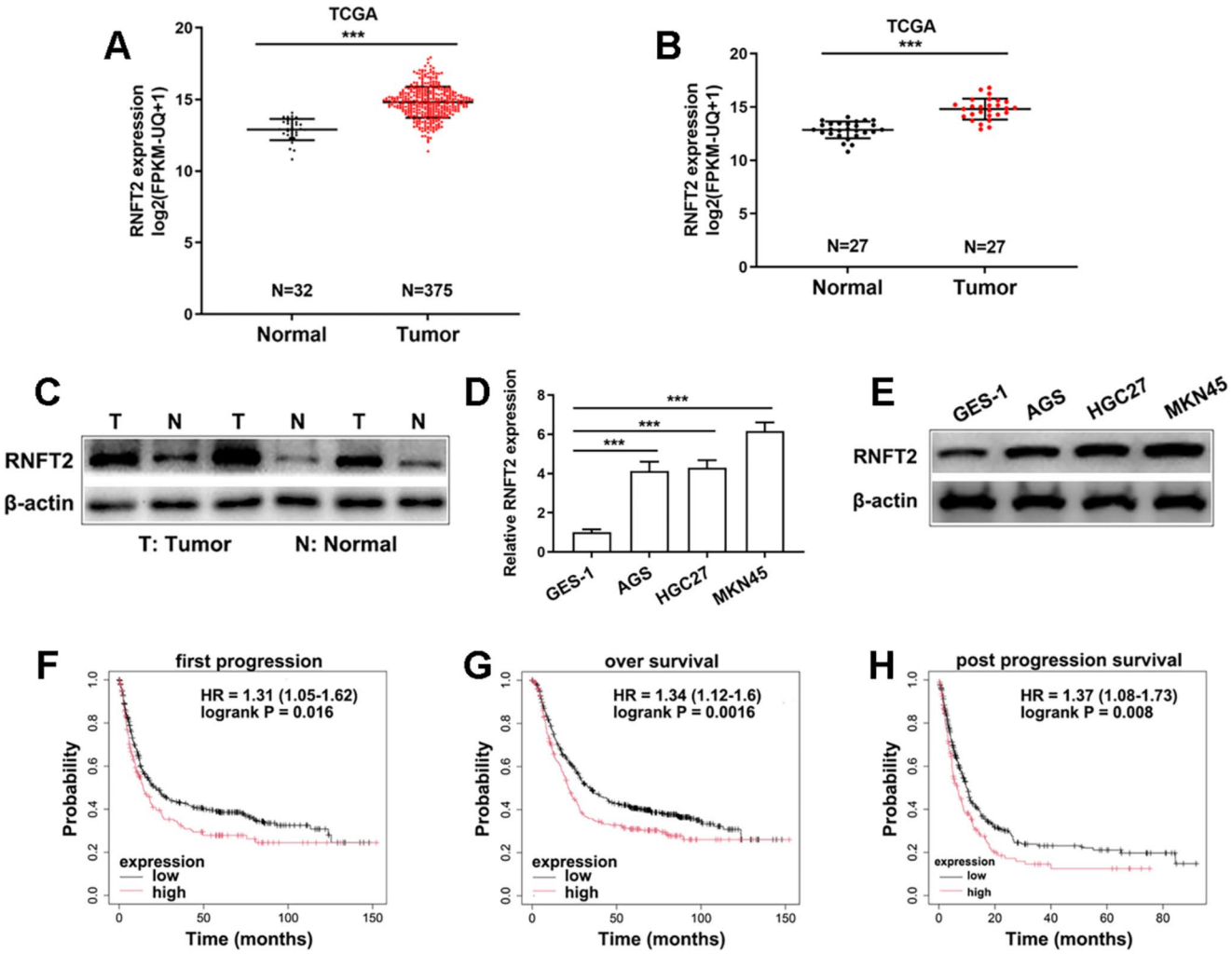


Fig. 1 RNFT2 was remarkably upregulated and correlated with poor prognosis in GC. **A**, **B**. RNFT2 was remarkedly overexpressed both in unpaired and paired gastric cancer tissues according to TCGA database. **C**. Western blot analysis to detect RNFT2 expression in protein levels in our own paired GC tissues. **D**, **E**. Western blot analysis and qRT-PCR analysis to detect RNFT2 expression in GC cell lines both in protein and mRNA levels. **F**-**H**. Kaplan-Meier analysis showed that gastric cancer patients with high expression of RNFT2 generally had worse prognosis than those with low expression of RNFT2. ***$p<0.001$

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated and extracted from tissues and cells by using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions, and the mRNA was transcribed into complementary DNA by using a PrimeScript RT Master Mix Kit (Takara, Japan). qRT-PCR was performed by using a 7500 Real-Time PCR System (Applied Biosystems, USA). β-actin was used as internal control. The specific primer sequences of RNFT2 could be found in supplementary Table 1.

Cell counting kit-8 (CCK-8) assay

The Cell counting kit-8 assay (CCK-8) (Djingo, Japan) was utilized to analyze cell viability in accordance with the manufacturer's recommendations as described in a previous study(Lin et al. 2019). When cells were treated differently, the microplate reader (Thermo Fisher Scientific) was used to measure the absorbance at 450 nm to determine the proliferation rate each day for 5 days.

5-Ethynyl-2′-deoxyuridine (Edu) assay

The EdU assay kit (RiboBio, China) was employed to analyze cell proliferation ability in accordance with the manufacturer's recommendations as described in a previous study(Lin et al. 2019). When cells were cultured in different

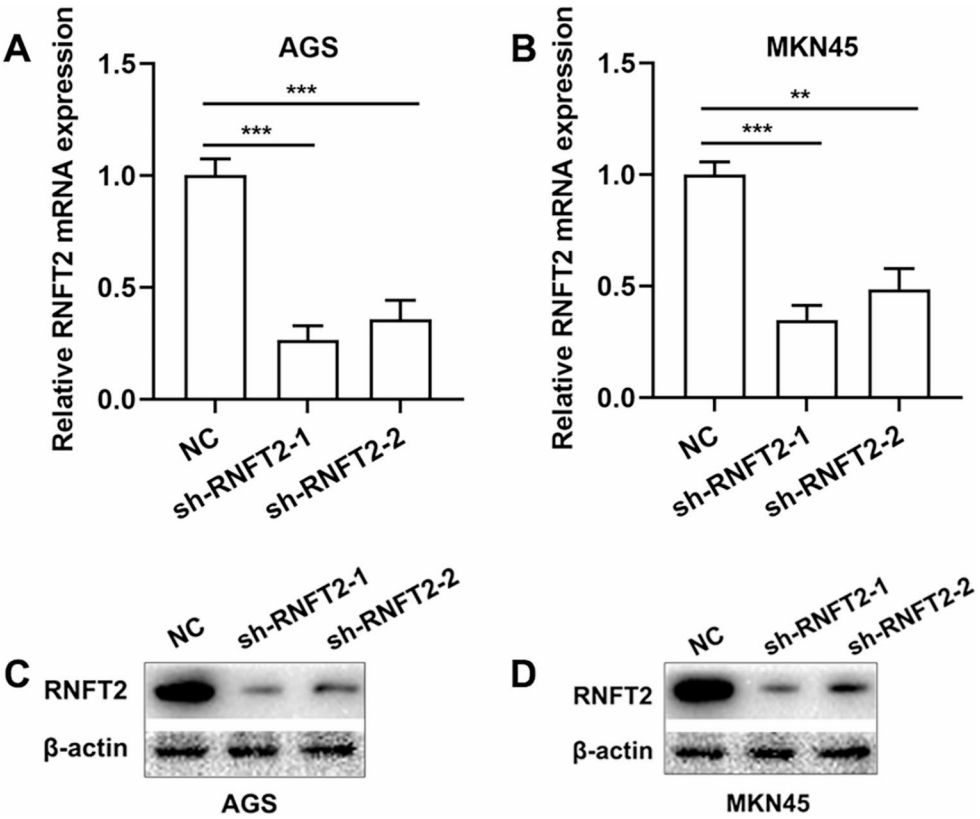


Fig. 2 Analysis of RNFT2 mRNA and protein expression in GC cells treated with sh-RNFT2. **A**, **B**. qRT-PCR analysis to detect the RNFT2 expression after transfecting GC cells with sh-RNFT2. **C**, **D**. Validate the knockdown efficiency by using western blot analysis after knockdown of RNFT2 expression. ***$p<0.001$

groups, fluorescence microscopy (Nikon, Japan) was used to determine the rate of EdU-positive cells after using the Hoechst 33,342 to stain the nucleus.

Wound healing assay

A scratch on the cell layers was created in different groups of cells, which were seeded and cultured in six-well plates, by using the 200 µl sterile pipette tip along the lines on the back of plates, then, each well was washed with phosphate-buffered saline (PBS). Images were taken at 0 h and 48 h, respectively, we used ImageJ software to calculate the cell healing rates by the fraction of cell coverage across the line.

Transwell assay

The transwell system (Corning, USA) was used to analyze cell migration and invasion capacity in accordance with the manufacturer's recommendations as described in a previous study(Lin et al. 2019). Medium with 1% antibiotics and 10% FBS was added to the lower chamber, and cells treated differently were cultured with the serum-free medium in the upper chamber. Matrigel (BD Biosciences, USA) should be covered in the upper membrane when it comes to evaluating invasive ability. Cells were observed using an inverted microscope after being stained with crystal violet.

Xenograft model

4-week-old female BALB/c nude mice were purchased and raised in specific pathogen-free barrier facilities in the Laboratory Animal Center of Nanjing Medical University (Nanjing, Jiangsu, China). Cells (1×10^6 cells/100 µl of PBS) treated differently were injected into the axilla of the mice (five mice for each group), we observed and measured the size of tumor each week. Tumor volume was calculated as: 0.5 x length x width x width. Four weeks later, the tumors from the mice were harvested and weighed. All animal procedures were approved by the Affiliated Cancer Hospital of Nanjing Medical University.

Statistical analysis

Experimental data were analyzed by GraphPad Prism (GraphPad Software, USA) or SPSS 22.0 (IBM, USA) and shown as mean ± standard deviation (SD). Statistical significance (p value < 0.05) was determined by the two-tailed student t test and analysis of variance (ANOVA).

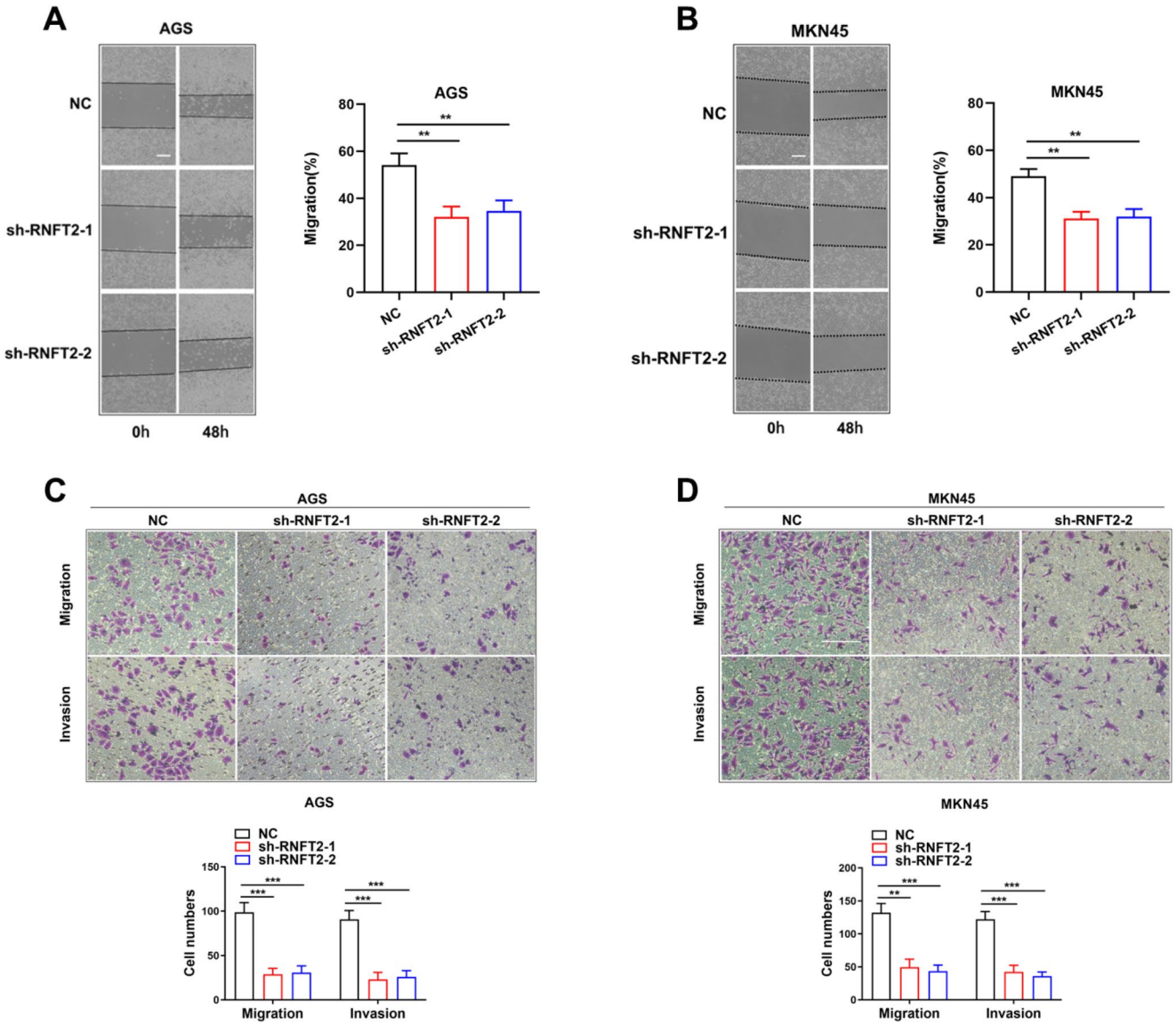
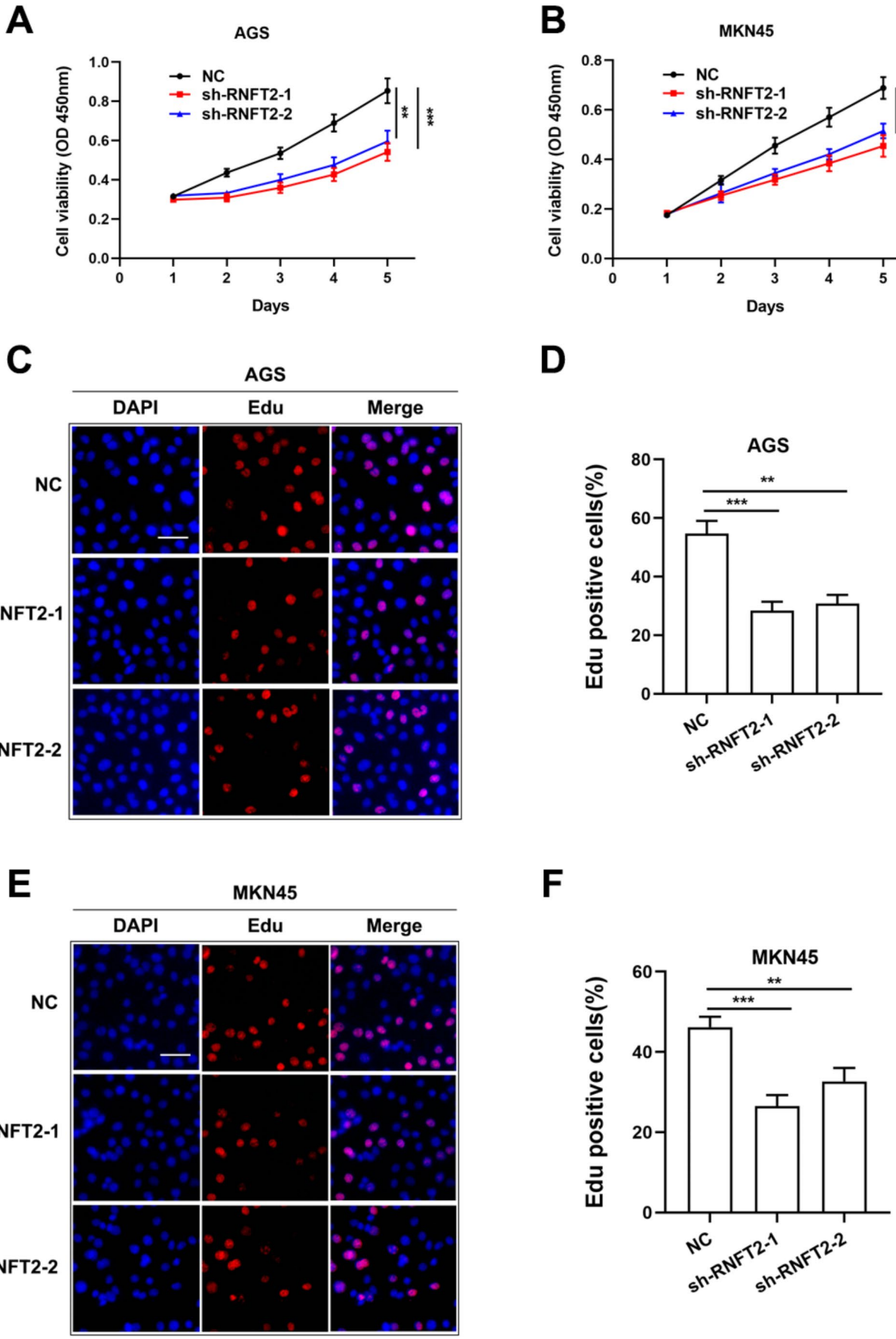


Fig. 4 Silencing RNFT2 suppressed migration and invasion of GC cells. **A**, **B**. Results from wound healing assay indicated that knockdown of RNFT2 could significantly reduce cell migration ability compared with the negative control through the wound healing assay (scale bar: 100 μm). **C**, **D**. Transwell assay showed that the migration and invasion ability of the GC cells was dramatically suppressed by downregulation of RNFT2 (scale bar: 200 μm). $^{**}p<0.01$, $^{***}p<0.001$

Results

RNFT2 was remarkably upregulated and correlated with poor prognosis in GC

To investigate the function of RNFT2 in GC, we firstly extracted and analyzed the RNA sequencing data of 32 adjacent non-tumor tissues and 375 GC tissues from TCGA and found that RNFT2 was remarkedly upregulated both in paired and unpaired gastric cancer tissues (Fig. 1A and B). To further validate its expression, we employed western blot analysis and qRT-PCR analysis to detect RNFT2 expression in protein and mRNA levels, and results suggested that RNFT2 also remained upregulated in our own paired GC tissues and cell lines both in protein and mRNA levels (Fig. 1C-E). In addition, the relationship between RNFT2 expression levels and prognosis was analyzed through Kaplan-Meier analysis (http://www.kmplot.com) in GC, and the results suggested that GC patients with RNFT2 overexpression generally had worse prognosis than those with low expression of RNFT2 (Fig. 1F-H).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AGS
Cell viability (OD 450nm)
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Fig. 3 Knockdown of RNFT2 expression inhibited cell proliferation in GC. **A**, **B**. Results from CCK8 assay suggested that knockdown of RNFT2 could significantly reduce cell proliferation ability compared with the negative control through the growth curves of CCK8 experiments. **C**-**F**. Edu assay indicated that the proliferation ability of the GC cells was dramatically suppressed by downregulation of RNFT2 (scale bar: 100 μm). ***$p<0.001$

Knockdown of RNFT2 expression inhibited cell proliferation in GC

Given that RNFT2 was significantly upregulated in GC tissues and cells and associated with poor prognosis, we wondered to further figure out the biological functions of RNFT2 in GC cells. In order to knockdown of RNFT2 expression, we transfected GC cells with shRNA and validated the knockdown efficiency by using qRT-PCR analysis and western blot analysis, which showed the satisfactory transfection efficiency (Fig. 2A-D). Subsequently, we employed CCK-8 assay and Edu assay to detect the cell proliferation ability. We observed that knockdown of RNFT2 could significantly reduce cell proliferation ability compared with the negative control through the growth curves of CCK8 experiments (Fig. 3A and B). Similarly, Edu assay indicated that the proliferation ability of the GC cells was dramatically suppressed by downregulation of RNFT2 (Fig. 3C-F). We also upregulated RNFT2 expression in gastric cancer cells (Fig.S1A), and the results showed that overexpression of RNFT2 could promote the proliferation activity of gastric cancer cells (Fig.S1B and C).

Silencing RNFT2 suppressed migration and invasion of GC cells

Based on our discovery that downregulation of RNFT2 could inhibit the proliferation of GC cells, we continued to investigate its effects on migration and invasion ability by employing transwell assay and wound healing assay. We found that knockdown of RNFT2 could significantly reduce cell migration ability compared with the negative control through the wound healing assay (Fig. 4A and B). Similarly, transwell assay indicated that the migration and invasion ability of the GC cells was dramatically suppressed by downregulation of RNFT2 (Fig. 4C and D). After upregulation of RNFT2, we found that overexpression of RNFT2 could accelerate the migration of gastric cancer cells (Fig.S1D and E).

Knockdown of RNFT2 suppressed malignant phenotypes in GC by inhibiting the phosphorylation of mTORC1 signaling pathway

Through a series of in vitro functional experiments, we discovered that silencing RNFT2 could suppress the proliferation, migration and invasion of gastric cancer cells. Subsequently, we further investigated the underlying mechanism by which RNFT2 caused the above malignant phenotypes. GSEA is a powerful and knowledge-based analytical method used to interpret gene expression data(Subramanian et al. 2005). GSEA via GSE51575 dataset suggested that the expression of RNFT2 was remarkably positively correlated with the mTORC1 signaling pathway in GC (Fig. 5A). Accumulating evidence demonstrated that mTOR signaling is crucial for regulating many cellular processes involved in cell growth and metabolism(Saxton and Sabatini 2017; Wu et al. 2021). To explore the relationship between RNFT2 and the mTORC1 pathways, the expression of key proteins including mTOR, pS6K and S6 were detected by performing western blot analysis. Results indicated the phosphorylation of mTOR, pS6K and S6 significantly reduced after knockdown of RNFT2 expression compared with the negative control groups (Fig. 5B and C). After overexpression of RNFT2 in GC cells, we found that the mTORC1 pathway was activated and this activation could be partially rescued by the mTORC1 pathway inhibitor rapamycin (Fig.S2A and B).

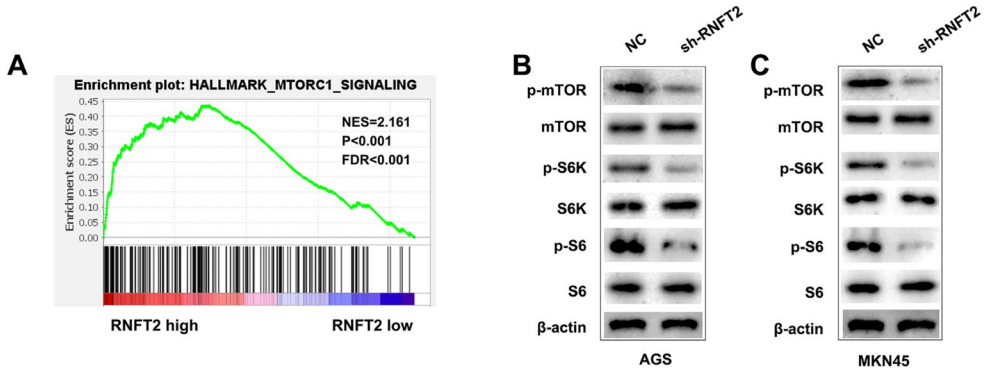

Fig. 5 Knockdown of RNFT2 suppressed malignant phenotypes in GC by inhibiting the phosphorylation of mTORC1 signaling pathway. (**A**) GSEA via GSE51575 dataset suggested that the expression of RNFT2 was remarkably positively correlated with the mTORC1 signaling pathway in GC. (**B**) Results from western blot analysis indicated that the phosphorylation of mTOR, pS6K and S6 significantly reduced after knockdown of RNFT2

Knockdown of RNFT2 suppressed GC tumor growth in vivo

To determine whether RNFT2 could influence tumor growth in vivo, the GC cells including AGS and MKN45 stably transfected with sh-RNFT2 or their negative control groups were inoculated subcutaneously into female nude mice. The tumor volume was observed and recorded regularly to evaluate the growth of different groups. The results suggested that the tumor size was remarkably smaller in sh-RNFT2 groups (Fig. 6A and B), the growth of tumors generated from sh-RNFT2 groups were slower (Fig. 6C and D), and the average weight were also lower in sh-RNFT2 groups than the negative control groups (Fig. 6E and F).

Discussion

Over the years, diagnostic and therapeutic techniques for gastric cancer have been constantly developing, but surgery remains the main method for radical treatment of GC(Sasako 2003). However, due to the lack of obvious symptoms and signs in early GC patients, many patients present with advanced disease at the initial diagnosis, missing the best opportunity for radical surgery to improve survival(Cervantes et al. 2008). Although we have made some progress in the fields of gastric cancer related treatments such as chemotherapy, immunotherapy, surgical techniques and molecular targeted therapy in recent decades, the overall therapeutic effect of gastric cancer is still unsatisfactory. This is because we know very little about the specific mechanisms of carcinogenesis and development of GC at the cellular and molecular levels(Liu et al. 2020; Lu et al. 2024; Shen et al. 2013; Van Cutsem and Ducreux 2016). Therefore, elucidating the molecular mechanism of GC pathogenesis and searching for effective diagnostic and therapeutic targets for gastric cancer are of great significance. Recently, RNFT2 has been rarely reported to be involved in biological behavioral processes. However, the effects of RNFT 2 on the phenotype of GC cells and the associated mechanisms remain unknown and have not been reported.

Most of the human genome is transcribed into ncRNAs, while only a small proportion of genes that encode proteins with biological functions are transcribed(Consortium 2012). The Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA) database are widely utilized in the study of various tumors, including gastric cancer. They contain large-scale molecular aberration data at the DNA, RNA, protein and epigenetic levels, as well as relevant clinical data(Figueiredo et al. 2022; Naqash et al. 2023; Pan et al. 2024). In this study, we employed TCGA human GC datasets to analyze gene expression in GC tissues and normal tissues, and RNFT2 was found highly expression and identified as a potential oncogene. Furthermore, we observed that the expression of RNFT2 was also upregulated in our paired GC tissues and cells, and survival analysis also showed that high expression of RNFT2 was associated with worse prognosis based on clinical information from TCGA and GEO databases (http://www.kmplot.com). These results showed that RNFT2 played a role as an oncogene in gastric cancer, and its intrinsic molecular mechanism and functional behavior needed to be further explored. Cell proliferation, migration and invasion are important phenotypes to evaluating the malignant biological behavior of tumors(Etienne-Manneville 2008; Liu et al. 2018). In terms of malignant biological behavior of gastric cancer cells, we employed CCK8 experiment and Edu experiment to analyze the effect of RNFT2 on the proliferation of GC cells, and our results indicated that knockdown of RNFT2 expression could significantly suppress the growth of GC cells. We used wound healing assay and transwell assay to examine the effects of RNFT2 on the invasion and migration of gastric cancer cells, and discovered that knockdown of RNFT2 could significantly suppress the migration and invasion of GC cells. In addition, more efforts were needed to analyze the potential molecular mechanisms by which RNFT2 accelerated the malignant biological phenotype of gastric cancer cells. We used GSEA to predict the active signaling pathway and observed that RNFT2 expression was positively correlated with the mTORC1 signaling pathway. The mechanistic target of rapamycin (mTOR) has two complexes, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2), and is an evolutionarily conserved serine/threonine protein kinase(Saxton and Sabatini 2017; Wu et al. 2021). Cells must achieve growth and division by increasing the synthetic metabolic pathway while inhibiting the catabolic pathway, and mTORC1 plays a central role in regulating all of these processes(Saxton and Sabatini 2017). When activated, mTORC1 phosphorylates and activates its key downstream effector ribosomal protein S6 kinase (S6K), and then S6K phosphorylates ribosomal S6 protein, ultimately exerting a series of biological functions, including cell proliferation, migration and invasion(Magnuson et al. 2012; Min et al. 2024; Saxton and Sabatini 2017). The mTORC1 pathway has also been widely demonstrated to play an important role in promoting tumor progression in gastric cancer(Geng et al. 2017; Kim et al. 2017). In our study, western blot was used to evaluate the exact relationship between RNFT2 and mTORC1 signaling pathway, and we found that knockdown of RNFT2 could downregulate the phosphorylation levels of mTOR, S6K and S6, further demonstrating that the effects of silencing RNFT2 expression on GC cell proliferation, migration and invasion were achieved through inhibition of mTORC1 signaling pathway. After overexpression

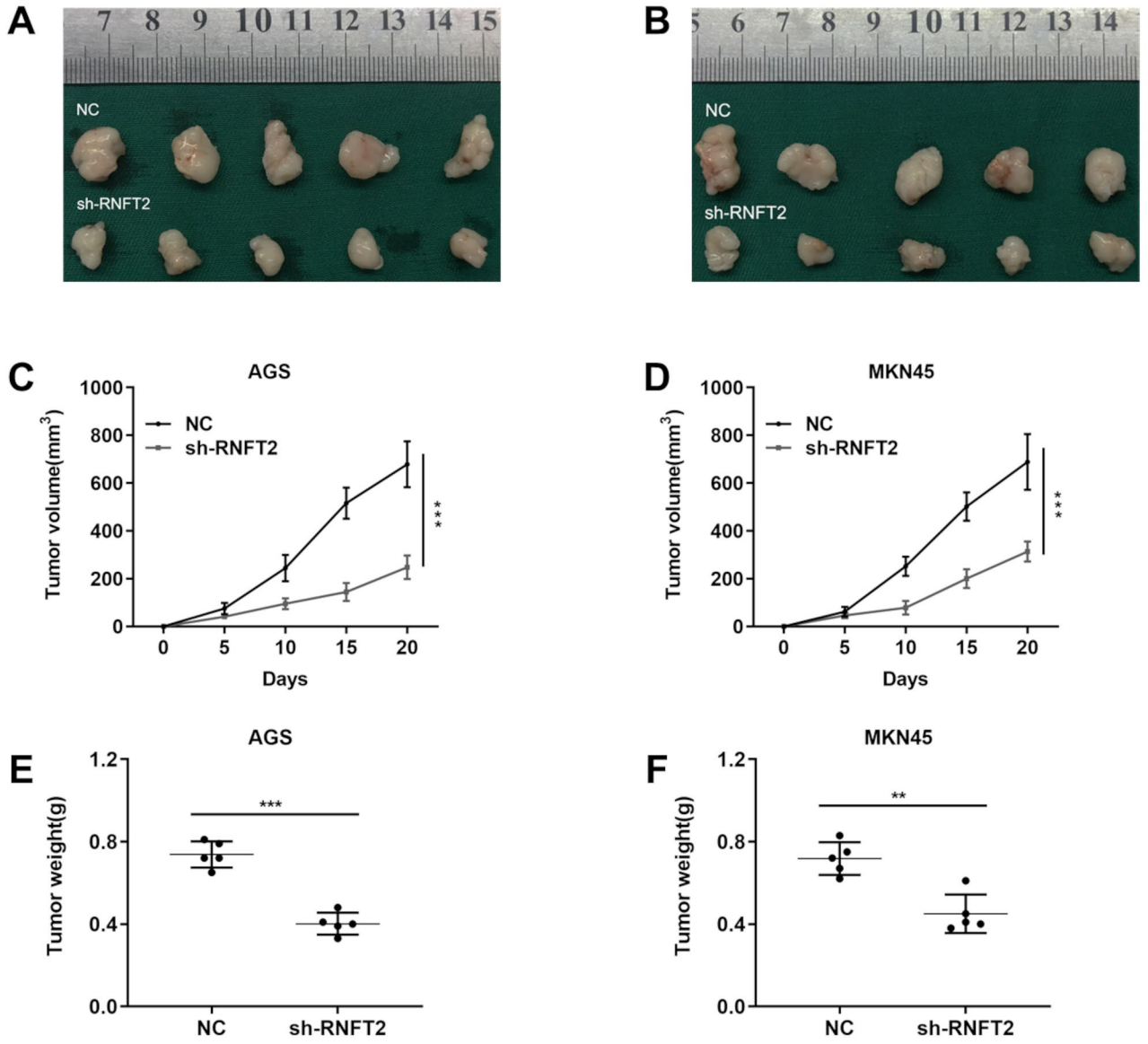

Fig. 6 Knockdown of RNFT2 inhibited GC tumor growth in vivo. **A**, **B**. Xenograft tumors harvested from the nude mouse model under different treatments. **C**, **D**. The tumors were remarkably smaller in sh-RNFT2 groups, the growth of tumors generated from sh-RNFT2 groups were slower. **E**, **F**. The average weight were also lower in sh-RNFT2 groups than the negative control groups. $^{**}p<0.01$, $^{***}p<0.001$

of RNFT2 in GC cells, we found that the mTORC1 pathway was activated and this activation could be partially rescued by the mTORC1 pathway inhibitor rapamycin, which also demonstrated that RNFT2 affected gastric cancer progression by regulating the mTORC1 pathway. In addition, it has been reported that RNFT2 could play a significant role in the innate immune response chain of lung disease via influencing ubiquitin-mediated degradation of IL-3Ra(Tong et al. 2020). We cannot rule out that RNFT2 may affect the mTORC1 signaling pathway by participating in the proteolytic process.

However, there are some limitations in our study which need further consideration. Due to the complex interactions between molecules in cells, despite the above research results, we cannot rule out the possibility that RNFT2 affects the malignant biological behavior of GC cells through other signaling pathways. Secondly, a deeper mechanism by which RNFT2 activates the mTORC1 signaling pathway still needs to be explored. Finally, relevant research needs to be expanded to other gastric cancer cell lines rather than the two representative cells involved in this study. Further research is needed in the future to fill these gaps.

In summary, we observed that RNFT2 was significantly upregulated in GC and influenced the proliferation, invasion and migration of GC cells through the mTORC1 signaling pathway. Our results clarified the carcinogenic role of RNFT2 in GC progression, provided inspiration to further understand the molecular mechanism of GC and make RNFT2 as a potential target for GC diagnosis and therapy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00726-025-03449-2.

Author contributions ZL designed the study. YW, QM, ZZ and HF performed the experiments. YW, HS, HF and ZL analyzed and interpreted the data. YW wrote the manuscript. QM, ZZ and HS have done a lot of work in the process of manuscript revision, including experiments and fund support. All authors read and approved the final version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that they have no conflict of interest.

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