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Tumor Cells Inhibit the Activation of ILC2s Through Up-Regulating PD-1 Expression

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Up-regulating programmed cell death ligand-1 (PD-L1) expressed on tumor cells and tumor-infiltrating myeloid cells interacting with up-regulated programmed cell death-1 (PD-1) expressed on tumor-infiltrating lymphoid cells greatly hinder their tumor-inhibiting effect. It is necessary to explore the deep mechanism of this negative effect, so as to find the potential methods to improve the immunotherapy effect. Here we found that the expression of PD-1 in colon cancer-infiltrating type II innate lymphoid cells (ILC2s) was highly up-regulated, which greatly restrained the activation and function of ILC2s. Furthermore, anti-PD-1 could restore the function inhibiting and effective cytokine secretion of ILC2s when co-cultured with tumor cells. In vivo studies proved that anti-PD-1-treating promoted the activation of tumor-infiltrating ILC2s and delayed the tumor growth of MC38-bearing mice. Our studies demonstrate a new PD-1/PD-L1 axis regulating mechanism on innate immune cells, which provide a useful direction to ILC2s-based immunotherapy to cancer diseases.

Keywords: Colon Cancer; PD-L1; PD-1; ILC2s; Cancer microenvironment.**Introduction**

Cancer diseases have become a global challenge, and the number of newly diagnosed cancer patients is increasing every year [1]. Due to the undefined pathogenesis of cancer diseases and the complexity of the cancer microenvironment, there has been no effective treatment for multiple cancer diseases, such as colon cancer [2]. Up to date, colon cancer is mainly treated by surgery, accompanied with radiotherapy and chemotherapy, but the therapeutic effect is unsatisfactory, especially for patients with advanced metastases [3]. However, with the advancement of medical science, treatment methods such as immunotherapy, targeted therapy, intervention, and radiofrequency continue to

emerge, providing new treatment avenues for cancer patients [4, 5]. As an innovative treatment method, tumor immunotherapy has become a hot spot in the field of tumor treatment research.

The concept of «cancer immunotherapy» is to fight the growth and development of tumors with enhancing the body's own immune system [6]. So far, cancer immunotherapy is mainly divided into four categories, adoptive cell therapy, non-specific immune activators, cancer vaccines and immune checkpoint inhibitors (ICBs) [7]. Among these therapeutic approaches, ICBs treatment stands at the point, and that can equip tumor treatment with novel and powerful «weapons» playing a huge role in the treatment of various tumors [8]. In general, tumor-infiltrating tumor-killing

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lymphocytes, mainly is anti-tumor T lymphocytes, up-regulate the immune checkpoints, such as PD-1, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and lymphocyte activation gene-3 (Tim-3) expression, which mediate the inhibitory signal greatly extinguish the activation and effect exert of tumor-killing lymphocytes, thus promotes the tumor immune invasive [9-11]. PD-1, as an inhibitory receptor, was first discovered in T cells, and that serve a role of inhibitory signaling to prevent T cell excessive activation in physiological state. However, in cancer diseases, PD-1 expression on anti-tumor cytotoxic T cells significantly up-regulated, and inter-act with PD-L1 expressed on tumor or tumor-infiltrating myeloid cells, greatly hinder the activation of cytotoxic T cells and their anti-tumor function [12-14].

Except the adaptive T cells, PD-1 is also found expressed on innate lymphoid cells (ILCs), such as ILC2s. In physiological situation, PD-1 expressed on ILC2s delivers inhibitory signaling, which prevent the inflammatory cytokine secretion, thus sustaining the homeostasis of body [15,16]. ILC2s, a subset of ILCs, do not express antigen-specific receptor, but express transcription factors ROR α and GATA3, and secrete type 2 cytokine IL-4, IL-5, IL-9 and IL-13. Previous studies have shown that ILC2s mainly participate in the anti-helminth response and the development of allergic diseases like asthma [17,18]. Recent years, the role of ILC2s in tumor diseases has been discovered, and studies have proved that ILC2s serve as a double-edged sword in the development of tumor diseases [19,20]. Here, we found that tumor cells can up-regulate the expression of PD-1 on ILC2s, which greatly inhibits the activation and function of ILC2s. Neutralizing antibody PD-1 significantly prevent the tumor growth and restore the ILC2s activation inhibitory, which demonstrates the potential anti-tumor role of ILC2s, and that maybe provide a useful direction to ILC2s-based immunotherapy to cancer diseases.

Material and methods

Reagents

Mouse anti-CD45, anti-Lineage, anti-CD90.2, anti-ST2, anti-PD-1 flow cytometry antibodies were purchased from Biolegend (San Diego, CA, USA). IL-4, IL-5, IL-9, IL-13 primer synthesis by Shengsong Biotech Co., Ltd (Shanghai, China). IL-4, IL-5, IL-9, IL-13 ELISA kits and the FITC-AnnexinV Apoptosis Detection Kit were bought from Multi Sciences (Lianke, China) Biotech Co., Ltd. (Hangzhou, China). Mouse PD-1 neutralizing antibody was bought from R&D systems (Minnesota, USA). Cell culture medium (1640 and DMEM) and fetal bovine serum (FBS) was obtained from Gibco Life Technologies (New York, USA).

Cells and animals

6-8 weeks old female BALB/c mice and nude mice were purchased from Yangzhou University animal center (Yangzhou, China) and Jiangsu university animal center (Zhenjiang, China). All mice were housed in the Animal Center of Jiangsu University in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. All methods and protocols were approved by the Animal Care and Use Committee of Jiangsu University. MC38 were cultured in DMEM medium supplemented with 10% FBS and 10 U/mL penicillin, and 10 U/mL streptomycin (Gibco, NY, USA). ILC2s were isolated from mouse spleen by magnetic bead sorting and flow cytometry sorting, and the purity

was identified by flow cytometry (FCM) analysis. Isolated ILC2s were cultured in RPMI-1640 medium supplemented with 10% FBS and 10 U/mL penicillin, and 10 U/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Construction of the MC38 tumor-bearing model

To building the MC38 transplantation mice tumor model, we treated 5×10⁵ MC38 tumor cells to mice by subcutaneous injection. Monitoring the tumor size from day 7 and anti-PD-1 were treated to the MC38 tumor-bearing mice to regulate the tumor growth by intravenous injection. The length (L), diameter, and width (W) of the tumor were tested with a vernier caliper, and the volume of the tumor calculated using formula $V = \pi \times L \times W^2/6$.

Enzyme-linked Immunosorbent Assay (ELISA)

Cell culture supernatants were collected. IL-4, IL-5, IL-9 and IL-13 were measured by ELISA kits according to the manufacturer's instructions.

RNA extraction and real-time fluorescence quantitative PCR (RT-qPCR)

Total RNA was extracted from ILC2s using TRIzol reagent according to the manufacturer's protocol and transcribed into first-strand cDNA. After cDNA synthesis, real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad, Shanghai, China) and a Real-Time PCR System (Bio-Rad, Hong Kong). β -Actin was included as an internal control, and gene expression was quantified relative to β -actin. The primer sequences are shown: IL-4: forward: 5'-GCTAT-TGATGGGTCTACCC-3' reverse: 5'-CAGGACGTCAAG-GTA-CAGGA-3'; IL-5: forward: 5'-AGGATGCTTCTGCACTTGAG-3' reverse: 5'-CCTCATCGTCTCATTGCTTG-3'; IL-9: forward: 5'-CACAAATGCACCTTCTGGGACA-3' reverse: 5'-TCACTCCAAC-GATACGGTCTCT-3'; IL-13: forward: 5'-TGAGCAACATCACAAAGACC-3' reverse: 5'-AGGCCATGCAATATCCTCTG-3'.

Apoptosis analysis

Collecting MC38 tumor cells in the co-culture system, MC38 cells were stained with AnnexinV/PI Apoptosis Detection Kit according to the manufacturer's protocol. Briefly, MC38 cells were resuspended in binding buffer. Then, 10ul of FITC-AnnexinV and 20ul of APC-PI were mixed with the cells and incubated for 20 min at room temperature. Adding another 200ul wash buffer to resuspend the incubated cells for FCM detecting, and the data were analysed using Flow Jo software.

Flow cytometry

Tumor tissues were digested by the solution containing 0.5mg/mL collagenase V, 0.2 mg/mL hyaluronidase, and 0.015 mg/mL DNase I (Sigma, St. Louis, USA) for half an hour in cell incubator. Then tumor tissue digestion solution were filtered through 200 um strainer to get the digested single cells. Getting 5×10⁵ digested single cells to stain with fluorescence antibodies. Mouse FITC-CD45, BV421-Lineage, PE-CD90.2 and APC-ST2 were applied to detect the percentage of ILC2s in the tumor tissue of tumor-bearing mice and human samples. PEcy7-PD-1 was used to analyze the expression level of PD-1 on tumor-infiltrating ILC2s. All the procedures according to the standard flow cytometry staining instructions and surface-stained samples were kept at 4°C for 15 min. Then add 1 ml PBS to wash the samples, cells were analysed

by FCM using a BD FACSCalibur. The data were analysed using FlowJo software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. Data are expressed as the mean SD. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction. values less than 0.05 were considered statistically significant.

Results

PD-1 is highly up-regulated in tumor-related ILC2s

Recent years, a great number of studies have demonstrated that PD-1 serves as an important role in the regulation of immune response especially in tumor diseases, which greatly hinder the anti-tumor role of tumor-killing cells by delivering the inhibitory signal [21]. Previous studies were all focused on the role of PD-1 on adaptive immune response T cells, but little known about the PD-1 expression to ILCs, including ILC2s[22]. Here, we found that the PD-1 expression in MC38 tumor-bearing mice tumor draining lymph nodes (dLNs) ILC2s highly up-regulated (Figure 1 A and B). Furthermore, the percentage of ILC2s in MC38-bearing mice dLNs showed a low percentage compared to normal mice corresponding dLNs (Figure 1C and D). RT-PCR results were also showed that ILC2s-related cytokines IL-4, IL-5, IL-9 and IL-13 in MC38-bearing mice dLNs were greatly down-regulated compared to control mice (Figure 1E). These results indicated that tumors have an effect on the PD-1 expression on ILC2s, and that might inhibit the activation and function exertion of ILC2s.

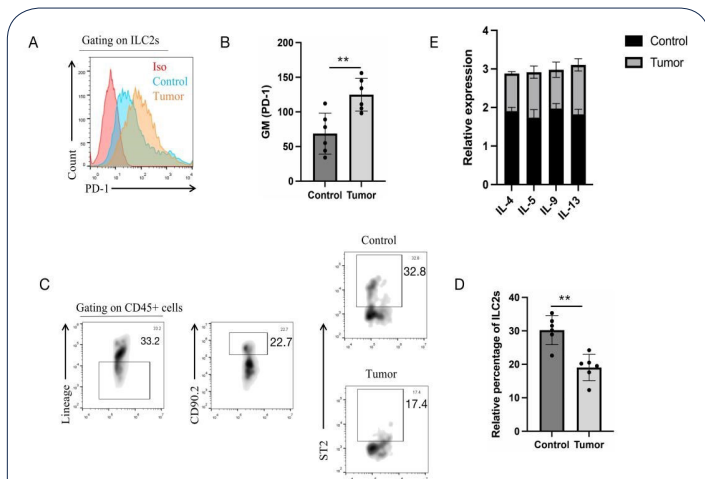


Figure 1: PD-1 is highly up-regulated in tumor-related ILC2s. (A-B): Flow cytometry was used to detect the PD-1 expression on MC38-bearing and control mice dLNs ILC2s (ILC2s as Lineage-CD90.2+ST2+). (C-D): Flow cytometry was used to detect the percentage of ILC2s in MC38-bearing and control mice dLNs. (E): RT-PCR was used to detect the relative expression of ILC2s-related cytokines IL-4, IL-5, IL-9 and IL-13 in dLNs. Data are presented as mean \pm SD, unpaired t-test, **P < 0.01, compared to controls.

Tumor cells inhibit the function of ILC2s by up-regulating PD-1 expression in vitro

To investigate the function of ILC2s from MC38 tumor-bearing mice, we isolated the ILC2s from the dLNs of MC38 tumor-bearing mice and normal mice. Isolated ILC2s cultured in the 96 plates for 3 days, ELISA was used to detect the effect or cytokine secretion level

of ILC2s. The results showed that ILC2s from MC38 tumor-bearing mice dLNs greatly down-regulated the secretion ability of IL-4, IL-5, IL-9 compared to ILC2s from normal mice corresponding dLNs (Figure 2A, B and C), but it has no significant difference of IL-13 secretion was found (Figure 2D). To further mimic the effect of tumor microenvironment on ILC2s, we co-cultured isolated ILC2s with

MC38 cells for 24 h, flow cytometry was used to detect the PD-1 expression on ILC2s. The result showed that PD-1 expression greatly up-regulated on ILC2s when co-cultured with MC38 cells (Figure 2E and F). Addition of anti-PD-1 could restore the function of ILC2s by up-regulating the effect or cytokine secretion (Figure 2G, H and I). These results demonstrated that in tumor microenvironment, tumor cells could inhibit the function of ILC2s by increasing the expression of PD-1 on ILC2s.

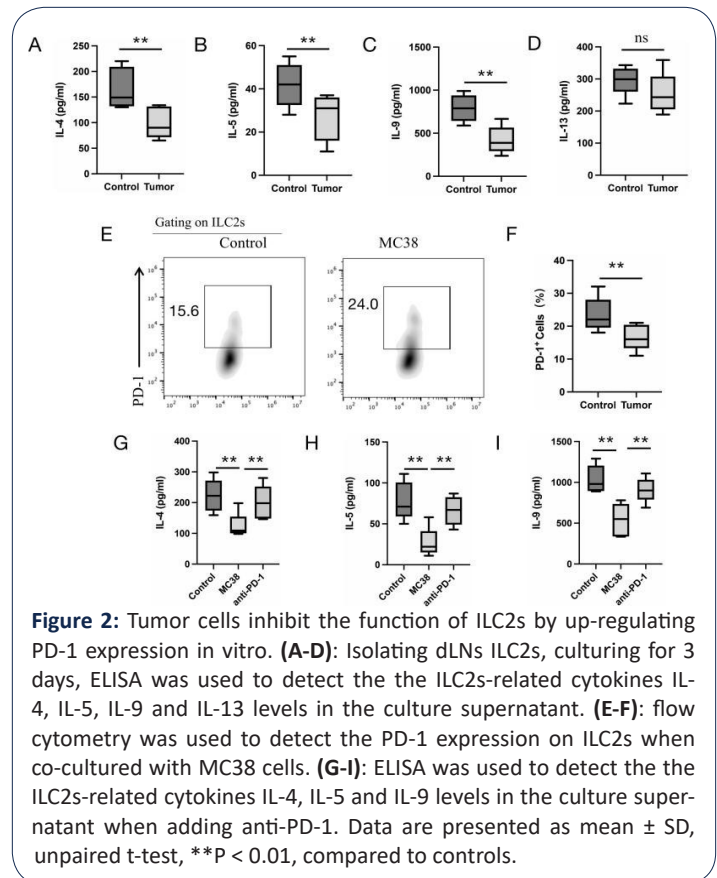
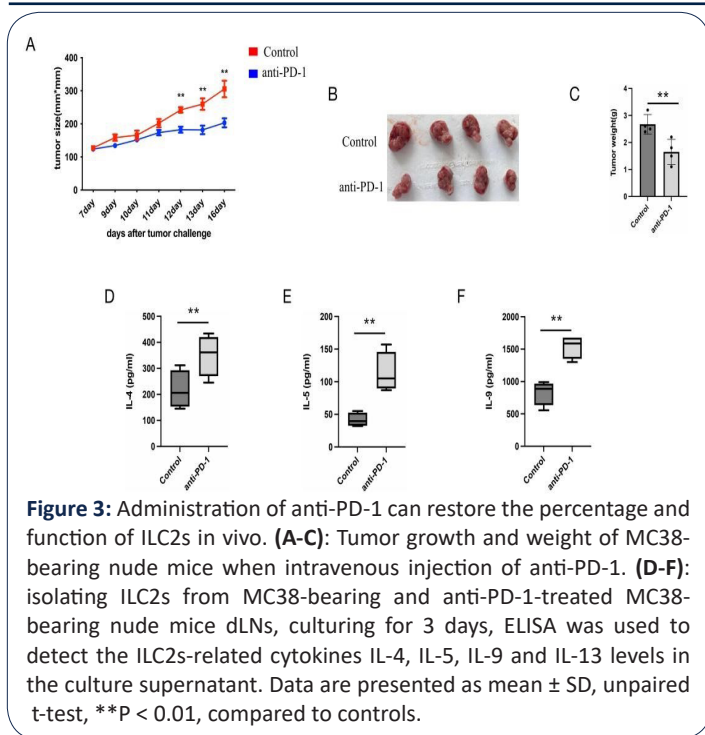


Figure 2: Tumor cells inhibit the function of ILC2s by up-regulating PD-1 expression in vitro. (A-D): Isolating dLNs ILC2s, culturing for 3 days, ELISA was used to detect the the ILC2s-related cytokines IL-4, IL-5, IL-9 and IL-13 levels in the culture supernatant. (E-F): flow cytometry was used to detect the PD-1 expression on ILC2s when co-cultured with MC38 cells. (G-I): ELISA was used to detect the the ILC2s-related cytokines IL-4, IL-5 and IL-9 levels in the culture supernatant when adding anti-PD-1. Data are presented as mean \pm SD, unpaired t-test, **P < 0.01, compared to controls.

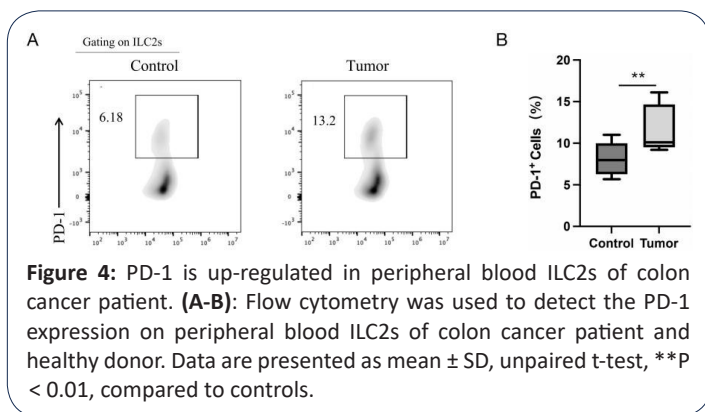
Administration of anti-PD-1 can restore the percentage and function of ILC2s in vivo

To further investigate the up-regulation of PD-1 on ILC2s could restrain the ILC2s function, we treated MC38 tumor-bearing nude mice (lacking T and B cells) with anti-PD-1 antibody, at day 5 to day 10 after tumor implanted. The results showed that the administration of anti-PD-1 greatly inhibit the growth of tumors of MC38 tumor-bearing nude mice (Figure 3A, B and C). To detect if anti-PD-1 reverse the ILC2s function inhibition effect, we isolated the ILC2s from dLNs and cultured for 3 days. ELISA was used to detect the ILC2s-related cytokines IL-4, IL-5, IL-9, result showed that these cytokine were up-regulated in the anti-PD-1 treated MC38 tumor-bearing nude mice dLNs ILC2s compared to control mice (Figure 3D, E and F). These results preliminarily proved the negative effect of tumor cells on tumor-infiltrating ILC2s by up-regulating PD-1 expression on them.



PD-1 is up-regulated in peripheral blood ILC2s of colon cancer patient

To explore whether PD-1 was up-regulated on human colon cancer patient, we detected the PD-1 expression on the peripheral blood ILC2s of colon cancer patient. Flow cytometry result showed that PD-1 was greatly up-regulated on the peripheral blood ILC2s of colon cancer patient compared to healthy donor (Figure 4A and B), which is consistent with mice research results. Our results preliminarily demonstrated that tumor can regulate the PD-1 expression on ILC2s, and inhibited ILC2s function, thus affect the tumor-regulating role of ILC2s.



Discussion & conclusion

In this study, we found that the PD-1 expression on tumor-infiltrating ILC2s greatly up-regulated, which push these ILC2s towards to an exhausted phenotype, such as the down-regulation of effect or cytokine IL-4, IL-5, IL-9 and IL-13. Furthermore, in vitro study showed that colon cancer cell line MC38 could up-regulate the expression of PD-1 on ILC2s when co-cultured with isolated ILC2s, and prevent the cytokine secretion from ILC2s, which could reversed by adding anti-PD-1. In vivo study demonstrated that the injection of neutralizing antibody PD-1 prevented the tumor growth of MC38-bearing nude mice, and greatly release tumor-

infiltrating ILC2s from inhibitory situation. Our studies firstly demonstrated that the regulation role of tumor cells on the PD-1 expression on tumor-infiltrating ILC2s, providing a new insight in the ILC2s-based tumor immunotherapy.

PD-1, as an inhibitory molecular, mainly expresses on lymphoid cells, and that interacts with PD-L1 or PD-L2 to deliver the inhibitory signal to prevent the excessive activation of immune cells in physiology situation [23]. As an immune checkpoint, PD-1 prevents the immune cells excessive activation-induced autoimmune diseases through two mechanism, that promotes the apoptosis (programmed cell death) of antigen-specific T cells in lymph nodes and reduces the apoptosis of immunosuppressive regulatory T cells. It is indeed benefit in the physiology body. However, recent studies have proved that PD-1 and their ligands are all up-regulated in multiple tumors, and that greatly inhibits the anti-tumor function of tumor-inhibiting Cytotoxic T Lymphocytes (CTL) and promotes the tumor growth [24-26]. Up to date, the study on PD-1-based immunotherapy has achieved great success, and some PD-1 inhibitors showed powerful therapeutic effects on several cancer diseases [27,28]. Although it is also necessary to further explore the full mechanism on the PD-1 related tumor immune inhibition. Here we found that tumor cells could also up-regulate the PD-1 expression on innate lymphoid cells, ILC2s, that provide a new insight in the interact of tumor cells with innate lymphoid cells in tumor microenvironment.

ILC2s, a subset of innate lymphoid cells, were initially proved to have an important role in the development of allergic diseases and anti-helminth response [29]. Recent years, the role of ILC2s in tumor diseases was achieved more and more attention. Studies have proved that ILC2s have a dual role in tumors, so more studies need to be done to detect the real or exactly function in the development of tumor diseases [30,31]. ILC2s are characterized by secreting type 2 cytokines, and the role of ILC2s-derived IL-4, IL-5, IL-9 and IL-13 have been reported. It is reported that IL-13 secreted by ILC2s can promote the activation of Myeloid-Derived Suppressor Cells (MDSCs) and tumor-promoting M2 macrophage, which greatly push the emergence of immunosuppressive tumor microenvironment and promote the tumor growth [32]. Furthermore, ILC2s-derived IL-5 can recruit anti-tumor CD8 T cells to tumor core to exert tumor-killing effect by activating tumor-infiltrating eosinophils to secret T cell chemokines. Recent a publication showed that ILC2s-derived IL-9 could inhibit the colorectal cancer development by activating CD8 T cells [16,19]. These studies demonstrated that ILC2s-derived cytokines played an important role in the regulation of tumor development. PD-1 expressed on ILC2s, delivers inhibitory signal greatly prevent the activation and effect or cytokine secretion of ILC2s. Here, we found that tumor cells up-regulated PD-1 expression on ILC2s significantly inhibited the activation of ILC2s and greatly decreased the cytokine secretion from ILC2s, thus greatly hindering the regulating role of ILC2s on tumors.

In conclusion, our results demonstrated that colon cancer cells inhibited the activation of ILC2s by up-regulating the PD-1 expression on them, and inhibited the tumor development regulating factors secretion, thus lose the anti-tumor function to colon cancer. Our results preliminary clarified the regulatory role of tumor cells on ILC2s, providing a new approach on ILC2s-based immunotherapy.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: All authors provided consent for publication.

Availability of supporting data: The conclusion figures have been uploaded and the tables supporting the view of this review have been listed in the article.

Conflict of interests: The authors declare that they have no competing interests.

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Authors' contributions: XX and HGZ wrote the manuscript. BM, JC and JXD draw the figures and tables. WJZ designed the study, drafted and revised the manuscript. All authors read and approved the final manuscript.

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