Original Article

Knockdown of LRRK2 inhibits the progression of lung cancer by regulating TLR4/NF-κB pathways and NLRP3 inflammasome

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(Received 24 October, 2022; Accepted 10 December, 2022; Released online in J-STAGE as advance publication 18 August, 2023)

Leucine-rich repeat kinase 2 (LRRK2) plays an important role in a variety of inflammatory diseases, as well as peripheral and central immune responses. At present, there are few reports about the role of LRRK2 in lung cancer, and need to be further explored. The main purpose of this study is to explore the role and mechanism of LRRK2 in lung cancer. The results revealed that the expression of LRRK2 was increased in the tissues of lung cancer patient and lung cancer cells. Further studies found that interference with LRRK2 expression significantly induced the apoptosis, and promoted the expression of caspase-3, caspase-9, and Bax. More importantly, si-LRRK2 inhibited the expression of VEGF and P-gp, indicating inhibition of cell proliferation and drug resistance. What's more, LRRK2 regulated TLR4/NF-κB signaling pathways and NLRP3 inflammasome, and TLR4/NF-κB pathways was involved in the molecular mechanism of LRRK2 on lung cancer cells. In conclusion, this study suggested that the mechanism of si-LRRK2 inhibiting the progression of lung cancer is to regulate the TLR4/NF-κB signaling pathways and NLRP3 inflammasome.

***Key Words*: LRRK2, TLR4/NF-κB, drug-resistance**

Lung cancer is the most common malignant tumor in the lung, with a high incidence rate and mortality rate, and poses a threat to human health and life.[1,2] Clinically common lung cancer includes small cell lung cancer (SCLC, 20%) and non-small cell lung cancer (NSCLC, 80%).[3,4] There are many factors leading to lung cancer, among which smoking is the main cause of lung cancer.[5] In recent years, the incidence rate and mortality rate of lung cancer have increased significantly, and the 5-year survival rate is less than 20%.[6]

Leucine rich repeat kinase 2 (LRRK2) plays an important role in a variety of inflammatory diseases and may mediate the immune response of Parkinson's disease (PD).[7,8] The cellular signal mediated by LRRK2 is not only very important for neuron function (including neuron development and internal environment stability), but also very important for peripheral and central immune response.[9,10] The catalytic activity of LRRK2 is regulated by autophosphorylation, protein monomer/dimer and upstream kinase, which affects its subcellular localization and downstream signal transduction.[11] It has been found that LRRK2 can regulate p38 MAPK and NF-κB signaling pathways downstream.[10,12] At present, the research of LRRK2 protein is more limited in neurological diseases and inflammatory diseases, and there are few reports on the occurrence and development of human cancer. It has been reported that the down regulation of LRRK2 inhibits the proliferation and migration of thyroid cancer cells and promotes apoptosis by inhibiting the activation of JNK signaling pathway.[13] However, the role of LRRK2 in the development of lung cancer has not been reported.

Toll like receptors (TLRs) are important protein molecules involved in non-specific immunity (innate immunity).[14] TLR can recognize external stimuli and activate immune cell response, which are the bridge between non-specific immunity and specific immunity.[15] TLR4 belongs to the TLR family, which is a highly conserved receptor family.[16] It recognizes the conservative pathogen related patterns and therefore represents the first line of defense. Therefore, TLR4 is a key receptor in the inflammatory response mediated by exogenous and endogenous ligands, and plays a key role in inflammatory response amplifier.[17,18] NF-κB is a transcription factor that induces the expression of various cytokines and regulates the inflammatory cascade.[19] More importantly, TLR4/NF-κB signaling pathway plays an important regulatory role in the occurrence and development of various diseases, including cancer and inflammatory diseases.[20,21]

As an important component of innate immunity, NLRP3 inflammasome plays an important role in immune response and disease occurrence.[22,23] NLRP3 inflammasome plays a key role in many diseases due to its ability to be activated by a variety of pathogens or danger signals, including initially identified familial periodic autoinflammatory response, type 2 diabetes mellitus, Alzheimer's disease and atherosclerosis.[24,25] Therefore, as the core of inflammatory response, NLRP3 inflammasome may provide a new target for the treatment of various inflammatory diseases.

In the present research, the results revealed that si-LRRK2 significantly induced the apoptosis, and inhibited cell proliferation and drug resistance in lung cancer cells. What's more, LRRK2 regulated TLR4/NF-κB signaling pathways and NLRP3 inflammasome, and TLR4/NF-κB pathways were involved in the molecular mechanism of LRRK2 on lung cancer cells. It is suggested that si-LRRK2 inhibits the progression of lung cancer via regulating the TLR4/NF-κB signaling pathways and NLRP3 inflammasome.

Materials and Methods

Tissue. From December 2018 to September 2019, 52 cases of lung cancer patients were treated, aged 45–80 years (average 59 years). Pathological examination confirmed the diagnosis. In addition, fresh tumor tissue samples were collected from all these patients and matched adjacent tissue samples were used as controls. This study was approved by the ethics committee of Shaanxi Provincial People's Hospital. For research in humans,

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doi: 10.3164/jcbn.22-122

each participant gave written informed consent and the research was conducted in accordance with the principles of the Declaration of Helsinki.

Cells. Normal pulmonary epithelial cell BEAS-2B and lung cancer cells H520, H1975, H1299, and A549 were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI1640 medium (containing 10% fetal bovine serum and double antibody) and placed in a cell incubator at 37°C and 5% CO_2. The cells were transferred to the third generation for subsequent experiments when they fused to 80–90%.

Reagents. Antibodies including anti-LRRK2, anti-VEGF, anti-P-gp, anti-caspase-3, anti-caspase-9, anti-Bax, anti-Bcl-2, anti-TLR4, anti-p65, and anti-NLRP3, were purchased from Cell Signaling Technology (CST, Boston, MA). Anti-GAPDH was purchased from Abcam Inc. (BD Biosciences, San Jose, CA).

Transfection. The H1299 and A549 cells were inoculated with 1×10^5 cells/well on the 24 well plate. When the cell growth convergence rate reached about 60%, the si-LRRK2 was transfected into H1299 or A549 cells according to the instructions of Lipofectamine 3000 transfection reagent. After 48 h culture in 37°C and 5% CO_2 incubator, cells of each group were collected for following experiment.

MTT assay. The IC50 for 5-Fu of H1299 and A549 cells was measured using an MTT assay kit (Abcam, Cambridge, UK). The cell proliferation was measured at specified time points using microplate reader (Thermo Fisher Scientific, Waltham, MA) by spectrophotometry at 570 nm.

RT-qPCR. Total RNA samples from the pulmonary epithelial cell BEAS-2B and lung cancer cells H520, H1975, H1299, and A549 cells were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA). Using specific miRNA RT primers to the reverse transcription reaction (Invitrogen, Carlsbad, CA). The thermocycling conditions of RT-qPCR were as follows: 95°C for 1 min; 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 20 s. Relative transcriptional levels were calculated by the $2^{-\Delta\Delta CT}$ method with β-actin as a normalizing gene.

Western blotting. When the cells fused to 80–90% were transfected with si-LRRK2 or siRNA. After 48 h, the cells in each group were collected, and were lysed with Ripa buffer (Sigma, St. Louis, MO) for 15 min. Then were centrifuged at 4°C and 12,000 rpm for 30 min (the centrifugation radius was 9.5 cm). The supernatant was determined by BCA protein quantitative detection kit (Solarbio, Beijing, China). Then, polyacrylamide gel electrophoresis (SDS-PAGE, 10%; Bio-Rad, Hercules, CA) was used to separate the total protein and transfer to the PVDF membrane (Millipore, Boston, MA). The membrane was placed in 5% zero fat milk powder and incubated with the corresponding primary antibody. After cleaning, the membrane was incubated with secondary antibody (Thermo Fisher Scientific). Finally, protein complexes were displayed by enhanced chemiluminescence (ECL) substrate and detected by Carestream molecular imaging system (Bio-Rad).

Statistical analyses. In the present research, all statistical analysis were performed using SPSS 22.0, (Chicago, IL). Data are represented as means ± SD and each experiment was

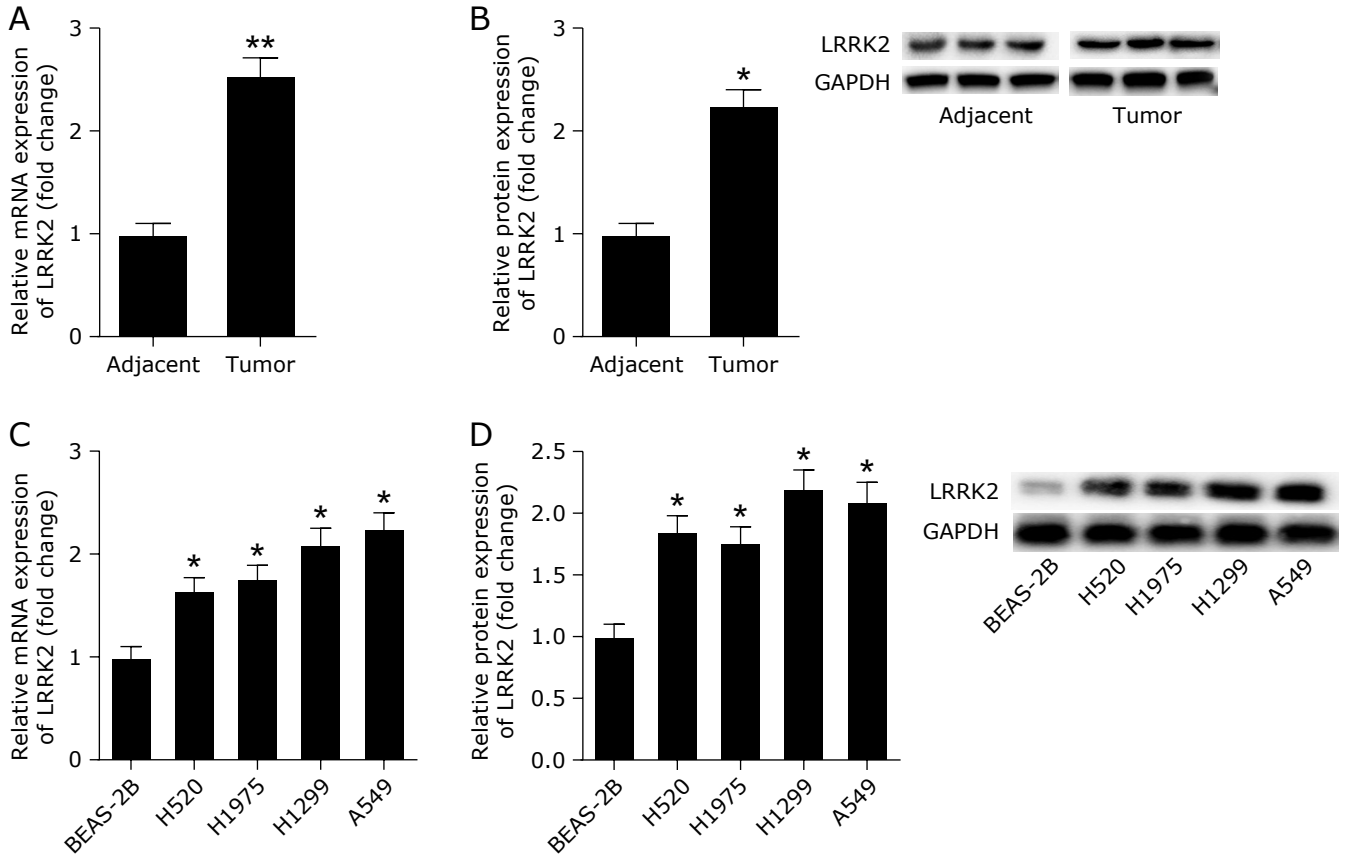

Fig. 1. The expression of LRRK2 in tumor tissue and cells is increased significantly. (A) The mRNA expression of LRRK2 in adjacent and tumor tissues. (B) The protein expression of LRRK2 in adjacent and lung cancer tissues. (C) The mRNA expression of LRRK2 in normal pulmonary epithelial cell BEAS-2B and lung cancer cells H520, H1795, H1299, and A549. (D) The protein expression of LRRK2 in normal pulmonary epithelial cell BEAS-2B and lung cancer cells H520, H1795, H1299, and A549. * means compared with BEAS-2B group or Adjacent group $p<0.05$, and ** means compared with Adjacent group $p<0.01$.

performed in triplicate in this study. One-way ANOVA and Student's unpaired *t* test were used to analyze statistical significance. All statistical analyses were performed by SPSS 20.0 software (SPSS, Inc., Chicago, IL). *P* value <0.05 was considered to be significant.

Results

The expression of LRRK2 is increased significantly in tumor tissue and cells. We first analyzed the expression of LRRK2 in tumor and adjacent tissues. The results showed that the expression of LRRK2 in tumor tissue was significantly increased than that in adjacent tissue, including mRNA and protein (Fig. 1A and B). As expected, the expression of LRRK2 in lung cancer cell lines (H520, H1975, H1299, and A549) was significantly increased compared with normal lung epithelial cells (BEAS-2B) (Fig. 1C and D).

si-LRRK2 inhibits proliferation and drug-resistance of lung cancer cells. The H1299 and A549 cells were transfected with the control si-RNA (si-RNA group) or si-LRRK2 (si-LRRK2 group) respectively. As shown in Fig. 2A, the protein expression of LRRK2 was significantly decreased in si-LRRK2 group compared with control and si-RNA group (Fig. 2A). The results showed that si-LRRK2 significantly inhibited cell viability and cell proliferation (Fig. 2B). In addition, the protein expression of VEGF was significantly down-regulated in si-LRRK2 group. Further study showed that the IC50 for 5-Fu was significantly reduced in si-LRRK2 group compared with control and si-RNA group (Fig. 2D). As expected, si-LRRK2 significantly inhibited the expression of P-gp (Fig. 2E and F).

si-LRRK2 induces apoptosis of A549 and H1299 cells. Next, we investigate the effect of LRRK2 on apoptosis of lung cancer cells. As shown in Fig. 3A, the results showed that si-LRRK2 significantly increased the apoptosis of A549 and H1299 cells (Fig. 3A). In addition, the protein expression of caspase-3 and caspase-9 was significantly increased in si-LRRK2 group compared with control and si-RNA group (Fig. 3B and C). As expected, the expression of Bax was increased significantly, while Bcl-2 was decreased significantly in si-LRRK2 group (Fig. 3D and E).

si-LRRK2 inhibits the activation of TLR4/NF-κB pathway and NLRP3 inflammasome. We further explored the mechanism of LRRK2 regulating lung cancer progression. As shown in Fig. 4A, si-LRRK2 significantly decreased the expression of TLR4 and p65 compared with control and si-RNA group (Fig. 4A–C). Furthermore, the expression of NLRP3 was significantly down-regulated after transfected with si-RNA (Fig. 4A and D).

TLR4/NF-κB pathway and NLRP3 participate in the mechanism of LRRK2 on lung cancer cells. The A549 cells were transfected with si-LRRK2, pcDNA3.1-TLR4 or pcDNA3.1-NLRP3 for 48 h. As shown in Fig. 5A, si-LRRK2 significantly decreased the cell viability, while pcDNA3.1-TLR4 or pcDNA3.1-NLRP3 increased the cell viability and reversed the role of si-LRRK (Fig. 5A). In addition, pcDNA3.1-TLR4 or pcDNA3.1-NLRP3 significantly up-regulated the expression of VEGF (Fig. 5B). We found that pcDNA3.1-TLR4 or pcDNA3.1-NLRP3 significantly promoted the IC50 for 5-Fu of A549 compared with the control group (Fig. 5C). What's more, the apoptosis was significantly increased in si-LRRK2 group, and further transfected with pcDNA3.1-TLR4 or pcDNA3.1-NLRP3

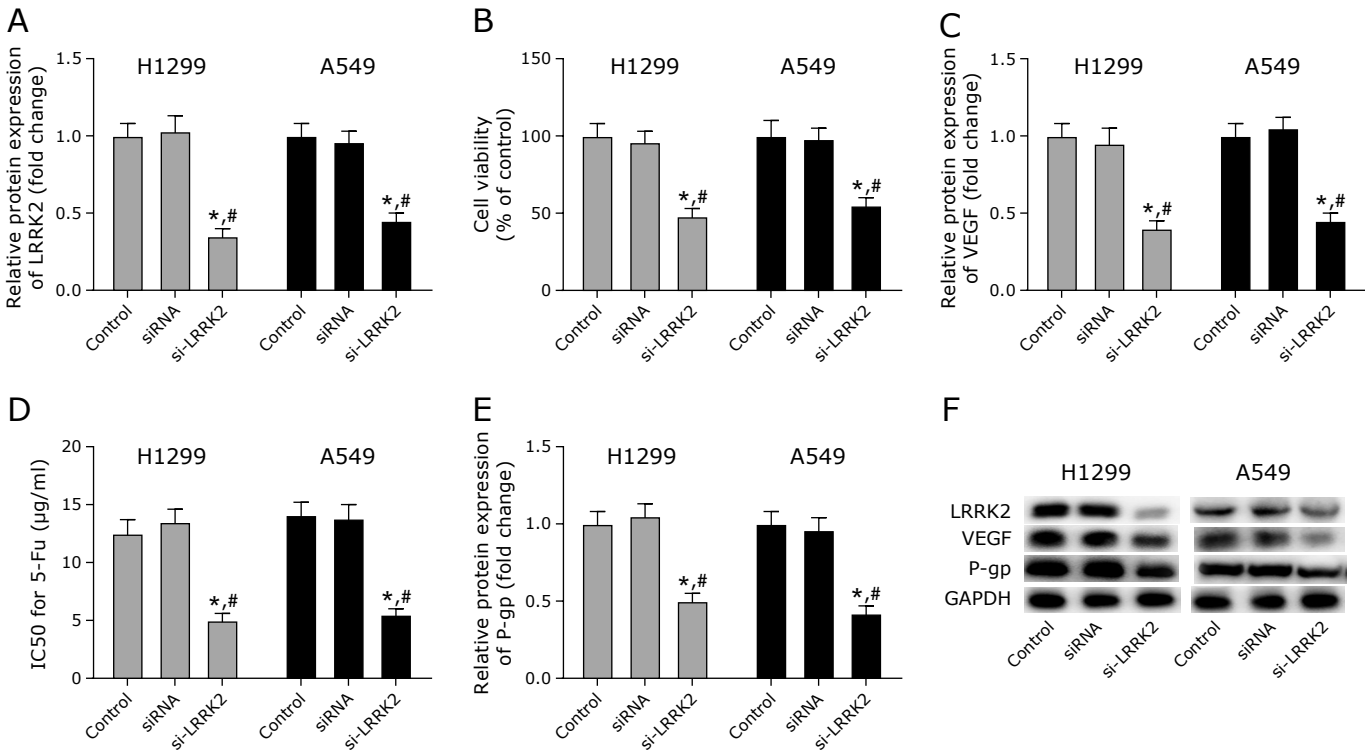

Fig. 2. si-LRRK2 inhibits proliferation and drug-resistance of lung cancer cells. The H1299 and A549 cells were transfected with the control si-RNA (si-RNA group) or si-LRRK2 (si-LRRK2 group) respectively. (A) The protein expression of LRRK2 in control group, si-RNA group and si-LRRK2 group. (B) The cell viability of H1299 and A549 cells in control group, si-RNA group, and si-LRRK2 group. (C) The protein expression of VEGF in control group, si-RNA group and si-LRRK2 group. (D) The IC50 for 5-Fu in control group, si-RNA group and si-LRRK2 group. (E) The protein expression of P-gp in control group, si-RNA group and si-LRRK2 group. (F) Western blot was performed to confirm the protein expression of LRRK2, VEGF and P-gp in H1299 and A549 cells. * means compared with control group $p<0.05$, and # means compared with si-RNA group $p<0.05$. GAPDH was used as an invariant internal control for calculating protein-fold changes.

doi: 10.3164/jcbn.22-122

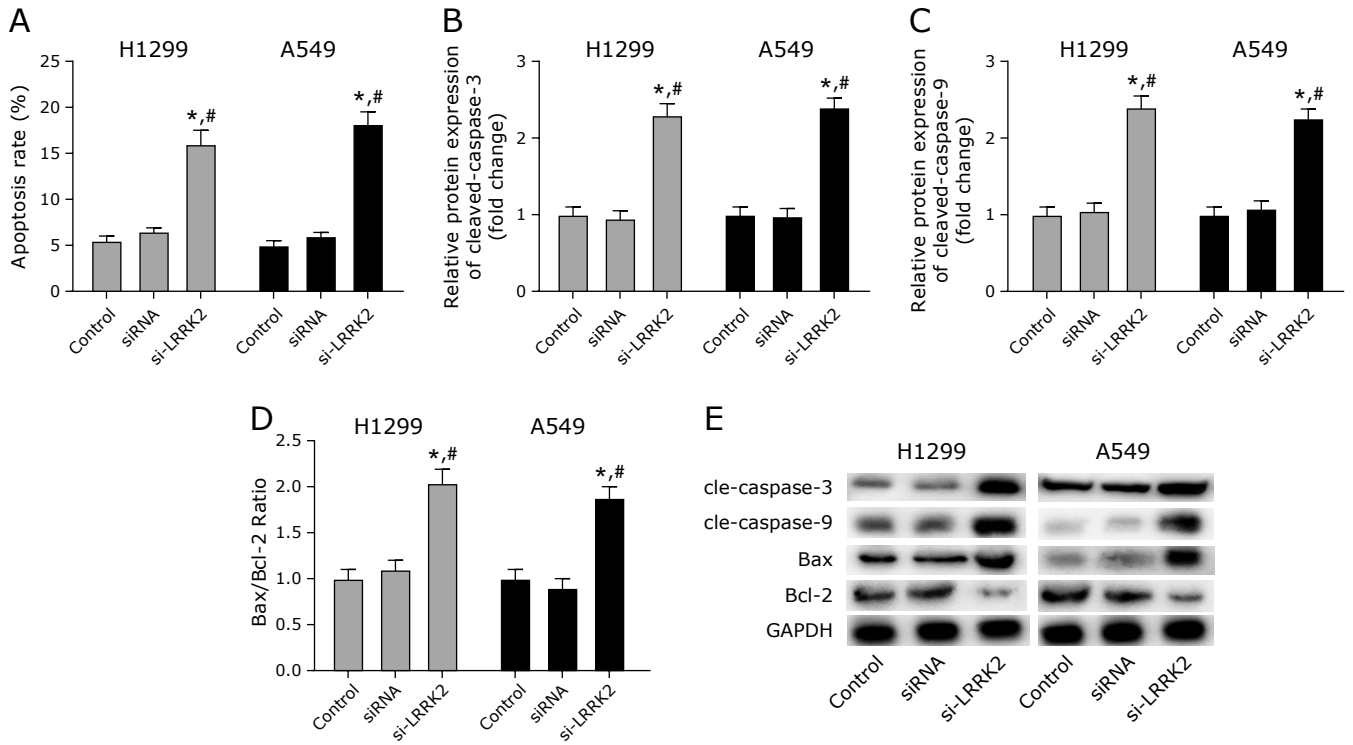

Fig. 3. si-LRRK2 induces apoptosis of A549 and H1299 cells. The H1299 and A549 cells were transfected with the control si-RNA (si-RNA group) or si-LRRK2 (si-LRRK2 group) respectively. (A) The apoptosis rate (%) of H1299 and A549 in control group, si-RNA group and si-LRRK2 group. (B) The protein expression of cleaved-caspase-3 in control group, si-RNA group and si-LRRK2 group. (C) The protein expression of cleaved-caspase-9 in control group, si-RNA group and si-LRRK2 group. (D) The ratio of Bax/Bcl-2 in control group, si-RNA group and si-LRRK2 group. (E) Western blot was performed to confirm the protein expression of cleaved-caspase-3, cleaved-caspase-9, Bax and Bcl-2 in H1299 and A549 cells. * means compared with control group $p<0.05$, and # means compared with si-RNA group $p<0.05$. GAPDH was used as an invariant internal control for calculating protein-fold changes.

significantly decreased the apoptosis (Fig. 5D). Furthermore, the expression of caspase-3 was down-regulated in pcDNA3.1-TLR4 or pcDNA3.1-NLRP3 group compared with si-LRRK2 group (Fig. 5E and F).

Discussion

Studies have shown that LRRK2 plays an important role in various diseases including cancer.[26] We found that LRRK2 is an important regulator of proliferation, apoptosis and drug-resistance in lung cancer. In addition, we found that LRRK2 regulates the TLR4/NF-κB signaling pathways and NLRP3 inflammasome. It is suggested that si-LRRK2 inhibits the progression of lung cancer via regulating the TLR4/NF-κB signaling pathways and NLRP3 inflammasome.

Genetic studies have found that LRRK2 deletion is closely related to neurodegenerative diseases, such as Parkinson's disease (PD).[27] In recent years, there are more and more studies on the function of LRRK2 in the development of Parkinson's disease.[28] It has been reported that LRRK2 promotes the phosphorylation of MAPK, activates MAPK signaling pathway, and promotes neurological dysfunction and neuroinflammation, further promotes the progress of PD.[8] It is worth noting that LRRK2 plays an important role in inflammatory diseases, such as inflammatory bowel disease and inflammatory cancer.[29] Wallings *et al.*[30] found that LRRK2 was highly expressed in macrophages, regulating inflammatory related pathways and the release of inflammatory factors. Moehle *et al.*[11] found that inhibition of LRRK2 reduces the inflammatory response of microglia. As we all know, inflammation plays an important role in the development of cancer, and LRRK2 is closely related to inflammation.[9,31] Therefore, we speculate whether LRRK2 also plays a key role in the development of lung cancer. As expected, we found that knockdown of LRRK2 inhibited proliferation and drug-resistance as well as induced apoptosis of lung cells, which blocked the further deterioration of lung cancer. Coincidentally, Jiang *et al.*[13] reported that inhibition of LRRK2 promoted cell cycle arrest and apoptosis, inhibited cell proliferation and migration, and further inhibited the progress of thyroid cancer. Therefore, targeting LRRK2 may be an attractive potential therapy for human diseases.

LRRK2 is involved in the regulation of multiple signaling pathways, including AMPK/mTOR, Wnt, TLR4/NF-κB signaling.[32] As we all know, TLR4/NF-κB is involved in the regulatory process of various diseases, including cancer, inflammation, neurologic diseases, and involved in various physiological processes, such as cell proliferation, apoptosis, migration.[18,33] It has been found that the activation of TLR4/NF-κB signaling by HMGB1 enhances the migration ability of lung cancer cells and promote the metastasis of lung cancer.[34] In addition, Zhou *et al.*[16] confirmed that TLR4, as a ligand of galectin-3, induces the activation of TLR4 signaling pathway and activates downstream p65 nuclear translocation, which ultimately affects the proliferation and migration of lung adenocarcinoma cells. Coincidentally, this study found that activation of TLR4/NF-κB pathway promotes the further deterioration of lung cancer. Therefore, inhibiting the activation of TLR4/NF-κB signaling pathway may be a promising strategy to inhibit the development of human cancer.

The abnormality of NLRP3 inflammasome is involved in

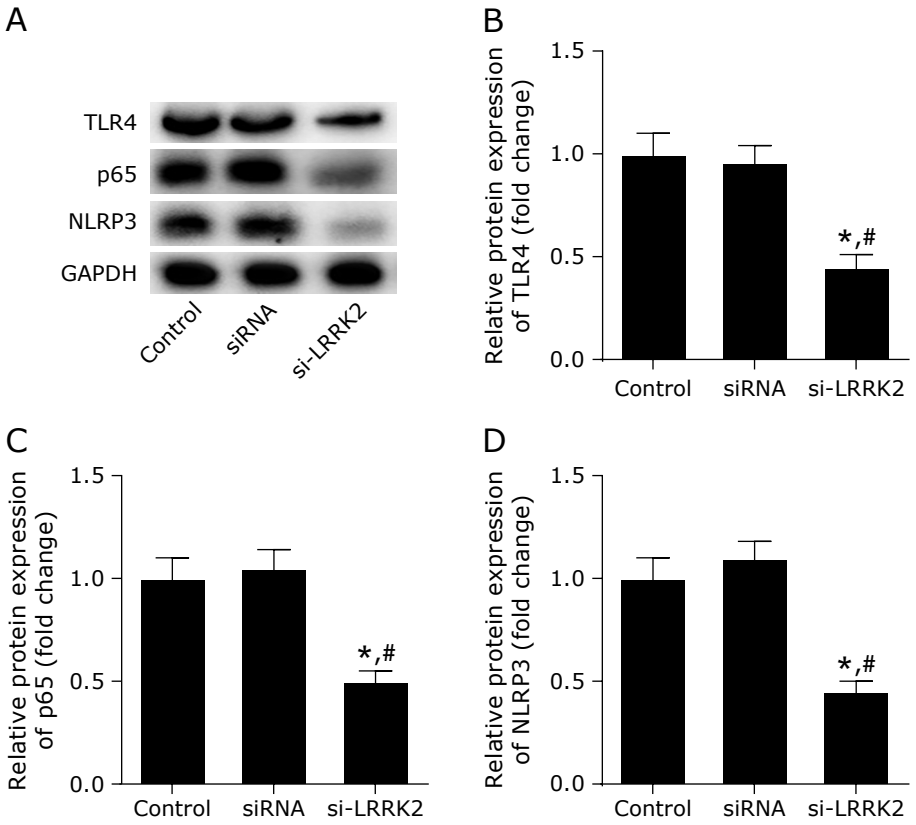

Fig. 4. si-LRRK2 inhibits the activation of TLR4/NF-κB pathway and NLRP3 inflammasome. The A549 cells were transfected with the control si-RNA (si-RNA group) or si-LRRK2 (si-LRRK2 group) respectively. (A) Western blot was performed to confirm the protein expression of TLR4, p65 and NLRP3 in A549 cells. (B) The protein expression of TLR4 in control group, si-RNA group and si-LRRK2 group. (C) The protein expression of p65 in control group, si-RNA group and si-LRRK2 group. (D) The protein expression of NLRP3 in control group, si-RNA group and si-LRRK2. * means compared with control group $p<0.05$, and # means compared with si-RNA group $p<0.05$. GAPDH was used as an invariant internal control for calculating protein-fold changes.

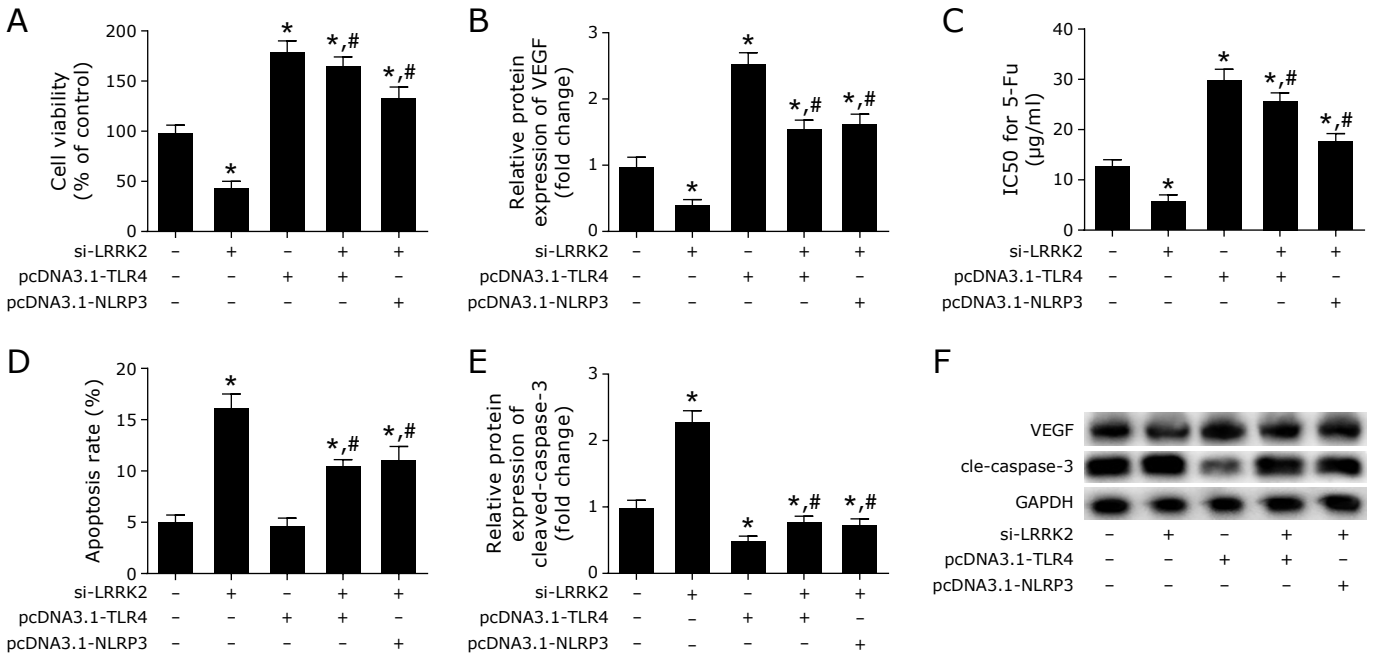

Fig. 5. TLR4/NF-κB pathway and NLRP3 participate in the mechanism of LRRK2 on lung cancer cells. The A549 cells were transfected with si-LRRK2, pcDNA3.1-TLR4 or pcDNA3.1-NLRP3 respectively. (A) The cell viability in each group. (B) The protein expression of VEGF in each group. (C) The IC50 for 5-Fu in each group. (D) The apoptosis rate (%) of A549 in each group. (E) The protein expression of cleaved-caspase-3 in each group. (F) Western blot was performed to confirm the protein expression of VEGF and cleaved-caspase-3 in A549 cells. * means compared with untreated group $p<0.05$, and # means compared with si-LRRK2 group $p<0.05$. GAPDH was used as an invariant internal control for calculating protein-fold changes.

doi: 10.3164/jcbn.22-122

tumor development, although its role in cancer development and progression is still controversial.[35] More and more attention has been paid to the role of NLRP3 inflammasome in different types of tumors. Studies on AOM/DSS induced colitis and colon cancer show that NLRP3 inflammasome has a protective effect on carcinogenesis.[36] On the contrary, NLRP3 inflammasome promotes tumor growth, proliferation, invasion and metastasis in lung cancer, melanoma cancer and breast cancer.[37] In addition, NLRP3 inflammasome is associated with chemotherapy resistance in glioblastoma and oral squamous cell carcinoma. In the present study, we found that activation of NLRP3 inflammasome promotes the proliferation and drug resistance of lung cancer cells, and promotes the further development of lung cancer. The evidences that NLRP3 inflammasome promotes cancer progression is still at a preliminary stage, which needs to be further confirmed and to determine their potential therapeutic effects in human malignant tumors.[38]

In summary, the results reveal that si-LRRK2 inhibits the progression of lung cancer via regulating the TLR4/NF-κB signaling pathways and NLRP3 inflammasome, which suggests that LRRK2 might be a potential marker for the treatment of lung cancer. In the future, looking for a reasonable mechanism for the role of LRRK2 will help us to understand its function more comprehensively, and finally find a new way for the treatment of human malignant tumor.

Author Contributions

YM involved in Conceptualization, Methodology. JFW involved in Formal analysis and Writing - original draft. SMY and HW involved in Software and Validation. YCH involved in Data curation and Writing - review & editing.

Conflict of Interest

No potential conflicts of interest were disclosed.

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