RIPK3 Suppresses the Progression of Spontaneous Intestinal Tumorigenesis

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Receptor-interacting protein 3 (RIPK3), a member of the family of serine/threonine protein kinases, emerged as a critical regulator of necroptosis. Downregulated expression of RIPK3 is correlated with poor prognosis in multiple tumor types. Here, we show that RIPK3 is involved in the progression of spontaneous intestinal tumorigenesis. As a clinical correlate, reduced expression of RIPK3 is positively associated with histological grade, lymphatic metastasis and poor prognosis in CRC patients. RIPK3-deficient (Ripk3-/-) mice exhibit increased tumor formation in Apcmin/+ spontaneous intestinal tumorigenesis. Apcmin/+Ripk3-/- tumors promote hyperactivation of IL-6/STAT3 signaling, which exacerbates proliferation and inhibits apoptosis. Blocking IL-6 signaling suppressed tumor formation and reduced STAT3 activation in Apcmin/+Ripk3-/- mice. Thus, our results reveal that RIPK3 is a tumor suppressor in spontaneous intestinal tumorigenesis, and implicate targeting the IL-6/STAT3 signaling axis as a potential therapeutic strategy for intestinal tumor patients with reduced RIPK3.

Keywords: RIPK3, intestinal tumorigenesis, APC mutant mouse model, STAT3 (signal transducer and activator of transcription 3), IL-6 receptor antibody

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide, and the number of deaths is rising in developing countries (1, 2). An increased frequency of CRC patients is usually diagnosed at an advance stage (3). Histologically, over 95% of CRC cases are adenocarcinomas, while squamous cell carcinomas, lymphomas and sarcomas are rarer (4). Increasing evidence reveals that excessive neoplastic transformation of colonic epithelial cells have the ability to initiate and develop CRC (5, 6). Moreover, CRCs arise in intestinal epithelial cells upon loss of tumor suppressor genes such as adenomatous polyposis coli (APC) (7). Thus, investigation of the molecular mechanisms in CRC tumorigenesis is urgently needed to prevent and treat CRC.

Inflammation is a dynamic and complex process to prevent overwhelming dampens and maintain tissue homeostasis (8, 9). Accumulation of the inflammatory environment drives intestinal cell proliferation and tissue regeneration through activation of pro-oncogene factors...
such as signal transducer and activator of transcription 3 (STAT3) (10, 11). Elevated levels of interleukin 6 (IL-6) are linked with chronic intestinal inflammation and tumorigenesis (12, 13). The role of IL-6/STAT3 signaling in intestinal cell proliferation and tumorigenesis is well established (14–16). A better understanding of the regulation of IL-6/STAT3 activation would facilitate the development of novel approaches for CRC therapy.

Necroptosis is a form of necrotic-regulated cell death upon extracellular and intracellular stimuli in certain pathologies (17–19). Receptor-interacting protein kinase 1 (RIPK1) and RIPK3 are critical regulatory molecules during necroptosis (20–22). RIPK1 interacts with RIPK3 via the RIP homotypic interaction motif (RHIM) domains and then forms the necrosome (23). RIPK1–RIPK3 necrosome complex results in the recruitment and phosphorylation of mixed-lineage kinase domain-like (MLKL), which leads to impair membrane integrity and drive necroptosis (24, 25). Several studies have shown that RIPK3 is involved in several inflammatory diseases, including intestinal inflammation, acute pancreatitis and skin inflammatory diseases (23, 26, 27). In fact, RIPK3 also exhibits a necroptosis-independent function in intestinal inflammation and tumorigenesis (28, 29). Reduced RIPK3 expression has been shown in human CRC tumors, and low expression of RIPK3 is significantly correlated with poor progression (30, 31). In contrast, overexpression of RIPK3 is susceptible to intestinal tumorigenesis in inflammation-associated colon cancer models (31, 33). However, colitis-associated tumorigenesis represents only 1% of colorectal cancer, and sporadic intestinal tumors are more prevalent (34). Thus, it is necessary to address the function of RIPK3 in the progression of spontaneous intestinal tumorigenesis.

In the present study, we revealed a tumor suppressor role for RIPK3 in spontaneous intestinal tumorigenesis. Expression analyses of RIPK3 expression showed that RIPK3 is significantly downregulated in CRC tumors, which predicted a poor prognosis in CRC. The absence of RIPK3 exhibits dramatically increased tumor numbers in Apcmin/+ mice through the hyperactivation of IL-6/STAT3 signals. Anti-IL-6R antibody therapy suppressed STAT3 activation and attenuated tumor burden in Apcmin/+Ripk3−/− mice. Our findings highlight that reduced RIPK3 predicts a more aggressive disease and worse outcome in CRC.

**Determination of Clinical Scores**

Clinical scores were calculated as described in our previous studies (35). The disease activity index (DAI) was quantified with a clinical score assessment based on stool consistency and fecal blood. Briefly, stool scores were determined as follows: 0 = well-formed pellets; 1 = semi-formed stools in the anus and 2 = liquid stools adhered in the anus. The bleeding scores were determined as follows: 0 = no observed blood; 1= visible blood traces in stool; and 2= gross bleeding in rectal tissue.

**Tumor Load**

Tumor load was determined according to a published protocol (36). Tumor load was calculated based on tumor numbers and tumor diameter.

**Tissue Homogenization and Western Blotting**

Isolated intestinal tissues were homogenized using a Mini-Rotor (Thermo Scientific) and lysed in complete radioimmunoprecipitation assay (RIPA) buffer containing Roche’s Complete™ protease inhibitor cocktail. The protein concentrations were quantified by a quantitative BCA protein kit (P0010S; Beyotime Biotechnology, Shanghai, China). The proteins were separated by SDS-PAGE and detected using chemiluminescent substrate (Thermo Scientific). The following primary antibodies were used: rabbit anti-RIPK3 (#ab152130, Abcam; 1:1000), mouse anti-STAT3 (#9139, CST; 1:1000), rabbit anti-phospho-STAT3 (Tyr705) (#9145, CST; 1:1000), rabbit anti-PCNA (#13110, CST; 1:1000), rabbit anti-Cleaved-Caspase-3 (#9661, CST; 1:1000), and rabbit anti-Cyclin D1 (#2978, CST; 1:1000), and rabbit anti-C-myc (#9402, CST; 1:1000), and anti-GAPDH (#5174, CST; 1:5000).

**Quantitative Reverse-Transcriptase PCR (qRT-PCR)**

Intestinal tissues were homogenized in TRIzol reagent (Life Technologies) to obtain RNA. RNA was reverse-transcribed to complementary DNA using HiScript III RT SuperMix for qPCR (gDNA wiper) (# R323, Vazyme Biotech Co., Ltd), and the levels of genes were measured using quantitative RT-PCR using SYBR Premix Ex Taq™ (#RR420, TAKARA) according to the manufacturer’s instructions. mRNA quantities were normalized against that of GAPDH.

**Isolation of Colon Crypt Cells**

Intestines were cleared and then cut into pieces. The colon crypt cells were collected following shaking after incubation in cold PBS with 3 mM EDTA/1.5 mM DTT.

**Intestinal Tissue for the Analysis of Cytokine Production**

The intestinal tissues were collected and then homogenized in RIPA buffer containing Roche’s Complete™ protease inhibitor cocktail for 30 mins on ice. The protein concentration of IL-6 was measured by enzyme linked immunosorbent assay (ELISA) (eBioscience) (37).

**MATERIALS AND METHODS**

**Animal Experiments**

All the mice were on a C57BL/6 background and maintained in a specific pathogen-free (SPF) facility. Ripk3−/− mice and Apcmin/+ mice have been described previously (29, 35). Ripk3−/− mice were crossed with Apcmin/+ mice to generate Apcmin/+Ripk3−/− and Apcmin/+Ripk3+/− mice. Then, Apcmin/+Ripk3−/− mice were crossed with Ripk3+/− mice to generate Apcmin/+ and Apcmin/+Ripk3+− mice. This study was conducted according the guidelines of the Institutional Animal Care and Use Committee of the Hubei University of Medicine.
Anti-IL-6R Antibody Treatment
IL-6 signaling was antagonized with the IL-6R antibody tocilizumab (#A2012, Selleckchem; Houston, TX, USA). Four-week-old Apc\textsuperscript{min/+} Ripk3\textsuperscript{-/-} mice were intraperitoneally injected with 4 mg/kg tocilizumab weekly. After 10 weeks of treatment, all mice were euthanized, and the intestinal tissues were collected for further analysis.

Statistical Analysis
The data are represented as the mean ± standard error of the mean (SEM) and were analyzed by GraphPad Prism software. Significant significance was determined by 2-tailed Student’s t-test, one-way ANOVA, Pearson’s correlation coefficients test, or log-rank test. P values below 0.05 were statistically significant.

RESULTS
RIPK3 Expression Is Reduced in Colorectal Cancer (CRC) Patients
To determine the role of RIPK3 in human CRC, we analyzed the levels of RIPK3 expression in containing 41 normal intestinal specimens and 286 CRC tumor samples from the Cancer Genome Atlas (TCGA) transcriptome database. CRC tumor samples showed significantly decreased expression of RIPK3 relative to normal intestinal tissue samples (Figure 1A). We next examined the levels of RIPK3 in different stages of CRC tumor progression. The results revealed that RIPK3 expression was higher in normal intestinal controls than in four CRC stages (Figure 1B). Interestingly, RIPK3 expression was lower in metastatic tumors than in normal counterparts (Figure 1C). More importantly, overall survival was significantly higher in patients with high RIPK3 expression than in patients with reduced RIPK3 expression in colorectal cancer (Figure 1D). These data indicate reduced RIPK3 expression in human CRC and suggest a suppressor role of RIPK3 in colorectal tumorigenesis.

Loss of RIPK3 Aggravates Tumor Burden in Apc\textsuperscript{Min/+} Mice
Mutations of the WT Apc allele (Apc\textsuperscript{min/+}) are found in ~85% of intestinal tumors and promote intestinal tumorigenesis. Apc\textsuperscript{min/+} mice are a suitable mouse model for the study of sporadic intestinal tumorigenesis. We hypothesized that RIPK3 deletion might increase the tumor burden in Apc\textsuperscript{min/+} mice during intestinal tumorigenesis. To address the role of RIPK3 in intestinal tumorigenesis, Apc\textsuperscript{min/+} Ripk3\textsuperscript{-/-} mice were obtained by crossing Apc\textsuperscript{min/+} mice with Ripk3\textsuperscript{-/-} mice. Tumor burden was significantly higher in Apc\textsuperscript{min/+} Ripk3\textsuperscript{-/-} mice compared to Apc\textsuperscript{min/+} controls (Figure 2A). We further examined the effects of RIPK3 deletion on tumor progression by analyzing the levels of tumor markers in colorectal tissues. The results revealed that RIPK3 deletion significantly increased the expression of tumor markers such as Ki67 and CD31 (Figure 2B and 2C). These findings suggest that RIPK3 deletion promotes tumor progression in colorectal cancer.
progression in Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} mice and those of Apc\textsuperscript{min/+} mice was analyzed. The results showed significantly more lesions in the Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} mice than in Apc\textsuperscript{min/+} mice (Figure 2A). Moreover, Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} mice exhibited increased tumor numbers and tumor loads compared with in Apc\textsuperscript{min/+} mice (Figures 2B, C). Of note, the size of the tumor distribution in Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} mice tended to be larger than Apc\textsuperscript{min/+} mice (Figure 2D). H&E staining also confirmed that there were larger polyps in Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} intestines than in Apc\textsuperscript{min/+} littermate controls (Figure 2E). Accordingly, anemia and thymus atrophy were significantly exacerbated in Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} mice compared to the Apc\textsuperscript{min/+} littermate controls (Figures 2F, G). Analyses of Kaplan-Meier survival found that the survival time of the Apc\textsuperscript{min/+}Ripk3\textsuperscript{-/-} mice was dramatically shorter, with a median survival of only 132 days relative to the median survival of the Apc\textsuperscript{min/+} mice, which was 195 days (Figure 2H).
Since loss of RIPK3 resulted in higher tumor burdens in the Apc<sup>min/+</sup> model, we hypothesized that RIPK3 inhibited tumor formation by regulating cellular proliferation. Compared with Apc<sup>min/+</sup> mice, Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> tumors and colon crypt showed increased PCNA and decreased cleavage of caspase-3 proteins (Figures 2I, J). However, there were no significant differences in proliferation and apoptosis found in WT and Ripk3<sup>-/-</sup> intestines (Figure S1), suggesting that loss of RIPK3 accelerates intestinal tumorigenesis by promoting proliferation and preventing apoptosis. These results demonstrate that loss of RIPK3 aggravates intestinal tumorigenesis in Apc<sup>min/+</sup> mice.

**Loss of RIPK3 Activates STAT3 Signaling Pathway in Apc<sup>min/+</sup> Mice**

Activation of the STAT3 signaling pathway is associated with colorectal carcinogenesis and progression. In Apc<sup>min/+</sup> mice, hyperactivation of STAT3 accelerates intestinal tumorigenesis (38). To evaluate the status of STAT3 in intestinal epithelial cells and tumors from Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice, we analyzed protein expression in intestinal tumors and colon crypt cells. The results indicated that the levels of pSTAT3 were higher in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> tumors than in Apc<sup>min/+</sup> tumors, WT and Ripk3<sup>-/-</sup> intestines (Figure 3A). We also found higher levels of pSTAT3 in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> colon crypts than in control mice (Figure 3B). As tumors from Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice display activation of STAT3 signaling, we determined the reason for STAT3 hyperactivation in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice. IL-6 maintains persistent activation of STAT3 signaling in CRC (16). We found significantly increased levels of IL-6 mRNA and protein in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> tumors (Figures 3C, D). Aberrant activation of STAT3 promotes intestinal tumorigenesis by upregulating STAT3 target genes, which are involved in cell survival and proliferation (39). The results showed that the expression of STAT3 target genes (Cyclin D1, C-myc and Survivin) was increased in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> tumors compared with Apc<sup>min/+</sup> tumors (Figure 3E). Consistently, we found higher levels of these target genes in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> colon crypts than in Apc<sup>min/+</sup> mice (Figure 3F). Similarly, qRT-PCR results also showed significantly increased expression of these genes in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> tumors and colon crypts (Figures 3G, H). However, no remarkable increase in expression of STAT3 target genes was observed either in the intestines of the WT or Ripk3<sup>-/-</sup> intestines (Figure S2). These results indicate that IL-6/STAT3 signaling is activated in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice during intestinal tumorigenesis.

**Inhibition of IL-6 Blocks Tumor Burden in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> Mice**

IL-6 is required for homeostasis of intestinal crypts (40, 41), and tumor formation is suppressed in the absence of IL-6 in the Apc<sup>min/+</sup> model (38, 42). To confirm the role of IL-6/STAT3 signaling in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice, we investigated the effect of IL-6 using an anti-IL-6 receptor (anti-IL-6R) antibody. We injected the anti-IL-6R antibody into Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice for 10 weeks (Figure 4A). Treatment with anti-IL-6R antibody resulted in a lower clinical score in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice compared to the untreated (UT) mice (Figure 4B). After IL-6R antibody treatment for 10 weeks, the anti-IL-6-R-treated Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice showed fewer intestinal polyps than UT Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice (Figure 4C). As expected, anti-IL-6R antibody therapy alleviated anemia and thymus atrophy in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice (Figures 4D, E). To elucidate whether STAT3 activation changes in anti-IL-6R-treated Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice, intestinal polyps were isolated and analyzed by western blotting. Anti-IL-6R-treated Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> intestines had decreased levels of p-STAT3 compared to UT Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice (Figure 4F). Consistently, the expression of STAT3 target genes Cyclin D1, C-myc and Survivin, was markedly suppressed in anti-IL-6R-treated Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> tumors (Figures 4G, H). These results demonstrate that increased IL-6/STAT3 signaling plays a causative role in the increased tumor burden in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice.

**DISCUSSION**

In this study, we found that loss of RIPK3 promoted the initiation and progression of spontaneous intestinal tumors. RIPK3 is reduced in human CRC patient cohorts and reduced RIPK3 is associated with poor prognosis in CRC. In the Apc<sup>min/+</sup> model, loss of RIPK3 elevated IL-6 levels, in turn hyperactivating the STAT3 signaling pathway and contributing to accelerated intestinal tumorigenesis. Notably, blocking IL-6 with a neutralizing IL-6 receptor antibody effectively attenuated tumor burden and STAT3 hyperactivation in Apc<sup>min/+</sup>Ripk3<sup>-/-</sup> mice. Our results highlight that RIPK3 plays a suppressive role in intestinal tumorigenesis by inactivating the IL-6/STAT3 signaling axis.

Necroptosis is involved in intestinal inflammation in mice and human IBD patients (43, 44). Abnormal RIPK3 expression is increased in Crohn’s disease (CD) and ulcerative colitis (UC) patients (44, 45). RIPK3 has a necroptosis-independent function by inhibiting inflammatory responses in DSS-induced acute colitis (28). In some types of tumors, reduced expression of RIPK3 appears to occur commonly and was found to correlate with reduced overall survival and poor prognosis (32, 46, 47). Previous studies have shown that RIPK3 suppresses inflammation-associated CRC (31, 33), while conflicting results suggest that RIPK3 promotes colitis-associated colorectal cancer (48). Recent work has examined the lack of an overt phenotype in Ripk3<sup>-/-</sup> mice in an inflammation-associated CRC model (49). These studies present conflicting results that might be due to differences in experimental conditions, commensal microflora and genetic background of mice. Thus, the results of a previous study indicate that the role of RIPK3 in the initiation and progression of CRC is still not clear. In addition, sporadic intestinal tumors are the majority of colon cancers, and the importance of RIP3 needs to be addressed in sporadic intestinal tumors. Here, we took advantage of genetically engineered mouse models (GEMs) and established that RIP3 deficiency drove increased tumor burden during intestinal tumorigenesis in Apc<sup>min/+</sup> mice. The present study revealed that the survival time was shorter in Apc<sup>min/+</sup> mice in the absence of RIP3, which is consistent with reduced RIPK3 correlated with poor clinical
FIGURE 3 | Loss of RIPK3 activates STAT3 signals in Apcmin/+ mice. (A) The expression of pSTAT3 in 6-week-old Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ intestinal tumors. (B) The levels of pSTAT3 in Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ colonic crypts. (C) Quantitative mRNA expression of IL-6 in Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ intestinal tumors. (D) The protein levels of IL-6 in Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ intestinal tumors as determined by ELISA. (E) The expression of STAT3 target genes in Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ intestinal tumors. (F) The expression of STAT3 target genes in Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ colonic crypts. (G, H) Quantitative mRNA expression of STAT3 target genes in Apcmin/+ and Apcmin/+ Ripk3⁻/⁻ intestinal tumors and colonic crypts. *p < 0.05, **p < 0.01, ***p < 0.001 versus Apcmin/+ mice.
outcomes in CRC. Consistently, we found reduced RIPK3 expression in human CRC compared with normal tissues using the TCGA database. RIPK3 expression was significantly decreased in four CRC stages versus in healthy controls. Importantly, significantly lower expression of RIPK3 predicted poor overall survival in CRC patients. In summary, these data reveal that RIPK3 exerts tumor-suppressive roles in intestinal tumorigenesis.

Intestinal tumors originate from epithelial cells through activation of multiple key signaling pathways for cell growth, differentiation, and survival. Compensatory proliferation and widespread apoptosis are known to contribute to promoting colorectal carcinogenesis. Here, our results indicated that Apc\textsuperscript{min/−}/Ripk3\textsuperscript{−/−} tumors exhibited higher proliferative rates and reduced apoptotic compared to Apc\textsuperscript{min/−} tumors. Notably, untransformed colon tissue and colon crypts of Apc\textsuperscript{min/−}/Ripk3\textsuperscript{−/−} mice also exhibited higher proliferation and lower apoptosis. Thus, cooperation with increased proliferation and decreased apoptosis contributes to accelerated intestinal tumors in Apc\textsuperscript{min/−}/Ripk3\textsuperscript{−/−} mice.

Inflammatory cytokines stimulate the activation of STAT3 signaling, which in turn contributes to promoted epithelial cell survival and resistance to apoptosis (5). STAT3 is critical for...
intestinal regeneration and tumorigenesis by regulating survival, cell cycle progression, and inflammation (50). Hyperactivation of STAT3 in epithelial cells has been linked to the development of CRC. Constitutively activated STAT3 in mice is resistant to intestinal tumor development (14). In contrast, IEC-specific STAT3-deficient mice exhibit decreased intestinal tumors (16).

In this study, we observed that loss of RIPK3 promoted the activation of STAT3, thereby accelerating intestinal tumorigenesis in Apcmin/+ mice. Moreover, RIPK3-deficient mice exhibited increased expression of STAT3 target genes, including Cyclin D1, C-myc and Survivin in the Apcmin/+ model. Enhanced levels of IL-6 play a key role in STAT3 signaling in tumor formation and the development of CRC (42, 51, 52). Indeed, the levels of IL-6 were significantly upregulated in Apcmin/+Ripk3−/− mice. As expected, decreased levels of pSTAT3 and target genes were observed in anti-IL-6R-treated Apcmin/+Ripk3−/− mice. Moreover, RIPK3-deficient mice exhibit decreased intestinal tumors (16).

In summary, we report that RIPK3 exerts a suppressive role in intestinal tumorigenesis by suppressing the IL-6/STAT3 signaling axis. Mechanistic analysis provides evidence to further understand the role of RIPK3 in intestinal tumorigenesis and identify that increasing RIPK3 may be considered as a potential therapeutic strategy for CRC therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.664927/full#supplementary-material

ETHICS STATEMENT

The animal study was reviewed and approved by Hubei University of Medicine. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

XY and QZ conceived the study and designed the experiments. XY, JG, XC, YB, YG, XZ and QZ performed experiments. XY, JG, XC, YL, YG, XW, WY, SJ, YT and QZ analyzed data. XY and QZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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