



Original research

Oncosuppressive role of MicroRNA-205–3p in gastric cancer through inhibition of proliferation and induction of senescence

Oncosuppressive role of MicroRNA-205 in gastric cancer

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ARTICLE INFO

Key words:

microRNA-205–3p

CXCL11

Gastric cancer

Senescence

Proliferation

A B S T R A C T

Background: Our previous study showed that CXCL11 could play an immunomodulatory role. In this study, we investigated the regulator (miR-205–3p) of CXCL11 and the mechanism of miR-205–3p as a tumor suppressor gene in gastric cancer (GC).

Materials and methods: A target relationship between miR-205–3p and CXCL11 was revealed by using the bioinformatics method. This study detected the expressions of miR-205–3p and CXCL11 through qRT-PCR and Western blotting. Moreover, the expressions of Akt, PD-L1, p16, p21, and senescence-associated secretory phenotype (SASP) factor were determined. The effects of miR-205 on proliferation, invasion, and senescence of GC cells were assessed by using methods, such as transfection, Transwell assay, tablet cloning, flow cytometry, and senescence-associated beta-galactosidase (SA-β-gal) staining. Furthermore, the effects were verified using methods, like immunohistochemistry, flow cytometry and SA-β-gal in animal experiments.

Results: Based on the study, it is found that the expression of miR-205–3p is down-regulated, while that of CXCL11 is up-regulated in GC cell lines. By regulating CXCL11, miR-205–3p inhibits Akt activation, reduces the proliferation and invasion of GC cells, promotes cell apoptosis, induces senescence of GC cells, and secretes immunostimulatory SASP factor. The animal experiments confirm that miR-205–3p promotes cell senescence, down-regulates the immunosuppressive signal induced by PD-L1, and promotes secretion of immunostimulatory SASP factor, so that more T cells are recruited in blood and tumors.

Conclusions: This study revealed the molecular mechanism of miR-205–3p in inhibiting proliferation and invasion and inducing senescence of GC cells by regulating CXCL11 and Akt pathways in animal and cell experiments.

Introduction

Gastric cancer (GC), as the fifth most common cancer, is the third leading global cause of cancer-related death [1]. Among all GC cases, those cases in China account for more than 50% thereof. According to the latest statistics, there were about 679,100 newly diagnosed GC cases, and about 498,000 patients died from GC in 2015 in China. Although the survival rate of patients with early-stage GC is significantly increased by surgery, radiation therapy, and chemotherapy, the incidence and mortality thereof remain high in China. The prognosis of GC is generally

poor, and the five-year survival rate of patients with advanced GC remains below 30% [2, 3]. At present, there is a lack of effective treatments. In recent years, the focus of research has mostly shifted to studying novel biomarkers and developing new targeted treatment strategies.

Chemokines are a type of small molecular proteins and the chemokine secreted by tumor cells plays an important role in tumor invasion and immune regulation [4, 5]. CXCL11 (chemokine 11) is a member in the sub-family of Glu-Leu-Arg (ELR)-negative CXC chemokines, also known as IFN-inducible T cell α chemoattractant. Its receptor CXCR3 is

Abbreviations: GC, Gastric cancer; MicroRNAs, Micro-ribonucleic acids; miR-205, MicroRNA-205; CXCL12, chemokine (C-X-C motif) ligand 12; MAPKs, mitogen-activated protein kinases; PI3K-Akt, phosphatidylinositol 3-kinase PI3K-Akt; STAT, signal transducers and activators of transcription; PD-L1, programmed cell death-ligand 1; SASP, senescence-associated secretory phenotype.

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Received 16 June 2021; Received in revised form 22 July 2021; Accepted 7 August 2021

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not only expressed on immune cells, but also on many tumor cells, such as breast cancer, malignant melanoma, kidney cancer, and colon cancer [6]. CXCL11/CXCR3 can trigger the activation of downstream pathways, such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase PI3K-Akt (PI3K-Akt), signal transducers and activators of transcription (STAT), and regulate tumor proliferation and metastasis [7]. Our previous study implied that CXCL11/CXCR3 signaling could play an immunomodulatory role by activating Akt and STAT pathways and inducing up-regulated expression of programmed cell death-ligand 1 (PD-L1) in GC cells [8], so the upstream microRNA which regulates CXCL11 has aroused our curiosity. Our study indicated that miR-205-3p was an upstream molecule of CXCL11.

Micro-ribonucleic acids (MicroRNAs), as a short non-coding RNAs that can regulate the expressions of key genes, are generally involved in regulating proliferation, invasion, and metastasis of tumor cells and promoting or inhibiting tumor development. MicroRNA-205 (miR-205) was first found in sequences of mouse and *Fugu rubripes*, and its expression was then detected in zebrafish and human [9]. MiR-205 was found to be expressed in human mammary, prostate, and thymus glands, indicating that miR-205 can regulate the normal human physiological function [10]. For example, miR-205 can repair skin damage by regulating the Akt pathway, and promote cell metastasis and adhesion by regulating chemokine (C-X-C motif) ligand 12 (CXCL12) [11]. In human cancer, abnormally expressed miR-205 is generally thought to play a role as a tumor suppressor gene in some tumors, such as prostatic cancer, breast cancer, and colon cancer [12]. Some studies show that miR-205 can induce senescence of melanoma and breast cancer cells, thereby inhibiting tumor progression [13, 14], which has aroused much interest. The senescence of tumor cells has always been a research hotspot, and is typically characterized by irreversible cell growth arrest and changes in the secretion of immunostimulatory cytokines and can be induced by a variety of factors. Cell senescence is considered to be an important way in which the body can fight oncogenesis [15, 16], however, the molecular biological mechanism of action of miR-205 in inhibiting tumor progression by inducing senescence has yet to be elucidated.

This research indicated that miR-205-3p was an upstream molecule of CXCL11 through bioinformatics. Moreover, through *in vitro* and *in vivo* experiments, the research revealed the molecular mechanism by which miR-205-3p down-regulates CXCL11 protein and inhibits activation of Akt signaling, thus hindering the proliferation and invasion of GC cells. In addition, the signal axis can induce senescence of GC cells, promote the secretion of immunostimulatory senescence-associated secretory phenotype (SASP) factor, and decrease the expression of immunosuppressive protein PD-L1. It can also recruit more T cells *in vivo* to inhibit the progression of GC in a synergistic manner. This finding revealed that miR-205-3p may become a potential target for the treatment of GC in the future.

Materials and methods

Bioinformatics analysis

The miRNAs which target CXCL11 were predicted by identifying overlapping microRNAs across different databases (Targetscan, http://www.targetscan.org/vert_71/; miRDB, <http://mirdb.org/miRDB/index.html>; miRwalk, <http://mirwalk.umm.uni-heidelberg.de/>; RNA22, <https://cm.jefferson.edu/rna22/> and DIANA, http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) (Table S1).

Cell culture, plasmids, and transfection

All the cell lines used in this study were purchased from the Type Culture Collection of the Chinese Academy of Sciences (China), and grown in RPMI-1640 (Hyclone) with 10% foetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere containing 95% air

and 5% CO₂ at 37 °C [17]. pEX-miR null control vector (pEX Null), pEX-hsa-mir-205 expression vector (pEX miR-205) and CXCL11 siRNA sequence (GenePharma, Inc., Shanghai, China) were purchased. The CXCL11 siRNA sequence was 5'-GAGAACAUUUCUGUCUCUAdTdT-3'. Transient transfection was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with the One-Step PrimeScript RT-PCR Kit (Takara, Japan) according to manufacturer's instructions. Samples were normalized to β -actin (Takara, Japan), as indicated. Primers used for qRT-PCR were:

miR-205 Fw 5'-TGGGCTGAGTCCCTCT-3'
 miR-205 Rev 5'-GAGGGACGGGTGATGGGCAGATTGG-3'
 CXCL11 Fw 5'-TGCCCAAAGGAGTCCAACAA-3'
 CXCL11 Rev 5'-TTTCCGACCAATGGTAGCCT-3'
 IL-1 α Fw 5'-ACCAC TG TTC TCT TCTTACCC-3'
 IL-1 α Rev 5'-TAGGAGGAAGGGAGAAA TCG TG-3'
 IL-1 β Fw 5'-CAGGATGAGGACCCAAGCAC-3'
 IL-1 β Rev 5'-GTCGTCATCATCCACGAGT-3'
 IL-8 Fw 5'-TTGGCAGCCTTCCTGATTC-3'
 IL-8 Rev 5'-AACTTCTCCACAACCCTCCTG-3'
 IL-6 Fw 5'-TTCGGTCCAGTTGCCTTCT-3'
 IL-6 Rev 5'-GTACTCATCTGGACAGCTC-3'
 MMP3 Fw 5'-ACAATGGACAAAGGATACAACAGG-3'
 MMP3 Rev 5'-CATCTTGAGACAGGCGGAACC-3'

Cell proliferation, invasion, and colony formation assays

The cell proliferation rate was determined using Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindo Laboratories, Japan). AGS cells were seeded onto 96-well plates at a density of 3000 cells per well. Cell proliferation was documented every 24 h for 3 days and absorbance at 450 nm was evaluated by a microplate absorbance reader (Bio-Rad). Cell invasion was determined by Transwell invasion assay. Briefly, AGS cells were plated in the upper chamber (Costar, Corning, USA) coated with Matrigel (BD Bioscience, USA) at a density of 3×10^4 cells per well. After 48 h, the non-invading cells in the upper chamber were removed, and the invaded cells under the filter were stained with crystal violet and counted in nine fields. For the colony formation assay, 200 cells were seeded in a 100-mm plate and cultured until visible colonies appeared. Colonies were stained with Giemsa and counted.

Cell cycle and apoptosis analysis

For cell cycle analysis, AGS cells were harvested, washed with PBS, and stained with propidium iodide (50 μ g/mL) in the presence of RNase (10 μ g/mL) for 2 h in the dark at 4 °C. The percentages of AGS cells in G₀/G₁, S and G₂/M phases of cell cycle were determined. Apoptosis was evaluated by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) Apoptosis Detection Kit (Solarbio) according to the manufacturer's protocol. Briefly, AGS cells were harvested, washed twice with PBS, and incubated in 200 μ L binding buffer with 10 μ L annexin V-FITC and 10 μ L PI for 15 min in the dark at room temperature. Flow cytometry was performed on a FACSCalibur, and data were analyzed using ModFit software (BD).

Western blot analysis

The Western blot analysis was performed as previously described [18]. The antibodies used were: anti-CXCL11 (ab216157, Abcam), anti-p-AKT (ab38449, Abcam), anti-AKT (ab8805, Abcam), anti-PD-L1 (ab205921, Abcam), anti-P16INK4A (ab201980, Abcam), anti-P21

(ab109520, Abcam), and anti-GAPDH (ab8245, Abcam).

Luciferase reporter assay

The wild-type and mutated 3'-UTR region of CXCL11 containing the binding site of miR-205 was cloned into pGL3 reporter vector (Promega USA). AGS cells were transfected with wild-type or mutant reporter plasmid and miR-205 mimics in 24-well plates. The miR-205 mimics were obtained from GenePharma. Firefly luciferase activities were measured with a Dual-Luciferase Assay (Promega, USA) kit some 48 h after transfection and normalized with Renilla luciferase. All experiments were conducted in triplicate.

Stable transfections and in vivo study

AGS cells transfected with pEX miR-205 or pEX Null as control and selected with puromycin (1 mg/mL). For *in vivo* studies, 1×10^7 cells (pEX miR-205 or pEX Null) were injected subcutaneously into nude mice (10 mice in each group), and the tumor size was monitored for 30 days using caliper measurements as an index. Tumor volume was calculated thus: $v = 0.5ab^2$ (v , the tumor volume; a , the major-axis diameter of the tumor; b , the minor-axis diameter of the tumor). At the end of the experiment, blood and tumor tissue were collected for further examination. This study was approved by the Institutional Animal Care and Use Committee of China Medical University.

Immunohistochemistry assays

Immunohistochemical assays were performed on nude mice xenograft tissue as described previously [19], using the anti-PD-L1 (ab205921), anti-P16^{INK4A} (ab201980), and anti-P21 (ab109520) antibodies (Abcam, USA). All histological and IHC slides were evaluated (in a single-blind manner) by two pathologists independently.

SA- β -Gal activity assays

SA- β -Gal staining was performed on AGS cells and nude mice xenograft tissue by using the Senescence Cell Histochemical Staining Kit (Sigma, USA) following the manufacturer's instructions. In brief, cells were fixed with fixation buffer, washed with PBS, and incubated with SA- β -Gal staining solution at 37 °C overnight. Cells were counted using a contrast phase microscope (Olympus, Japan).

Flow cytometry analysis of in vivo sample preparation

For *in vivo* sample preparation, blood and tumors were collected and allocated for 10% formalin fixation, and snap frozen tissue and flow cytometry analysis. To prepare single-cell suspensions of tumors for flow cytometry analysis, tumors were minced using scissors into small pieces, washed with PBS and digested with 0.05% collagenase/dispase (Roche) for 15 min at 37 °C. Samples were then transferred to a gentleMACS™ Octo dissociator (Miltenyi Biotec). Dissociated tissue was filtrated with a 50 μ m cell strainer, centrifuged at 800 rpm for 2 min and re-suspended with PBS. For blood samples, 3 mL of whole blood was layered onto Histopaque-1077 (Sigma), then centrifuged at 1500 rpm for 30 min at room temperature. After centrifugation, the opaque interface was transferred with a pipette into a clean centrifuge tube, thrice-washed with PBS, and re-suspended with PBS. Samples were incubated with the following antibodies for 30 min on ice: CD3-PeCy7 (Invitrogen), FITC-CD8, CD69-PE, and APC-CD44 (eBioscience, UK). Flow cytometry was conducted on a FACSCalibur, and data were analyzed using ModFit software (BD). This study was approved by the Institutional Animal Care and Use Committee of China Medical University.

Statistical analysis

All statistical analysis and graphs were generated using GraphPad Prism 8.0 software. Differences between two groups were evaluated by Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference. The level of significance was set to $*p < 0.05$ and $**p < 0.01$. Data are expressed as the mean \pm SD of measures used.

Results

Down-regulated expression of miR-205-3p and up-regulated expression of CXCL11 in GC cells

The expression of miR-205-3p in eight GC cell lines and one normal gastric cell line was first evaluated by the quantitative reverse transcription polymerase chain reaction (qRT-PCR). By doing so, it is found that the expression of miR-205-3p in the eight GC cell lines is significantly down-regulated compared with that in the normal cell line (Fig. 1 (A)) and the lowest expression is shown in AGS GC cells. This indicates that miR-205-3p is a potential GC inhibitor. By using the bioinformatics method, it is predicted that miR-205-3p is the precise upstream regulator of CXCL11. The seed sequence of miR-205-3p is complementary to the 3' untranslated region (3'UTR) of CXCL11, which is highly conserved in six different species (Fig. 1(B) and (C)). Furthermore, this research further investigated the relationship of expressions between miR-205-3p and CXCL11 and evaluated the mRNA and protein expressions of CXCL11 in the same aforementioned eight GC cell lines and one normal gastric cell line. It is found that, compared with the normal gastric cell line, CXCL11 shows significantly up-regulated expressions in the eight GC cell lines (Fig. 1(D) and (E)), although its absolute expressions vary across these GC cell lines. These data suggest that the expression of miR-205-3p exhibits the opposite trend to that of CXCL11 in GC cells, which supports the potential role of miR-205-3p as a regulator of CXCL11.

miR-205-3p promoting cell apoptosis and reducing proliferation and invasion of GC cells by regulating CXCL11

This study ascertained whether the 3'UTR of CXCL11 is a functional target of miR-205-3p. The 3'UTR reporter gene plasmids of wild-type and mutant CXCL11 complementary to the subsequence of miR-205-3p were constructed (Fig. 2(A)). Based thereon, it is found that luciferase activity in the group with overexpressed miR-205-3p significantly decreases compared with the negative control (NC) group in GC AGS cells with wild-type CXCL11-3'UTR structures subject to transient transfection, however, in GC AGS cells with mutant CXCL11-3'UTR structures subject to transfection, there is no significant difference in expressions of luciferase activity between the group with overexpressed miR-205-3p and the NC group (Fig. 2(B)). The results show that 3'UTR of CXCL11 is a direct target of miR-205-3p.

To study the role of miR-205-3p in GC cells, the following experiments were conducted: in the Cell Counting Kit-8 (CCK8) experiment, in comparison with the NC group, miR-205-3p overexpression significantly inhibits the growth rate of GC cells, and thus the inhibition of miR-205-3p is counteracted by adding CXCL11 (Fig. 2(C)). In the tablet cloning experiment, the clone-forming ability of GC cells transfected with miR-205-3p decreases compared with the NC group and is significantly restored after adding CXCL11 (Fig. 2(D)). To explore whether miR-205-3p induces apoptosis of GC cells or not, flow cytometry was used to reveal that apoptotic cells account for 4.9% of GC cells transfected with miR-205-3p, which is significantly increased compared with that (1.88%) in the NC group. In GC cells in the miR-205+CXCL11 group, the proportion (1.8%) of apoptotic cells decreases, suggesting that miR-205-3p overexpression promotes cell apoptosis and CXCL11 can block apoptotic signaling (Fig. 2(E)). These results show that miR-205-3p significantly weakens proliferation of GC cells by inhibiting CXCL11. The Transwell assay demonstrates that the number of GC cells

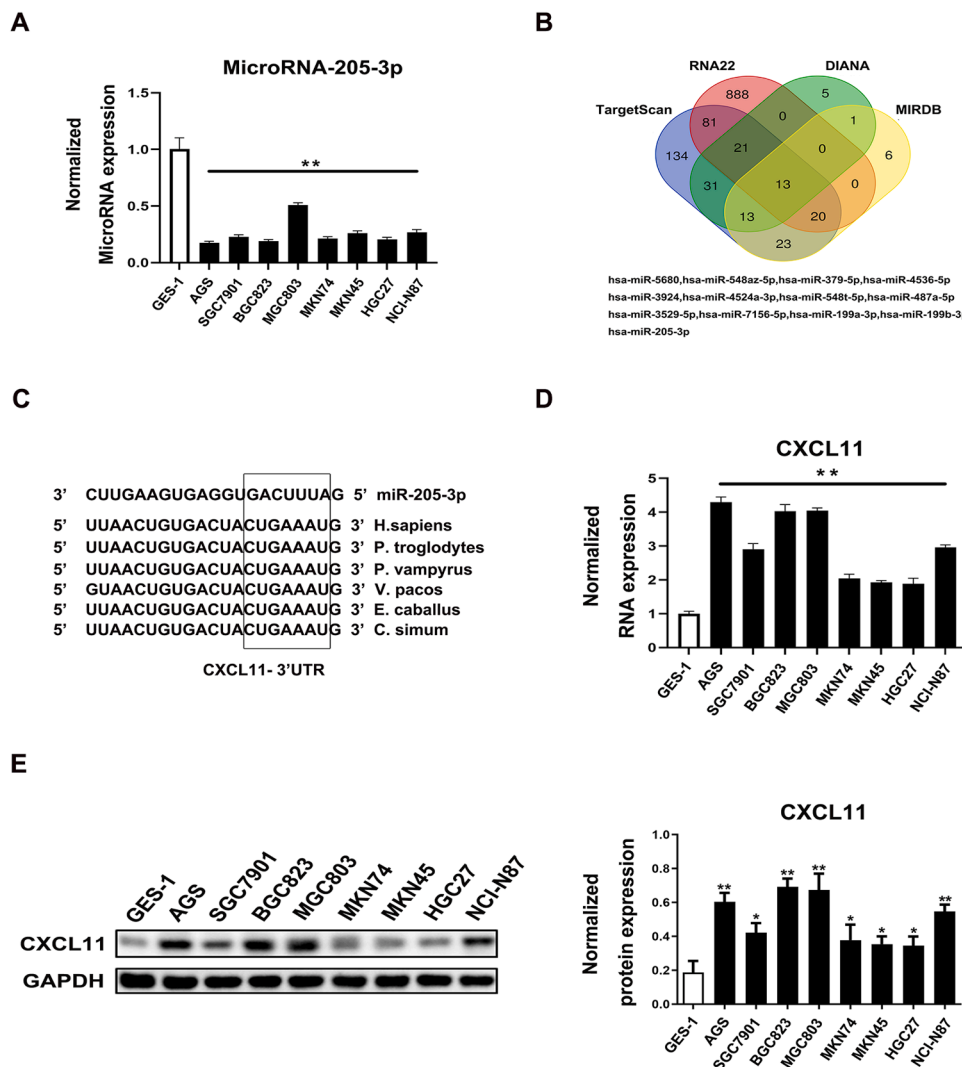


Fig. 1. Down-regulated expression of miR-205-3p and up-regulated expression of CXCL11 in GC cells

A: miRNA quantitative RT-PCR analysis of miR-205-3p expression level in eight GC cell lines and one normal gastric cell line. **B:** Venn diagram of the numbers of overlapping miRNAs from different databases (Targetscan, miRDB, RNA22 and DIANA). **C:** The miR-205-3p seed sequence is complementary to the 3'-UTR of CXCL11 and is conserved in six different species. **D:** Quantitative RT-PCR analysis of CXCL11 expression level in eight GC cell lines and one normal gastric cell line. **E:** CXCL11 expression was analyzed by Western blot in eight GC cell lines and one normal gastric cell line. * $p < 0.05$ and ** $p < 0.01$.

passing through compartments decreases significantly in the group with overexpressed miR-205-3p compared with the NC group. Based on this, after adding CXCL11, the number of cells passing through compartments increases again (Fig. 2(F)). These results show that miR-205-3p targeting CXCL11 can effectively inhibit the motility and invasion of GC cells.

Effects of miR-205-3p on cell cycle progression and cell senescence

To explore the mechanism of miR-205-3p in mediating senescence, GC cells in miR-NC, miR-205, and miR-205+CXCL11 groups were analyzed by the flow cytometry. The percentage (59.42%) of cells in the miR-NC group in cell cycle G1 is significantly lower than that (67.58%) of the miR-205 group, while the percentage of cells in the miR-205+CXCL11 group in cell cycle G1 is 58.37%, which reverses the inhibitory effect of miR-205 on cell cycle progression (Fig 3(A) and (B)). This implies that miR-205-3p blocks cell cycle progression, which reconfirms the regulation of CXCL11 by miR-205-3p. In the senescence-related β -galactosidase (SA- β -gal) staining experiment, the percentage of SA- β -gal-positive GC cells in the miR-205 group is significantly higher than that in the miR-NC group. Meanwhile, an interesting phenomenon can be observed in Fig. 3(C): GC cells in the miR-205 group show senescence-like cell enlargement (Fig 3(C) and (D)), suggesting that miR-205-3p induces senescence of GC cells.

miR-205-3p induces senescence of GC cells and secretes SASP factors by regulating CXCL11 and inhibiting Akt activation

It has been reported that both miR-205 and CXCL11 are related to the Akt pathway [8, 11], so this study speculated that miR-205 can induce senescence and secrete SASP factors by inhibiting CXCL11 and Akt. Western blotting (WB) demonstrates that in GC cells in the group with overexpressed miR-205, the expressions of CXCL11, pAkt, and PD-L1 are significantly down-regulated, while those of senescence marker factors P16^{INK4A} and P21^{KIP1/CIP1} are notably up-regulated. To assess whether miR-205 induces changes in downstream factors by regulating CXCL11 or not, CXCL11 was inhibited. It is found that the expressions of CXCL11, pAkt and PD-L1 are significantly down-regulated in GC cells, while those of P16^{INK4A} and P21^{KIP1/CIP1} are significantly up-regulated; however, after adding CXCL11 in GC cells in the group with overexpressed miR-205, the expressions of CXCL11, pAkt, and PD-L1 are remarkably up-regulated, while those of P16^{INK4A} and P21^{KIP1/CIP1} are greatly down-regulated (Fig. 4(A) and (B)). To explore whether Akt can cause changes to downstream factors or not, Akt phosphorylation was inhibited. It is found that the expressions of pAkt and PD-L1 are significantly down-regulated, while those of P16^{INK4A} and P21^{KIP1/CIP1} are significantly up-regulated in GC AGS cells after inhibiting Akt phosphorylation (Fig. 4(C) and (D)), proving that miR-205 can induce senescence by inhibiting CXCL11 and Akt.

In the present research, SASP factors were further detected by using

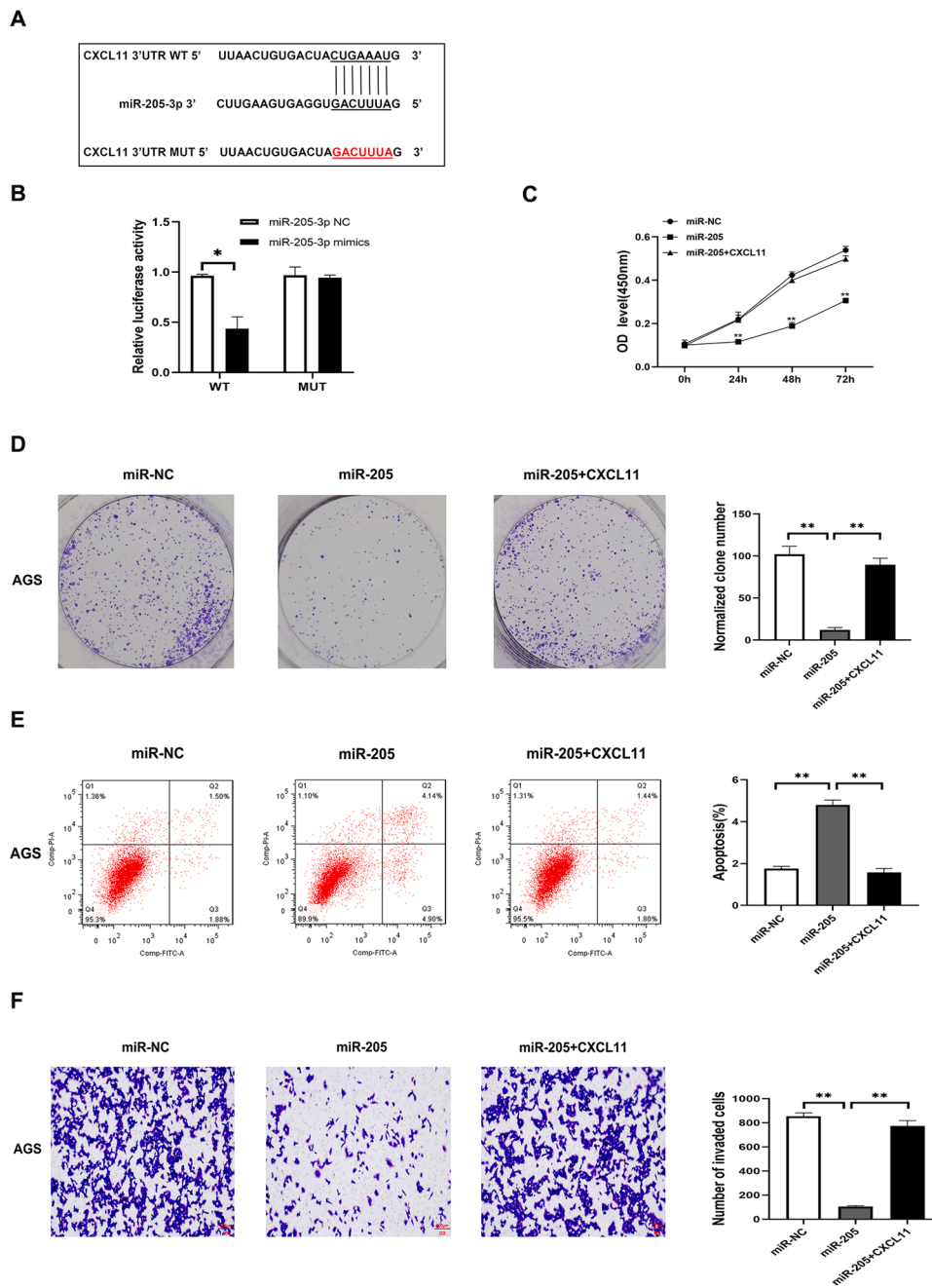


Fig. 2. miR-205-3p inhibits proliferation and invasion and promotes apoptosis by regulating CXCL11 in gastric cancer cells.

A: Putative miR-205-3p binding sequence on CXCL11 wild-type(WT) 3'-UTR and mutant 3'-UTR sequence that abolished binding. **B:** Luciferase assay shows decreases in reporter activity after co-transfection of CXCL11 wild-type(WT) 3'-UTR with miR-205-3p mimics in AGS cells. **C:** In CCK8 experiment, the growth rate of AGS cells after miR-205-3p transfection is reduced compared with the NC group(miR-NC), and significantly restores after adding CXCL11(100 ng/ml). **D:** miR-205-3p overexpression significantly inhibits the clone-forming ability of gastric cancer cells, and the inhibition is counteracted by adding CXCL11(100 ng/ml). **E:** Apoptotic analysis is performed on miR-205, miR-205+CXCL11 and NC groups. The percentage of apoptotic cells is reported. **F:** In Transwell invasion assay, miR-205-3p overexpression significantly reduces the number of invasive cells, and the reduction is counteracted by adding CXCL11(100 ng/ml). * $p < 0.05$ and ** $p < 0.01$.

qRT-PCR in GC cells. The results show that in the GC cells in the group with overexpressed miR-205, those including IL-1 α , IL-1 β , IL-6, IL-8, and MMP3 increase compared with the NC group. This further proves that miR-205-3p can induce senescence of GC cells and the senescent cells secrete SASP factors.

In vivo experiments verifying that miR-205-3p promotes senescence of GC cells and secretes SASP factor to recruit more T cells in blood and tumors

The cell experiment shows that the miR-205-3p signaling pathway inhibits proliferation and invasion of GC, induces senescence of GC cells, secretes immunostimulatory SASP factors, and reduces the expression of immunosuppressive protein PD-L1. Furthermore, it is verified in animal experiments that the tumor volume and growth rate of nude mice in the group with overexpressed miR-205 are significantly reduced (Fig. 5(A) and (B)) compared with the NC group. By performing

immunohistochemical analysis and SA- β -gal staining on tumors, the expressions of P16^{INK4A} and P21^{KIP1/CIP1} are up-regulated, while the expression of PD-L1 is down-regulated in the group with overexpressed miR-205. Moreover, the proportion of SA- β -gal-stained cells increases (Fig. 5(C) and (D)). qRT-PCR was used to detect SASP factors (IL-1 α , IL-1 β , IL-6, IL-8, and MMP3) in tumor cells. It is observed that the indicators of the group with overexpressed miR-205 are significantly up-regulated (Fig. 5(E)). Previous studies indicate that SASP factors can activate immune surveillance, produce innate and adaptive immune responses for removing senescent and proliferative tumor cells, and enhance the inhibition on senescent tumor [20]. This study carried out the flow cytometry on tumors and blood in nude mice to detect the number of CD44⁺CD8⁺T and CD69⁺CD8⁺T cells. Interestingly, both types of T cells are increased in the group with overexpressed miR-205 (Fig. 5(F)). The vivo experiments were approved by China Medical University Application for Laboratory Animal Welfare and Ethics

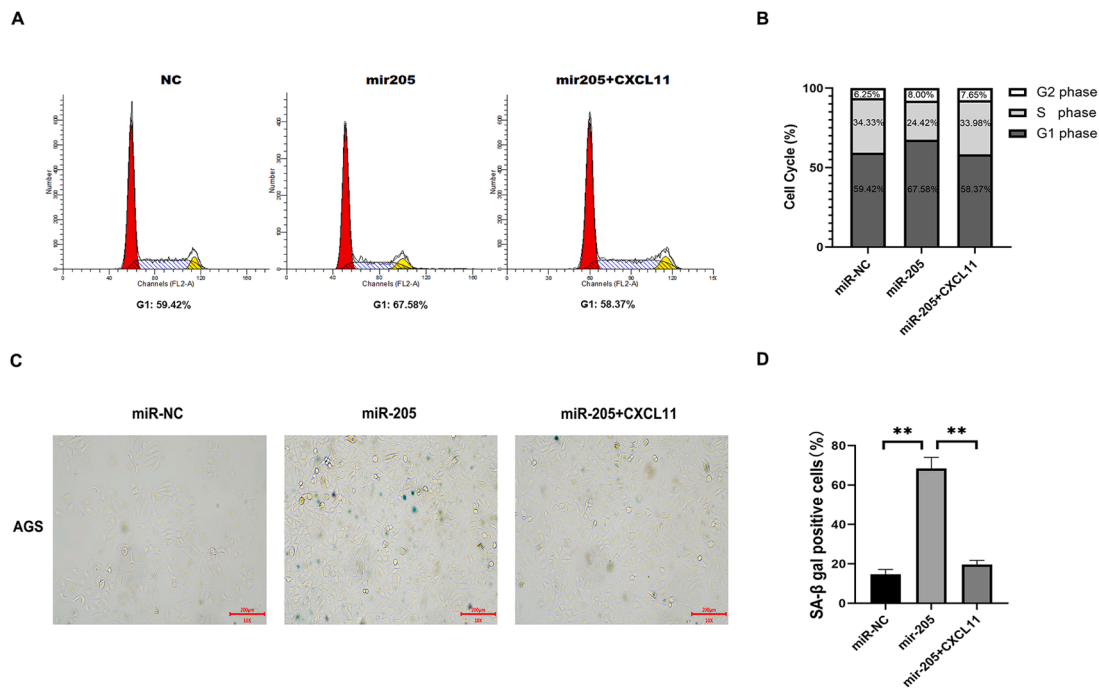


Fig. 3. Effects of miR-205-3p on cell cycle progression and cell senescence.

A, B: Cell cycle analysis shows increase in the G1 phase of AGS cells overexpressing miR-205 as compared with negative control (miR-NC), while additional CXCL11 (100 ng/ml) reverses the increase. C, D: SA-β-gal staining was performed on miR-205, miR-205+CXCL11 and NC groups and light microscope cell images were acquired. The percentage of SA-β-gal-positive cells is reported (D). ** $p < 0.01$.

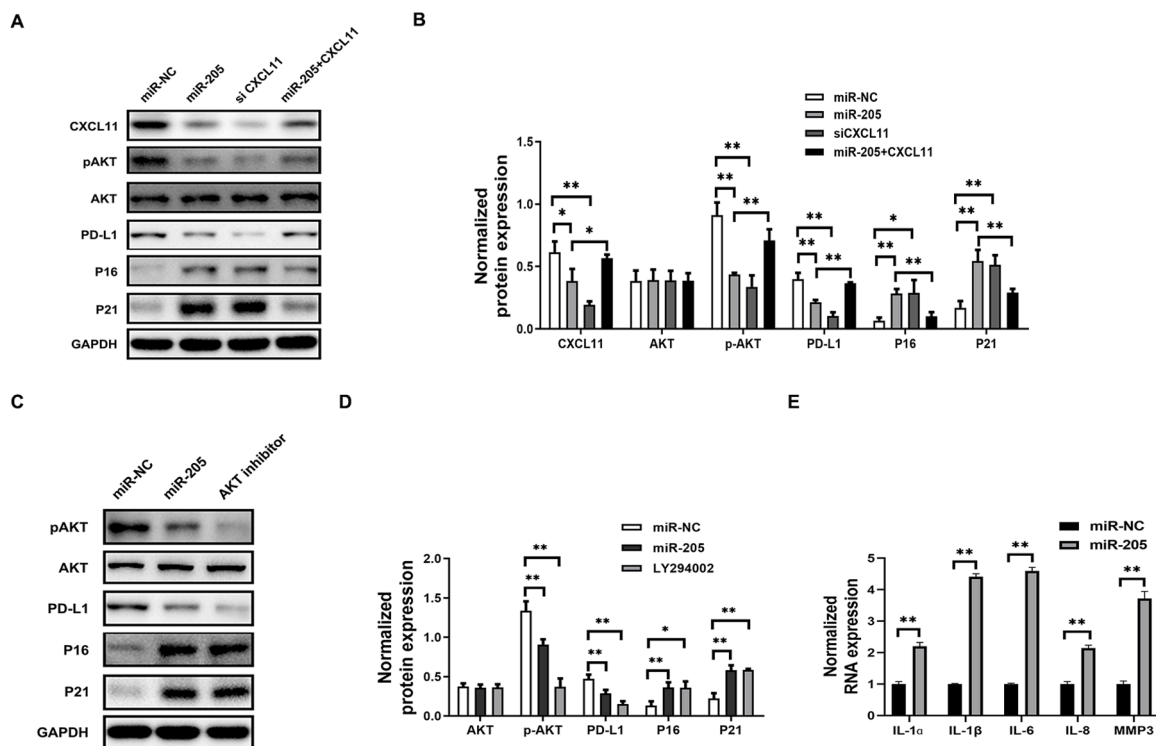


Fig. 4. miR-205-3p inducing senescence of gastric cancer cells and secreting SASP factors by regulating CXCL11 and inhibiting Akt activation

A, B: AGS cells are transfected with miR-205 alone for 48 h or with addition of CXCL11 (100 ng/mL) after 24 h of transfection for another 24 h, or transfected with CXCL11 siRNA for 48 h. and cell lysates were collected for Western blot analysis. C, D: AGS cells are treated with LY294002 (an Akt inhibitor, 25 μM) for 2 h or transfected with miR-205 for 48 h, The protein levels of indicated genes were measured by Western blot. GAPDH was used as an internal control for the total protein measurement. E: mRNA levels of the indicated genes in AGS cells transfected with negative control or miR-205. Actin was used for normalizing the expression of mRNA. * $P < 0.05$ and ** $P < 0.01$.

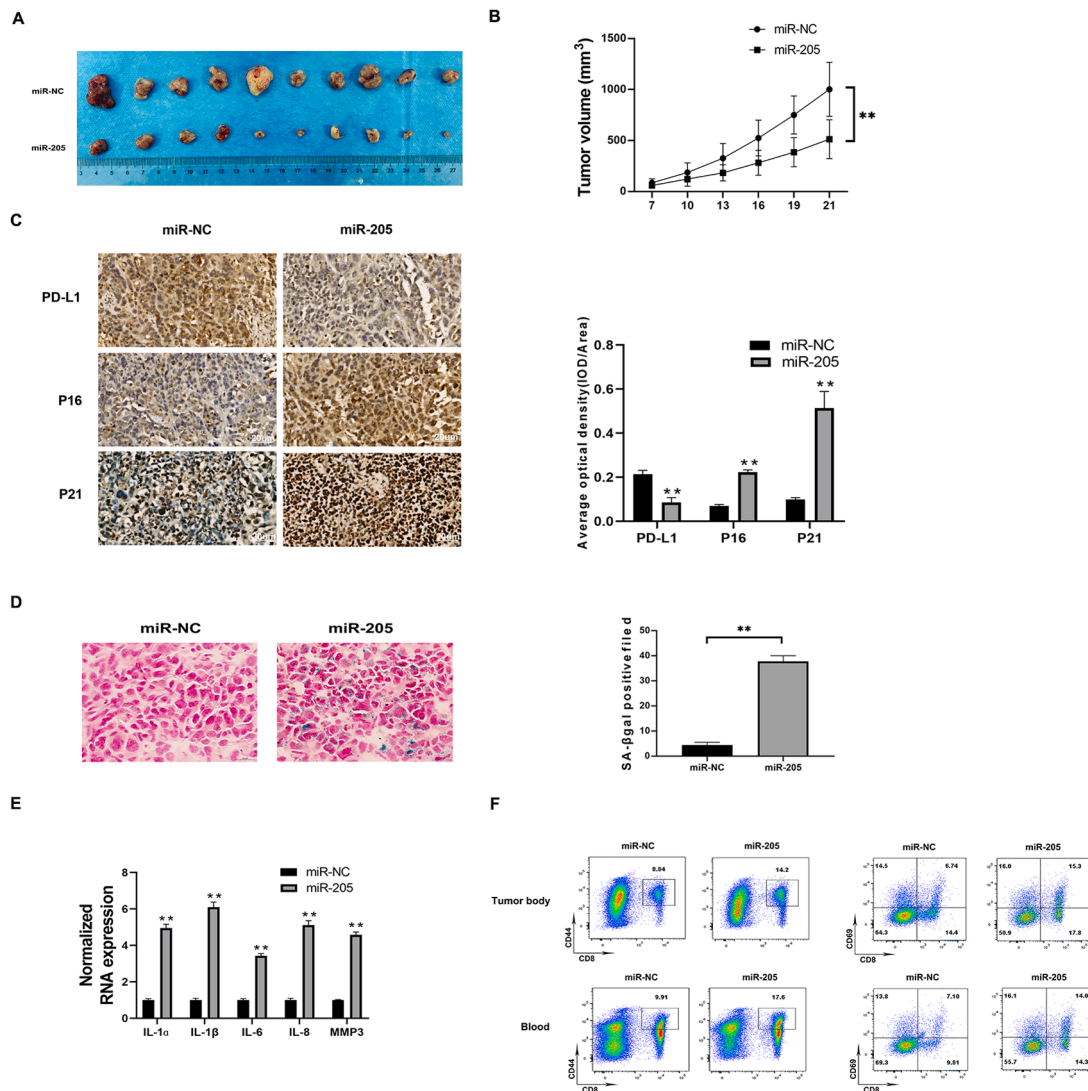


Fig. 5. *In vivo* experiments verifying that miR-205-3p promotes senescence of gastric cancer cells and secretes SASP factor to recruit more T cells in blood and tumors.

A: Representative tumor photos of AGS xenografts following subcutaneous injection of AGS cells expressing miR-205 or negative control in nude mice. B: Tumor volume was measured on the days indicated. (n = 10, each group). C: Representative immunohistochemical staining and quantitative analysis of P16, P21 and PD-L1 expression of AGS xenograft sections of miR-205 and negative control groups. D: Representative SA-β-gal staining and quantitative analysis of AGS xenograft sections of miR-205 and negative control groups. E: qRT-PCR assay is used to detect the mRNA levels of IL-1α, IL-1β, IL-6, IL-8 and MMP3 in AGS xenografts of miR-205 and negative control groups. F: Representative example of CD8⁺CD44⁺ and CD8⁺CD69⁺ T cells gated on live CD3⁺ T cells in blood and freshly dissociated xenograft tumors of miR-205 and negative control groups. **P < 0.01.

Committee.

Discussion

Accumulating evidence indicates that miR-205 can be used as a tumor suppressor to target the corresponding oncogenes in various tumors. For instance, the abnormally expressed miR-205 significantly inhibits the proliferation of breast cancer cells by inhibiting the expression of ErbB3 [21]. In addition, it has been reported that miR-205 can promote tumor formation and proliferation. The overexpression of miR-205 is observed in cervical cancer cells, and the expressions of its targets Cyr61 and CTGF are down-regulated [22]. Therefore, miR-205 is likely to play a dual role as both “friend” and “enemy” in tumors. This study suggests that the expression of miR-205-3p is down-regulated, while the expression of CXCL11 is up-regulated in GC cells, and 3’UTR of CXCL11 can pair with miR-205-3p sequence. Based on tablet cloning, Transwell assay, CCK8, and apoptosis experiments, it is observed that miR-205-3p

can significantly inhibit growth and invasion of GC cells, indicating that miR-205 plays the role of an enemy in GC, and targets and regulates downstream CXCL11 to play an anti-tumor role.

Cell senescence is an irreversible state of cell cycle arrest and the increased SA-β-gal activity is a key feature of senescence. In senescent cells, cell cycle arrest is closely related to the increased levels of cell cycle inhibitors including p16^{INK4a}, p21^{CIP1}, and p27, which are considered to be specific biomarkers of senescence [23, 24]. Due to its anti-proliferative function, senescence plays a role in preventing tumor development, which is considered to be an effective anti-tumor mechanism. With the increasing interest in the molecular mechanisms that drive cell senescence, miRNAs have gradually become key effectors used to stimulate senescence [25]. For instance, miR-34 is recognized as a tumor suppressor, which can induce senescence by inhibiting mRNA of genes, such as encoding E2F and c-Myc, so it is a potent inducer of senescence [26, 27]. It has been reported that miR-205 can induce senescence of breast cancer cells by targeting E2F1 [14], while the

mechanism of miR-205 in regulating senescence remains unclear. In this study, GC cells in the group with overexpressed miR-205 show characteristics of cell cycle arrest and increased SA- β -gal activity compared with the NC group, which is restored after adding CXCL11. In the follow-up WB assay, the expressions of CXCL11, pAkt, and PD-L1 are significantly down-regulated, while the expressions of P16^{INK4A} and P21^{KIP1/CIP1} are significantly up-regulated in GC cells in the group with overexpressed miR-205. In addition, in GC cells in the group with overexpressed miR-205, the expressions of IL-1 α , IL-1 β , IL-6, IL-8, and MMP3 are found to be significantly up-regulated as evinced by qRT-PCR compared with the NC group. In the animal experiment, immunohistochemistry and SA- β -gal staining on tumors with overexpressed miR-205 show that P16^{INK4A} and P21^{KIP1/CIP1} are positively expressed, while PD-L1 is negatively expressed compared with the NC group. Moreover, the proportion of SA- β -gal-positive cells increases. By performing the qRT-PCR on tumors with overexpressed miR-205, it is found that the expressions of IL-1 α , IL-1 β , IL-6, IL-8, and MMP3 are up-regulated. The research results demonstrate that, by inhibiting CXCL11 and Akt pathways, miR-205-3p induces senescence of GC cells and reduces the expression of negative immunoregulatory protein PD-L1. Moreover, senescent GC cells secrete SASP factors, decrease the degree of malignancy of GC and inhibit tumor progression.

Although senescent cells do not divide, they can maintain their vitality and metabolic activity for a long time and exhibit SASP. In recent years, it has been found that senescent cells can affect the cell micro-environment by secreting SASP factors, which is conducive to tumor inhibition, wound healing, embryonic development, and promotion of tumor generation [28]. Among many SASP factors, IL-1 α , IL-1 β , IL-6, and IL-8 are the most common immunostimulatory cytokines. For example, IL-6 and IL-8 can induce the senescence of adjacent cells by generation of paracrine, forming a barrier to inhibit tumor growth [29, 30]. SASP factors can activate immune surveillance, produce innate and adaptive immune responses for removing senescent and proliferative tumor cells, and enhance the ability of senescence-inhibiting tumors [31]. It has been reported that in breast cancer, senescent tumor cells secrete immunostimulatory SASP factors including IFN - γ , TNF - α , and IL-12P70, which can be used as anti-tumor vaccines [32]. The *in vivo* experiments in the present study demonstrate that the tumor body in nude mice in the group with overexpressed miR-205 significantly decreases, and the expressions of IL-1 α , IL-1 β , IL-6, IL-8, and MMP3 are up-regulated as revealed by detection with qRT-PCR. In the tumor body and blood of nude mice, detection by flow cytometry shows that the number of CD44⁺CD8⁺T and CD69⁺CD8⁺T cells increases significantly compared with the NC group. This indicates that senescent GC cells may secrete immunostimulatory SASP factors to inhibit tumor growth and recruit more T cells. More experiments such as co-staining with T cell marker and tumor cell marker were required to draw the conclusions. Meanwhile, immunohistochemical results suggest that overexpressed miR-205-3p significantly down-regulates the expression of PD-L1. PD-L1 expressed on the surface of tumor cells can bind with PD-1 on T cells to induce T-cell apoptosis, which leads to immune escape of tumor cells, however, blocking their binding can reactivate T cells and clear tumor cells [33]. It is speculated that the down-regulation of immunosuppressive signal induced by PD-L1 and up-regulation of immunostimulatory SASP factors induced by miR-205-3p may be jointly involved in immunomodulation to clear GC cells, which needs to be further verified in subsequent experiments.

Conclusion

In the present study, the molecular mechanism of miR-205-3p in suppressing the proliferation and invasion of GC and induction of senescence by inhibiting CXCL11 and Akt pathways was elucidated by animal and cell experiments. It was found that, in the immune micro-environment, senescent GC cells can secrete SASP factors, stimulate the immune system to recruit more T cells, and down-regulate the

immunosuppressive signal induced by PD-L1, which enriches the network of miR-205-3p for negatively regulating GC progression. Although many clinical trials are still needed for verification, miR-205-3p is likely to be a new target for the clinical treatment of GC.

Authors' contributions

Chenlu Zhang: Conceptualization, Methodology, Writing- Reviewing and Editing

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Declaration of Competing Interest

The authors have declared that no competing interest exists

Funding

This work was supported by National Natural Science Foundation of China (no. 81600693) and China Medical University "Youth Backbone Support Plan" Project (QGZ2018047).

Acknowledgments

To doctors Keyang Zhang and Jie Huang for their valuable suggestions and help in the study design.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2021.101199](https://doi.org/10.1016/j.tranon.2021.101199).

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