ORIGINAL RESEARCH

Effect of NLRP3 Inflammasome on Lung Cancer Immune Microenvironment Activation and Its Mechanism

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ABSTRACT

Objective • The NLRP3 inflammasome plays a dual role in the occurrence and development of tumors, and its role in lung cancer remains unclear. This study aims to investigate the impact of NLRP3 inflammasome activation on the proliferation and migration of lung cancer cells.

Methods • Data from the GEPIA, TCGA, and HPA databases were utilized to analyze the expression of NLRP3 in lung adenocarcinoma and its microenvironment. GO/KEGG enrichment analysis and GSEA analysis were employed to annotate the functions of differentially expressed genes related to NLRP3. The impact of NLRP3 inflammasome activation on the proliferation and migration of lung cancer cells was further investigated by CCK-8 assay and scratch assay. The effects of blocking NLRP3 inflammasome activation with IL-1RA and IL-18BP on the proliferation and migration of lung cancer cells were further assessed. Survival analysis was conducted to analyze the impact of NLRP3 expression on the prognosis of patients with lung adenocarcinoma.

Results • The expression of NLRP3 in lung cancer was lower than in normal tissues, with notably higher expression observed in macrophages compared to other cells. Patients with higher NLRP3 expression exhibit increased infiltration of M2 macrophages. Activation of the NLRP3 inflammasome using LPS+ATP promotes the proliferation and migration of A549 cells. Simultaneous use of IL-1RA and IL-18BP reverses the promoting effect of NLRP3 inflammasome activation on cell proliferation and migration. Survival analysis results indicate that patients with high NLRP3 expression have a poorer prognosis compared to those with low NLRP3 expression (Hazzard Ratio =1.44; 95% Confidence Interval: 1.21-1.71).

Conclusions • The activation of the NLRP3 inflammasome promotes the proliferation and migration of A549 cells through secretion of IL-1β and IL-18, potentially influencing patient prognosis. Simultaneously blocking IL-1β and IL-18 can reverse the pro-proliferative and migration-promoting effects. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Lung cancer is major health burden which stand as the third leading cause of death in China.[1] Proliferation is the most basic hallmark of cancer and is closely associated with other malignant cancer phenotypes.[2] Abnormal proliferation of cancer involves various abnormal signaling pathways, and inhibiting proliferation of tumors is a key strategy for cancer treatment.[3] Academic circles believe that chronic inflammation can promote the occurrence and development of tumors, and the accumulation of inflammatory cells and inflammatory mediators can promote the proliferation and survival of malignant cells, promote angiogenesis and tumor metastasis. As a core factor in initiating inflammatory response, inflammasome has received more and more attention in recent years.[4]

At present, NLRP3 inflammasome, which is the most studied, has been shown to play a dual role in different stages of tumorigenesis.[5] As a crucial component of anti-tumor inflammation, the NLRP3 inflammasome drives CD8 T cell-mediated cytotoxicity against transplantable tumors through IL-1β–IL-1 receptor signaling axis.[6] Additionally, it enhances natural killer (NK) cell-mediated cytotoxicity against colon tumors with liver metastasis by secreting IL-18.[7] However, NLRP3 inflammasome can impede NK cell activation, leading to reduced IFN-γ secretion and diminished anti-tumor activity.[8] The activation of the inflammasome, resulting in IL-1β secretion, has also been found to be associated with the

occurrence of gastric cancer.[9] While the role of NLRP3 in various cancers is gradually being revealed, its functions in lung cancer remain largely unknown. Therefore, it is necessary to further investigate the role of the NLRP3 inflammasome in lung cancer and the underlying mechanisms.

The present study aims to investigate the impact of NLRP3 inflammasome activation on the immune microenvironment of lung cancer, focusing on its effect on tumor cell proliferation and migration. Furthermore, we aimed to elucidate underlying value on patient's prognosis and evaluate the potential therapeutic targets related to NLRP3 inflammasome activity.

MATERIALS AND METHODS

Expression of NRPL3 in lung adenocarcinoma and its microenvironment

Comparison of NLRP3 expression between lung adenocarcinoma and normal lung tissues was conducted using the Gene Expression Profiling Interactive Analysis database (GEPIA).[10] The expression of NLRP3 in various cell types within lung tissues was analyzed using single-cell RNA sequencing data from the Human Protein Atlas (HPA) database (http://proteinatlas.org).[11] Bulk RNA sequencing data of lung adenocarcinoma were downloaded from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/), and CIBERSORT was employed to estimate immune cell infiltration scores for each patient. Based on the median expression of NLRP3, patients were divided into high and low expression groups, and the differences in cell infiltration abundance between the two groups were compared.

GO/KEGG and GSEA enrichment analysis

Patients were stratified into high M2 and low M2 groups based on the abundance of M2 macrophages, and DESeq2 package was utilized to analyze the differentially expressed genes (DEGs) between the two groups. Genes with a $Log_2FoldChange > 1$ and $P < .01$ were defined as DEGs. Functional enrichment analysis (GO and KEGG pathways analysis) was performed using the Metascape (http://metascape.org).[12] Additionally, based on the expression levels of NLRP3, Gene Set Enrichment Analysis (GSEA) software (Version 4.3.2) was employed for gene set enrichment analysis.

NLRP3 inflammasome activation and inhibition

Cell line A549 was obtained from the Cell Bank of Typical Cultures Preservation Committee, Chinese Academy of Sciences. A549 cells were cultured in dulbecco's modified eagle medium (DMEM, Gibco, USA, 8123539), supplemented with 10% fetal bovine serum (Gibco, USA, 10099-141), 1% of 10000 U/mL penicillin/streptomycin, and maintained in 5% CO_2 at 37°C. To activate the NLRP3 inflammasome, A549 cells were treated with 1μl/ml lipopolysaccharide (LPS) for 8 hours, with the addition or omission of 5 mmol/ml ATP in the final half an hour. Cell culture medium was collected, centrifuged at 5000 rpm for 30 minutes, and the supernatant was obtained. ELISA kits were then used to measure the levels of IL-1β and IL-18 in the supernatant. To inhibit the function of the NLRP3 inflammasome, cells were pre-treated with IL-1RA and IL-18BP separately or in combination 1.5 hours before LPS stimulation.

Cell proliferation assays and cell migration assays

Cell Counting Kit-8 (CCK-8, Beyotim, China, #COO38) was employed to assess cell proliferation. A total of 20,000 cells were seeded in 96-well plate, and cell proliferation was measured at 6, 12, 24, and 48 hours after drug treatment. 10μl of CCK-8 reagent was added to each well and incubated for 1 hour. Absorbance at 450 nm was measured using spectrophotometer. Scratch assay was conducted to assess the migration ability of A549 cells. Cells were seeded in 6-well plates, and when a monolayer reached 100% confluence, scratches were created in each well using 10μl sterile pipette tips. Cell migration was observed under phase-contrast microscope at 0h and 24h after scratching. Quantification of cell migration rates was performed using Image J software (Version 1.53t).

Statistical analysis

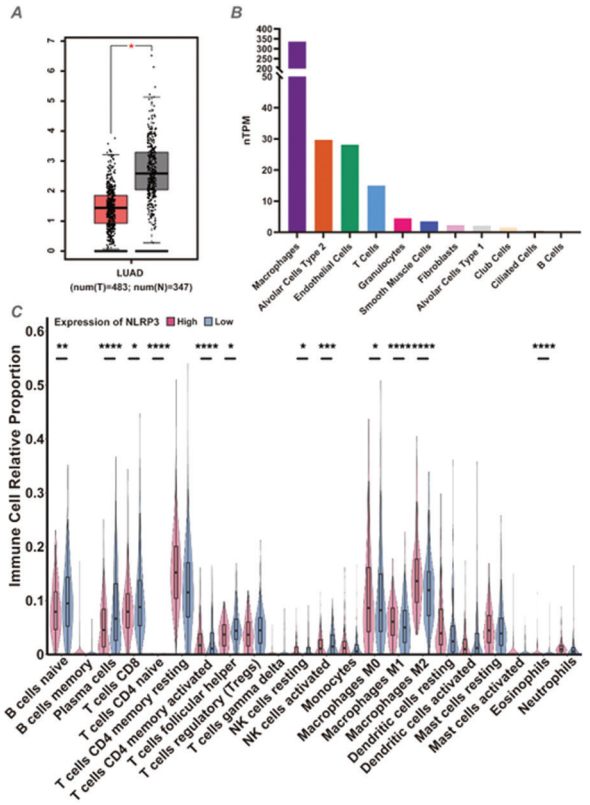
To analyze the prognostic value of NLRP3, Kaplan-Meier survival curves were generated and hazard ratios (HR) with 95% confidence interval (95%CI) were calculated using lung adenocarcinoma data from the Kaplan-Meier Plotter website (https://kmplot.com/analysis/). Overall Survival (OS) as the outcome was selected for patient prognosis analysis. Quantitative data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) or two-way ANOVA was used for comparison between multiple groups. $P < .05$ was defined as statistical difference in data comparison. Statistical analysis and plotting were conducted using R (Version 4.1.1) and GraphPad Prism (Version 9.5.1).

RESULTS

NLRP3 expression in lung adenocarcinoma and tumor microenvironment

Through the analysis of bulk sequencing data from the GEPIA database, it was observed that the expression of NLRP3 is higher in normal tissues compared to tumor tissues in lung adenocarcinoma (Figure 1A). Further analysis of single-cell sequencing data from normal lung tissues revealed that NLRP3 expression is significantly higher in lung macrophages compared to other cell types (Figure 1B). Consequently, we utilized TCGA lung adenocarcinoma data to calculate immune cell infiltration scores in tumor patients based on sequencing data. Patients were categorized into NLRP3 high-expression and low-expression groups according to the median expression of NLRP3, and differences in immune cell infiltration abundance between the two groups were further investigated. The results indicated that the NLRP3 high-expression group exhibited higher levels of macrophage infiltration, especially M2 macrophages, compared to the low-expression group. Additionally, plasma cells, eosinophils, and activated CD4-positive memory T cells also showed higher infiltration levels

Figure 1. The analysis of NLRP3 expression in lung adenocarcinoma and its microenvironment.



Note: Compared with the low-expression group, *P < .05, **P < .01, ***P < .001 ****P < .0001.

Abbreviations: LUAD, Lung adenocarcinoma; T, Tumor; N, Non-tumor; NK cells, Nature killing cells; TPM, Transcripts per million.

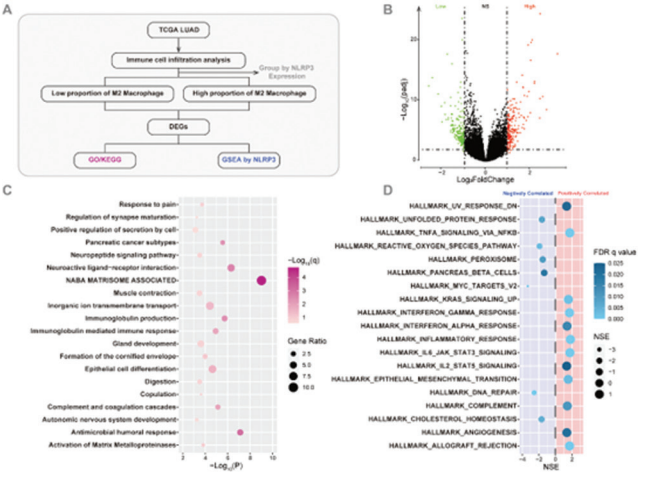
in NLRP3 high-expression group compared to low-expression group (Figure 1C).

Differential expression genes analysis and functional annotation

To + analyze the potential functions of NLRP3 in lung cancer and its microenvironment, we stratified TCGA lung adenocarcinoma patients into high-M2 and low M2-group based on the median infiltration abundance of M2 macrophages. Subsequently, we compared the differential gene expression analysis between these two groups, identifying a total of 302 differentially expressed genes (DEGs). Results from GO/KEGG enrichment analysis suggested that these DEGs might be involved in processes such as NABA MATRISOME, epithelial cell differentiation, and immunoglobulin production (Figure 2A-C).

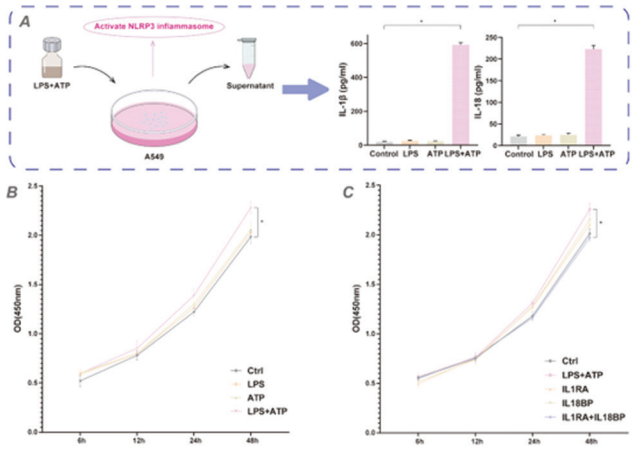
Moreover, we conducted GSEA analysis in accordance with the expression of NLRP3. The results revealed a positive correlation between NLRP3 expression and gene sets associated with KRAS signaling pathway, inflammatory response, epithelial-mesenchymal transition, and angiogenesis. Conversely, there was a negative correlation with gene sets related to DNA repair and peroxisome processes (Figure 2D).

Figure 2. Differential genes expression analysis and functional annotation of genes associated with NLRP3.



Abbreviations: DEGs, Differential Expression Genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; LUAD, Lung Adenocarcinoma; NS, No Difference; NES, Normalized Enrichment Score; FDR, False Discovery Rate.

Figure 3. NLRP3 inflammasome activation and its impact on the proliferation ability of A549 cells.



Note: Compared with the control group, *P < .05

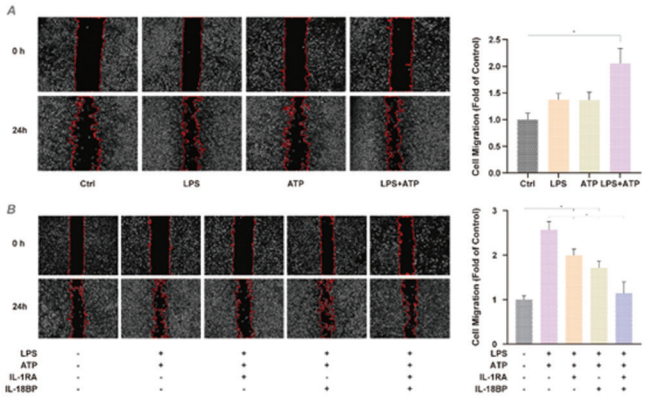
Abbreviations: Ctrl, Control; OD, Optical Density; LPS, Lipopolysaccharide.

The impact of NLRP3 inflammasome activation on the proliferation of A549 cells

To explore the potential role of NLRP3 in lung cancer, present study activated the NLRP3 inflammasome in A549 cells by co-treating them with LPS and ATP. Successful activation of the NLRP3 inflammasome was determined by measuring the levels of IL-1β and IL-18 in the cell culture medium supernatant (Figure 3A). Cell proliferation ability was compared between the control group and the drug-treated group, it was observed that the activation of the NLRP3 inflammasome promoted the proliferation of A549 cells (Figure 3B).

Furthermore, downstream signaling pathway of IL-1β and IL-18 was blocked by IL-1RA and IL-18BP, respectively,

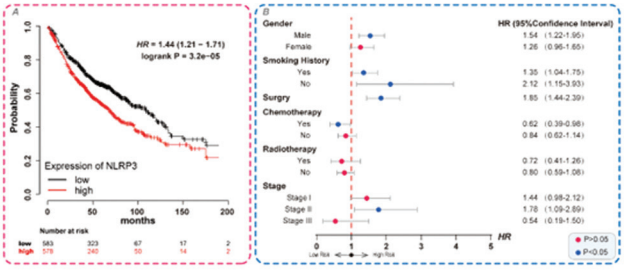
Figure 4. The impact of NLRP3 inflammasome activation on the migration ability of A549 cells



Note: Compared with the control group, *$P < .05$

Abbreviation: LPS, Lipopolysaccharide.

Figure 5. The relationship between NLRP3 expression levels and the prognosis of lung adenocarcinoma patients.



Abbreviation: HR, Hazzard Ratio.

following NLRP3 inflammasome activation. The results indicated that the promotion of A549 cell proliferation by NLRP3 inflammasome activation was partially attenuated by blocking either IL-1β or IL-18 signaling pathways. Moreover, simultaneous treatment with IL-1RA and IL-18BP completely blocked the proliferative effect induced by NLRP3 inflammasome activation in A549 cells (Figure 3C).

The effects of NLRP3 inflammasome activation on the migration of A549 cells

The scratch assay was conducted to examine the impact of NLRP3 inflammasome activation on the migration ability of A549 cells. The results revealed that the activation of the NLRP3 inflammasome enhanced the migration capability of A549 cells (Figure 4A). When IL-1RA and IL-18BP were used to individually block the IL-1β and IL-18 signaling pathways, the migration ability of A549 cells was partially attenuated. Notably, simultaneous treatment with IL-1RA and IL-18BP completely abolished the promotional effect of NLRP3 inflammasome activation on the migration ability of A549 cells (Figure 4B).

Survival analysis of NLRP3 in lung adenocarcinoma

Survival analysis was conducted to further explore the prognostic value of NLRP3 expression in lung adenocarcinoma. Patients were stratified into NLRP3 high-expression and NLRP3 low-expression groups based on the median expression of NLRP3. The analysis revealed that patients with high NLRP3 expression had poorer prognosis compared to those with low NLRP3 expression (Hazzard Ratio, HR=1.44; 95% Confidence Interval, 95%CI: 1.21-1.71, Figure 5A). Additionally, subgroup analysis showed that in male patients (HR=1.54, 95%CI: 1.22-1.95), those with (HR=1.35, 95%CI: 1.04-1.75) or without (HR=2.12, 95%CI: 1.15-3.93) a history of smoking, those who underwent R0 lung cancer surgical resection (HR=1.85, 95%CI: 1.44-2.39), and those in TNM stage II (HR=1.78, 95%CI: 1.09-2.89), patients with high NLRP3 expression had a worse prognosis than those with low NLRP3 expression. However, among patients who received chemotherapy, those with high NLRP3 expression had a better prognosis compared to those with low NLRP3 expression (HR=0.62, 95%CI: 0.39-0.98, Figure 5B)

DISCUSSION

Inflammation is closely related to the occurrence and development of cancer, playing a significant role in tumor proliferation, invasion, and metastasis.[13] The NLRP3 inflammasome is a crucial component of the innate immune system, and its overactivation is associated with several inflammatory diseases.[14] Recent research has found that the NLRP3 inflammasome plays a dual role in the pathogenesis of various metabolic diseases and cancers by mediating the activation of inflammatory responses and triggering the release of pro-inflammatory cytokines.[15] NLRP3 may exhibit diverse roles in different types of tumors. For example, NLRP3-mediated release of IL-18 has a tumor-suppressive effect in colorectal cancer,[16,17] while the activation of the NLRP3 inflammasome and the release of IL-1 may play a pro-tumorigenic role by recruiting macrophages into the tumor tissue.[18] However, its role and mechanisms in lung cancer are currently not well understood. Present study demonstrated that NLRP3 inflammasomes activation might promote the proliferation and migration of lung adenocarcinoma through IL-1β and IL-18 releasing, potentially impacting the prognosis of lung cancer patients. The results reveal the role of NLRP3 inflammasome in the microenvironment of lung cancer, providing evidence to support the development of novel cancer strategies targeting the NLRP3 inflammasome in the future.

A recent pan-cancer analysis revealed that the expression of NLRP3 inflammasome-related genes in various cancer tissues is significantly different compared to normal samples.[19] Present study found that the expression of NLRP3 in normal tissues was significantly higher than in tumor tissues, and higher expression of NLRP3 was associated with increased infiltration of M2 macrophages. M2 macrophages have been found to play a pro-tumorigenic role by secreting various cytokines, mediating tumor angiogenesis, and immune resistance in lung cancer.[20] Functional enrichment analysis revealed a positive correlation between NLRP3 expression and changes in the inflammation response pathway gene sets.

One study found that the NLRP3/caspase-1/IL-1β signaling axis within monocytes in the tumor microenvironment can independently promote the growth of tumor cells.[21] Based on the results of present study, we hypothesize that activation of the NLRP3 inflammasome could elevated levels of IL-1β and IL-18, recruit various inflammatory cells to produce pro-tumorigenic inflammation, further promoting the proliferation and invasion of tumor cells. Prognostic analysis also revealed that patients with high expression of NLRP3 have a worse prognosis compared to patients with lower expression. However, the role of NLRP3 in the tumor microenvironment remains largely controversial. Other studies have found that CD146 macrophages can enhance anti-tumor effects by activating NLRP3 inflammasome.[22] Therefore, the conclusions of present study and the underlying mechanisms require further exploration and validation.

IL-1β exhibits potent biological activities, including mediating inflammatory responses by inducing the expression of IL-6, iNOS, COX-2, and other factors, contributing to epigenetic changes in genes, and promoting neovascularization through the expression of IL-8, COX-2, and VEGF, thereby playing a crucial role in inflammation and tumor development.[23] Increased levels of IL-1β in the lung can significantly increase the risk of non-small cell lung cancer.[24-26] Tumor patients with increased levels of IL-1β have worse prognosis than those with normal levels of IL-1β. In Lewis lung cancer of mice, when the vector expressing IL-1β was introduced into cells, the tumor grew rapidly, that is to say, IL-1β secreted by tumor microenvironment could promote tumor growth.[27] The combination of the IL-1 receptor blocker Anar apigenin (blocking IL-1β signaling) and glucocorticoids (inhibiting IL-1β transcription and translation) significantly reduces myeloma proliferation.[28] IL-18 also belongs to the IL-1 family, and its biological activity is mainly regulated by enzymatic hydrolysis process. IL-18 signal transduction mainly depends on MAPK pathway, while IL-1β is mainly regulated by transcription level and its signal pathway mainly depends on NF-κB.[29] IL-18 has strong tumor-promoting effects which can promote the proliferation and migration of tumors, and up-regulate the pro-angiogenic factor thrombospondin-1 to enhance blood vessel formation.[30] IL-18 can activate PI3K-Akt signaling pathway and induce hypoxia-inducible factor-1alpha (HIF-1α) to promote tumor growth.[31] IL-18 can induce immune escape, enhance the adhesion to blood vessel wall, and promote programmed death-1 gene (PD-1) -dependent NK cell-mediated metastasis.[32] Serum IL-18 is significantly increased in patients with renal cell carcinoma and hepatocellular carcinoma, and is an independent risk prognostic factor.[33] In present study, activation of the NLRP3 inflammasome resulted in the secretion of IL-1β and IL-18, promoting the proliferation and migration of A549 cells. The pro-proliferative and pro-migratory effects were reversed when IL-1RA and IL-18BP were co-administered to collectively block IL-1β and IL-18. Currently, there were researches applying IL-1β inhibitors in clinical settings, and it has been found that IL-1β inhibitors can reduce the incidence of lung cancer and cancer-related mortality.[34,35] Mingyi et al. attempted to construct NLRP3 inflammasome score and found a positive correlation between the score and the expression of immune checkpoint genes in lung cancer patients.[19] This suggests that the NLRP3 inflammasome may be associated with the immune therapeutic response in patients. The NLRP3 inflammasome may serve as a crucial target for future cancer therapies.

The present study has following limitations: We focused on the effects of NLRP3 inflammasome activation on lung cancer cell proliferation and migration within a single cell line. The lack of validation across multiple cell lines and animal experiments limits the generalizability of our findings. Additionally, due to the complex nature of the tumor microenvironment and NLRP3 inflammasome effects, the study faced challenges in exploring how NLRP3 inflammasome activation affects various immune cells in microenvironment. Further investigation is necessary to acquire an in-depth understanding of the specific roles and mechanisms of NLRP3 inflammasome activation in the lung cancer microenvironment.

In conclusion, this study confirmed that the activation of the NLRP3 inflammasome promotes the proliferation and migration of A549 cells through secretion of IL-1β and IL-18, potentially influencing patient prognosis. Simultaneously blocking IL-1β and IL-18 can reverse the pro-proliferative and migration-promoting effects, providing further evidence to support the development of anti-tumor therapeutic strategies targeting NLRP3 in the future.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to report relevant to this article.

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AUTHOR CONTRIBUTIONS

Zhaoxun Li and Fujun Yang contributed equally to this work. ZL, FY and GJ designed the study and performed the experiments, XZ and BZ collected the data, KJ and JD analyzed the data, ZL, FY and GJ prepared the manuscript. All authors read and approved the final manuscript.

ETHICAL COMPLIANCE

Not applicable.

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