

Anisodamine Suppressed the Growth of Hepatocellular Carcinoma Cells, Induced Apoptosis and Regulated the Levels of Inflammatory Factors by Inhibiting NLRP3 Inflammasome Activation

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

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Introduction: Hepatocellular carcinoma (HCC) is a primary liver cancer with a 5-year incidence of over 70%. Anisodamine (ANI), an alkaloid extracted from *Anisodus*, has a good therapeutic effect in septic shock and morphine addiction. Our study designed to investigate the anticancer effect of anisodamine (ANI) on HCC.

Materials and Methods: HepG2 cells were subcutaneously injected into BALB/C nude mice and the tumor tissue was subcutaneously inoculated to construct the transplanted tumor. Mice were randomly divided into 10 groups (n = 5): control group, ANI-10 group, ANI-50 group, ANI-200 group, ANI-200+pcDNA-NLRP3 group, ANI-200+EV group, sh-NLRP3 group, ANI-200 + sh-NLRP3 group, normal group and normal+ANI-200 group.

Results: Studies indicated that ANI inhibited the growth of HCC xenografts and reduced liver damage in a dose-dependent manner. Besides, ANI increased the survival rate of tumor-bearing mice and suppressed the expression of NLRP3 in a dose-dependent manner. It is worth noting that NLRP3 overexpression reversed the inhibitory effect of ANI on HCC xenografts. In addition, TUNEL analysis showed that ANI-induced apoptosis of tumor cells, and NLRP3 overexpression reversed the inhibitory effect of ANI on HCC. Moreover, ANI further regulated the levels of IFN- γ , TNF- α , IL-4 and IL-27. Notably, low expression of NLRP3 enhanced the inhibitory effect of ANI on the development of HCC xenografts in mice.

Discussion: These findings indicate that ANI suppressed the growth of HCC cells, induced apoptosis and regulated the levels of inflammatory factors by inhibiting NLRP3 inflammasome activation.

Keywords: hepatocellular carcinoma, anisodamine, NOD-like receptor family pyrin domain containing 3, NLRP3, inflammatory factors, anticancer

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Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer, and its morbidity and mortality are second only to lung cancer and colon cancer.^{1,2} Worldwide, there are nearly 700,000 new cases of HCC diagnosed each year and 600,000 deaths.³ In China, the incidence of HCC accounts for about 40%.⁴ Early metastasis and high recurrence rate are important causes for poor prognosis of HCC.^{5,6} Despite the development of

many targeted drugs and therapies, the high cost of treatment, drug side effects and drug resistance have reduced the effective cure rate of HCC.^{7,8} Therefore, there is an urgent need to further develop economical, efficient and low side effect drugs for the treatment of HCC.

Anisodamine (ANI) is an alkaloid extracted from *anisodus* and has a good therapeutic effect on septic shock and morphine addiction.⁹ ANI has also been reported to have a significant anti-inflammatory effect, which can alleviate the damage of kidney and heart by inhibiting the activation of inflammasomes and the expression of inflammatory factors.^{10,11} Similarly, ANI showed an anti-apoptotic effect in myocardial ischemic injury.¹² However, the role of ANI in tumors has not been revealed.

Chronic inflammation plays an important role in the pathogenesis of cancer. The NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome is an important inflammatory complex discovered in recent years, and plays an important role in various diseases, such as atherosclerosis,¹³ diabetes¹⁴ and tumors.^{15,16} NLRP3 inflammasome is composed of NLRP3, ASC and pro-caspase-1. Activation of NLRP3 activates ASC and pro-caspase-1 and promotes the maturation and release of inflammatory cytokines (IL-1 β and IL-18), leading to various diseases. However, in HCC, NLRP3 inflammasome activation is thought to be associated with the occurrence and development of.^{17,18} Notably, ANI alleviated kidney damage by inhibiting the activation of NLRP3 inflammasome and reducing the expression of pro-inflammatory factors.¹¹ However, it is not clear whether ANI can regulate the progression of HCC by regulating the activation of NLRP3 inflammasome.

This study explored the role and molecular mechanism of ANI in HCC xenografts. It was found that ANI suppressed the growth of HCC cells, induced apoptosis and maintained the Th1/Th2 balance by inhibiting NLRP3 inflammasome activation. Collectively, ANI may be a promising potential drug for HCC therapy.

Materials and Methods

Cell Culture

The HCC cell line HepG2 was purchased from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Cells were cultured in DME medium containing 10% FBS (Thermo Fisher Scientific, Guangzhou, China) and 1% penicillin/streptomycin (Life Technology, Carlsbad, USA) at 37°C with 5% CO₂.

Animal Modeling and Administration

Animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals approved by Capital Medical University. BALB/C nude mice (4–5 weeks, weighing 18–20 g) were purchased from the Animal Center of the Capital Medical University. HepG2 cells (1.5×10^7) were subcutaneously injected into BALB/C nude mice. When the diameter of subcutaneous tumor was about 10 mm, the tumor was exfoliated. The isolated tumor tissue ($2 \times 2 \times 2$ mm³) was inoculated subcutaneously in the left subaxillary of the nude mice. When the tumor diameter was about 10 mm, tumor-bearing mice were randomly divided into 9 groups (n = 5): normal group (healthy mice), normal + ANI-200 (healthy mice were subcutaneously injected with 200 mg/kg ANI per day), control group (tumor-bearing mice were subcutaneously injected with normal saline), ANI-10 group (tumor-bearing mice were subcutaneously injected with 10 mg/kg ANI per day), ANI-50 group (tumor-bearing mice were subcutaneously injected with 50 mg/kg ANI per day), ANI-200 group (tumor-bearing mice were subcutaneously injected with 200 mg/kg ANI per day), ANI-200+pcDNA-NLRP3 group (nude mice were subcutaneously injected with HepG2 cells (with pcDNA-NLRP3) and 200 mg/kg ANI) and ANI-200+EV group (nude mice were subcutaneously injected with HepG2 cells (with EV) and 200 mg/kg ANI), sh-NLRP3 group (nude mice were subcutaneously injected with HepG2 cells transfected with sh-NLRP3). The survival curve of mice was drawn. After 30 days, the corresponding indexes were detected and the mice were sacrificed with 5% isoflurane. The transplanted tumor was removed, and the tumor volume was calculated. The transplanted tumor tissues were collected for subsequent study.

Detection of Liver Function Indexes

Mice were treated with ANI for 12 weeks and sacrificed by 5% isoflurane. The serum was collected by centrifugation. Triglyceride (TG), total cholesterol (TC), density lipoprotein (LDL), and high-density lipoprotein (HDL) were measured with the commercial kits according to the manufacturer's instructions (Wako Pure Chemical Industries, Japan).

H&E Staining

After 30 days of ANI treatment, the mice were sacrificed with 5% isoflurane. Liver tissue was removed and fixed with 10% neutral buffered formalin. The tumor tissue was

then dehydrated and embedded in paraffin. Next, the paraffin-embedded tumor tissue was cut into 5 μ m sections and stained with hematoxylin and eosin.

Immunohistochemistry (IHC)

The paraffin-embedded tissue was cut into 5 μ m-thickness sections. IHC staining was performed according to the previous method. Briefly, sections were dewaxed with xylene and hydrated with gradient ethanol. The sections were then repaired with trypsin and incubated with primary antibodies anti-Ki67 (#9027, 1:400, Cell Signaling Technology, USA) and anti-VEGF (ab36844, 1:500, Abcam, Cambridge, UK) at 4°C overnight. The sections were then probed with a secondary antibody. After staining, the sections were dehydrated, clarified with xylene and fixed with resin.

RT-qPCR

Total RNA was isolated from HCC tissues using Trizol (Thermo Fisher Scientific, USA). The RNA was then reverse-transcribed into cDNA using the GoScript Reverse Transcription Kit (Promega Corporation, USA) according to the supplier's instructions, and qPCR analysis was performed using SYBR-green (Takara, Japan). GAPDH was used as the housekeeping gene. The template of the qPCR was cDNA. The relative quantification of gene expression was calculated by $2^{-\Delta\Delta CT}$ method [24]. Primer sequence is as follows:

NLRP3, F 5'-TTCACCAAGGTCGCACCTG-3'; R 5'-A ACTGAACGTTGCAACTTAC-3'.

Ki67, F 5'-TGACTGGCCTACCGACTG-3'; R 5'-A TCAATCCTGCGCGTCGAC-3'.

ASC, F 5'-CATTGCAATGCTACGCGTG-3'; R 5'-CG GACTCGACATGCTAACT-3'.

Caspase 3, F 5'-CGAAGTCTACTGCATGC-3'; R 5'-T CGAACGGCATGCTGACG-3'.

IL-18, F 5'-TGCTACTGAGCTAGCAAC-3'; R 5'-CA TTCAGCTGACGGCTGACT-3'.

IL-1 β F 5'-GGACTGACAATGCTGGCAG-3'; R 5'-GG TCGTAATGCTACCGATC-3'.

Western Blotting

The tissue was ground with liquid nitrogen and lysed with RIPA buffer containing protease inhibitors (Beyotime, Beijing, China). The proteins were then collected by centrifugation, and protein concentration in the lysate was measured using the BCA kit (Takara, Dalian, China). The protein (20 μ g) was then added to the 10% (w/v)

polyacrylamide gel for separation. The separated proteins were then transferred to PVDF membrane (Millipore, Billerica, MA, USA) and blocked with 5% (w/v) skim milk for 30 min. Next, the membrane was hybridized with different primary antibodies (anti-VEGF, ab36844, 1:1000; anti-GAPDH, ab181602, 1:10,000; anti-ASC, ab155970, 1:5000; anti-cleaved caspase 3, ab32042, 1:500; anti-IL-18, ab71495, 1:100; anti-Ki67, ab92742, 1:500; anti-NLRP3, ab214185, 1:500 and anti-IL-1 β , ab9722, 1:200) and then incubated with secondary antibody. All the primary antibodies were purchased from Abcam (Cambridge, UK). Immunoreactive bands were visualized with enhanced chemiluminescence kits (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

Apoptotic cells in HCC tissues were examined using TUNEL staining (Roche Diagnostics, Mannheim, Germany) according to the instructions. After the paraffin-embedded sections were dehydrated with xylene and hydrated with gradient ethanol, the tissue was treated with 2% hydrogen peroxide for 5 min at room temperature to block endogenous peroxidase activity. After washing, the sections were exposed in the dark to a mixture containing terminal deoxynucleotidyl transferase and TUNEL for 1 h. Finally, the cells were counterstained with 0.05% diaminobenzene (DAB) and counted under a microscope (Olympus).

ELISA

Serum was collected and the levels of INF- γ , TNF- α , IL-4 and IL-27 were assayed using an ELISA kit according to the manufacturer's instructions.

Flow Cytometry

Peripheral blood of mice was obtained according to the previous method. The cells were subsequently stained with fluorescently labeled antibodies (CCR5-conjugated APC-eFluor 780, IL-12 β 2R-conjugated PE, CCR4-conjugated APC or CD30-conjugated PE) to detect Th1 and Th2 cells in peripheral blood. All the antibodies were purchased from eBioscience (San Diego, USA). The cells were then immobilized, permeated, stained and observed.

Statistical Analysis

Data are expressed as mean $\pm$ SD. All experiments were repeated three times. Statistical analysis was carried out using one-way ANOVA and post hoc tests via GraphPad Prism software. $P < 0.05$ was considered statistically significant.

Results

ANI Prevented the Development of HCC Xenografts and the Expression of NLRP3 in Mice

First, the study investigated the effect of ANI on tumor growth in HCC xenograft mice. As shown in [Figure 1A](#), ANI significantly inhibited tumor formation in a dose-dependent manner compared to the control group. Besides, ANI dose-dependently reduced the survival rate of HCC xenograft mice ([Figure 1B](#)). Further liver function analysis showed that ANI reduced the levels of TG, TC, HDL and LDL in serum in a dose-dependent manner ([Figure 1C–F](#)), but had no obvious toxic and side effects on healthy mice, demonstrating that ANI was committed to preventing liver dysfunction in HCC xenograft mice. In addition, H&E staining showed that, compared with the normal group and normal+ANI-200 group, the HCC cells in the control group were disordered and accompanied by lymphocyte infiltration. The cytoplasm was stained blue, and the nuclei were deeply stained. However, ANI treatment dose-dependently improved histopathological damage in HCC xenograft mice ([Figure 1G](#)). Notably, qPCR and Western blot analysis showed that, compared with the normal group and normal+ANI-200 group, the expression of NLRP3 in liver tissues of HCC xenograft mice was inhibited in a dose-dependent manner by ANI ([Figure 1H and I](#)). Collectively, these findings indicated that ANI prevented the development of HCC xenografts and the expression of NLRP3 in mice.

NLRP3 Reversed the Inhibitory Effect of ANI on the Development of HCC Xenografts in Mice

To investigate whether NLRP3 is associated with the anticancer effect of ANI, NLRP3 was forcibly overexpressed. As shown in [Figure 2A](#), NLRP3 overexpression significantly reversed the inhibitory effect of ANI on the growth of HCC xenografts, indicating that the high expression of NLRP3 promoted the growth of HCC xenografts. Besides, this study found that NLRP3 overexpression

affected the survival rate of HCC xenograft mice. As shown in [Figure 2B](#), the survival rate of mice in the ANI + NLRP3 group was decreased compared with the ANI group, indicating that NLRP3 overexpression inevitably counteracted the therapeutic effect of ANI on HCC. Further analysis showed that NLRP3 overexpression attenuated the improvement of ANI on liver dysfunction and liver damage ([Figure 2C–G](#)). In summary, these findings indicate that NLRP3 reversed the inhibitory effect of ANI on the development of HCC xenografts in mice.

ANI Suppressed Tumor Cell Growth and Motility and Induced Apoptosis by Inhibiting NLRP3 Expression

Next, this study further explored the potential molecular mechanisms of NLRP3 participating in the anticancer effects of ANI. RT-qPCR and immunohistochemical analysis showed that ANI treatment significantly inhibited the expression of Ki67 and VEGF compared with the control group. However, NLRP3 overexpression reversed the inhibitory effect of ANI on the expression of Ki67 and VEGF, indicating that ANI inhibited the proliferation and motility of tumor cells by inhibiting the expression of NLRP3 ([Figure 3A–D](#)). Secondly, TUNEL staining showed that ANI treatment induced apoptosis of tumor cells compared to the control group. Conversely, the overexpression of NLRP3 prevented ANI-induced apoptosis to some extent. However, the number of apoptotic cells in the ANI + EV group was not significantly changed compared with the ANI-200 group, while increasing compared with the ANI + NLRP3 group ([Figure 3E](#)). Taken together, these findings revealed that ANI suppressed the growth and motility of tumor cells and induced apoptosis by inhibiting the expression of NLRP3.

ANI Inhibited the Activation of NLRP3 Inflammasome

The abnormal activation of inflammasome can cause chronic inflammation and even induce tumors. Therefore, this study investigated whether the anticancer effect of ANI was related to the inactivation of NLRP3 inflammasome. This study found that ANI treatment inhibited the expression of NLRP3 ([Figure 1H and I](#)). Interestingly, the ANI treatment also significantly inhibited the expression of NLRP3 inflammasome-related proteins (ASC, caspase 1, IL-18 and IL-1 β). Notably, protein levels of ASC, caspase 1, IL-18 and IL-1 β were significantly increased after the overexpression of NLRP3, suggesting that the

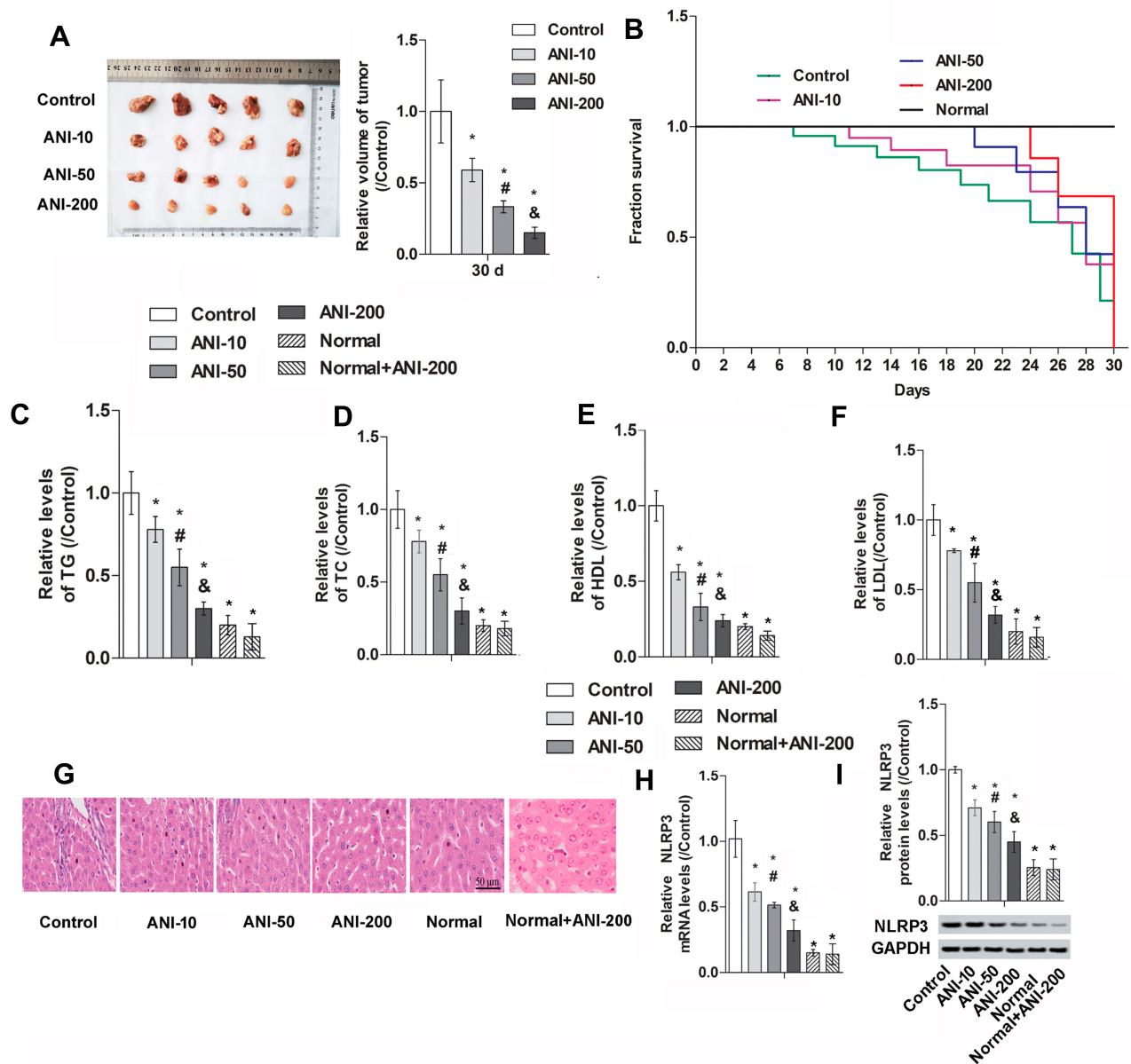


Figure 1 ANI hindered the development of HCC xenografts and the expression of NLRP3 in mice. HepG2 cells (1.5×10^7) were subcutaneously injected into BALB/C nude mice. Then, the subcutaneous tumor inoculated subcutaneously in nude mice to construct HCC xenografts. The mice were randomly divided into six groups ($n = 5$): Control group, ANI-10 group, ANI-50 group and ANI-200 group, normal group and normal+ANI-200 group. (A) The volume of tumors. (B) Survival curve. (C–F) The levels of TG, TC, HDL and LDL. (* $p < 0.05$ vs Control group, # $p < 0.05$ vs ANI-10 group, & $p < 0.05$ vs ANI-50 group) (G) Pathological damage of the liver is detected by H&E staining. (H) The mRNA level of NLRP3 was measured by RT-qPCR. (* $p < 0.05$ vs control group, # $p < 0.05$ vs ANI-10 group, & $p < 0.05$ vs ANI-50 group) I. The protein level of NLRP3 was measured by Western blotting. (* $p < 0.05$ vs control group, # $p < 0.05$ vs ANI-10 group, & $p < 0.05$ vs ANI-50 group).

anticancer effect of ANI could be achieved by inhibiting the expression of NLRP3 and further inhibiting the activation of NLRP3 inflammasome (Figure 4A–H).

ANI Regulated the Levels of Inflammatory Cytokines by Inhibiting NLRP3 Expression

The level of inflammatory factors is related to the malignancy of the tumor. Therefore, this study investigated

whether the anti-tumor effect of ANI is involved in the regulation of inflammatory factor levels. First, the levels of INF- γ , TNF- α , IL-4 and IL-27 were detected by ELISA. As shown in Figure 5A, ANI treatment significantly increased the levels of INF- γ and IL-27 and decreased the levels of TNF- α and IL-4 compared to the control group. However, the overexpression of NLRP3 reduced the levels of INF- γ and IL-27, while increasing the levels

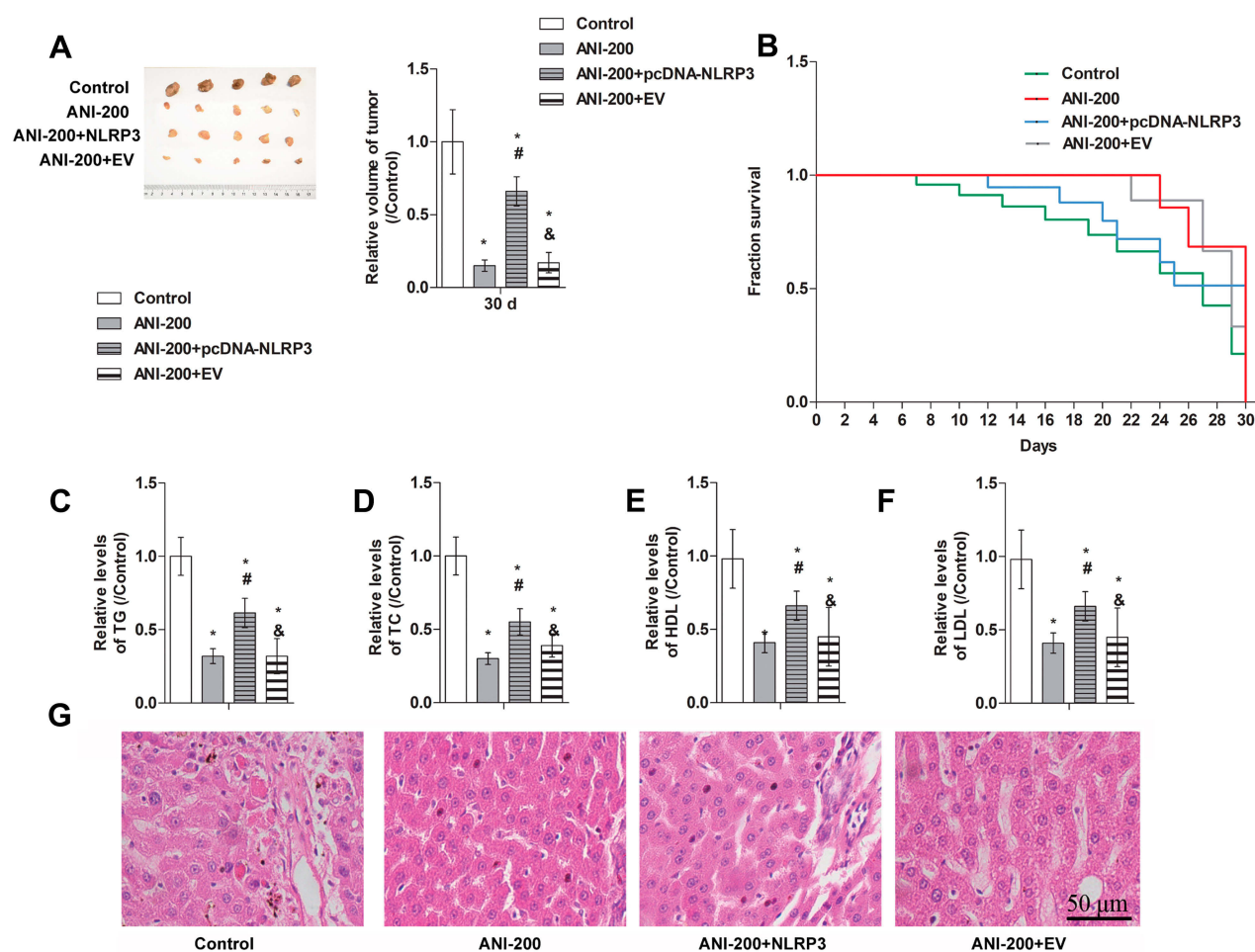


Figure 2 NLRP3 reversed the inhibitory effect of ANI on the development of HCC xenografts in mice. The mice were randomly divided into four groups ($n = 5$): Control group, ANI-200 group, ANI-200+pcDNA-NLRP3 group and ANI-200+EV group. (A) The volume of tumors. (B) Survival curve. (C–F) The levels of TG, TC, HDL and LDL. (G) Pathological damage of the liver is detected by H&E staining. (* $p < 0.05$ vs control group, # $p < 0.05$ vs ANI-200 group, & $p < 0.05$ vs ANI-200+pcDNA-NLRP3 group).

of TNF- α and IL-4, suggesting that ANI regulated inflammatory factor levels by inhibiting NLRP3 expression (Figure 5A–D). In addition, Th1 cells and Th2 cells were sorted by flow cytometry, respectively. As shown in Figure 5E–F, compared with the control group, the number of Th1 cells with CCR5 and IL-12 β 2R double-positive was significantly reduced in the ANI-200 group, while the overexpression of NLRP3 reversed the reduction in the number of Th1 cells induced by ANI. Similarly, compared with the control group, the number of CD30 and CCR4-positive Th2 cells in the ANI-200 group was significantly reduced, while overexpression of NLRP3 reversed the reduction in the number of Th2 cells induced by ANI. These findings indicate that ANI may achieve the anti-inflammatory and anticancer effects of ANI by inhibiting the expression of NLRP3.

Low Expression of NLRP3 Enhanced the Inhibitory Effect of ANI on the Development of HCC Xenografts in Mice

This study explored the effect of low expression of NLRP3 on the development of HCC xenografts in mice. After knocking down NLRP3 with shRNA, the expression of NLRP3 was significantly suppressed. ANI treatment further reduced the level of NLRP3 (Figure 6A). Besides, low expression of NLRP3 inhibited tumor growth and histopathological damage (Figure 6B and C). In addition, the low expression of NLRP3 also decreased the levels of TC, HDL and LDL in serum, and inhibited the expression of Ki67 and VEGF (Figure 6D–I). What's more, the low expression of NLRP3 inhibited the activation of inflammasome (Figure 6J). Further analysis showed

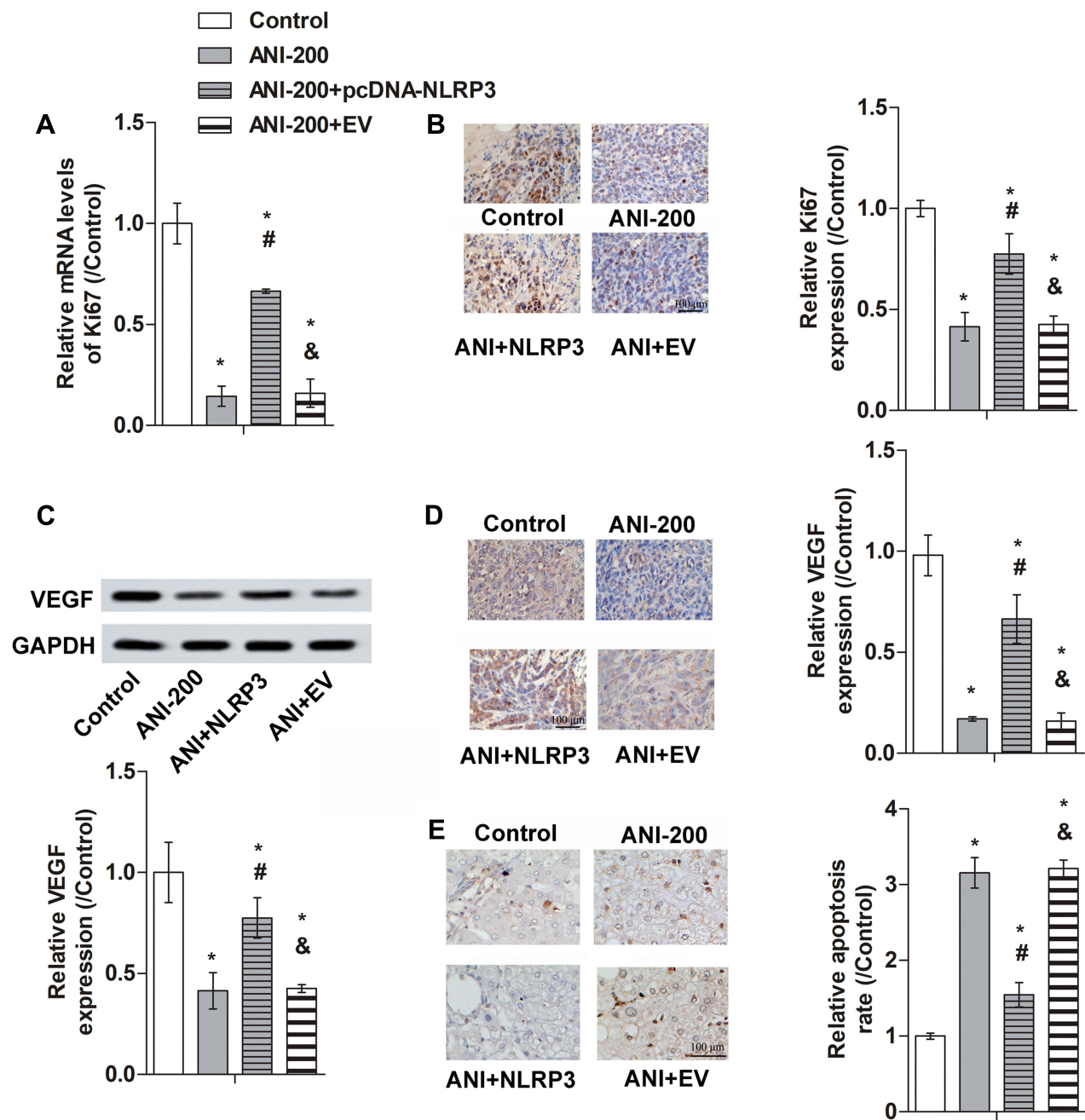


Figure 3 ANI suppressed tumor cell growth and motility of and induced apoptosis by inhibiting NLRP3 expression. The mice were randomly divided into four groups ($n = 5$): Control group, ANI-200 group, ANI-200+pcDNA-NLRP3 group and ANI-200+EV group. (A) The mRNA level of Ki67 was measured by RT-qPCR. (B) The expression of Ki67 in tumor tissues was measured by Immunohistochemistry. (C) The mRNA level of VEGF was measured by RT-qPCR. (D) The expression of VEGF in tumor tissues was measured by Immunohistochemistry. (E) Apoptosis was measured by TUNEL staining. (* $p < 0.05$ vs control group, # $p < 0.05$ vs ANI-200 group, & $p < 0.05$ vs ANI-200+pcDNA-NLRP3 group).

that the low expression of NLRP3 increased the levels of INF- γ and IL-27 and decreased the levels of IL-4 and TNF- α (Figure 6K and L). Notably, ANI treatment further enhanced the inhibitory effect of low expression of NLRP3 on the development of HCC xenografts in mice. In summary, low expression of NLRP3 enhanced the

inhibitory effect of ANI on the development of HCC xenografts in mice.

Discussion

As a primary liver cancer with a 5-year incidence of over 70%,¹⁹ HCC usually occurs in people with hepatitis

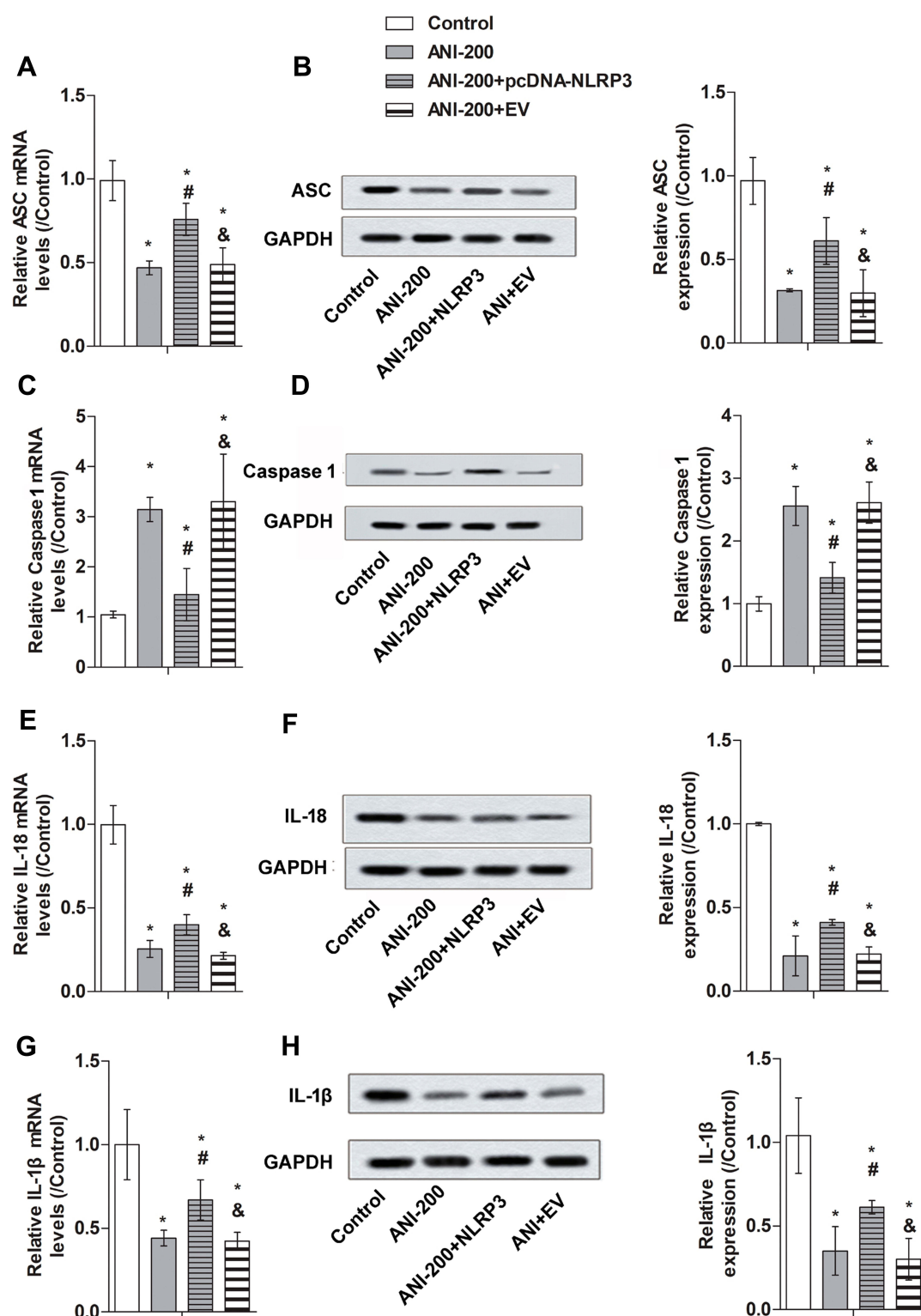


Figure 4 ANI inhibited NLRP3 inflammasome activation. The mice were randomly divided into four groups ($n = 5$): Control group, ANI-200 group, ANI-200+pcDNA-NLRP3 group and ANI-200+EV group. (A) The mRNA level of ASC was measured by RT-qPCR. (B) The protein level of ASC was measured by Western blotting. (C) The mRNA level of caspase 1 was measured by RT-qPCR. (D) The protein level of caspase 1 was measured by Western blotting. (E) The mRNA level of IL-18 was measured by RT-qPCR. (F) The protein level of IL-18 was measured by Western blotting. (G) The mRNA level of IL-1 β was measured by RT-qPCR. (H) The protein level of IL-1 β was measured by Western blotting. (* $p < 0.05$ vs control group, # $p < 0.05$ vs ANI-200 group, & $p < 0.05$ vs ANI-200+pcDNA-NLRP3 group).

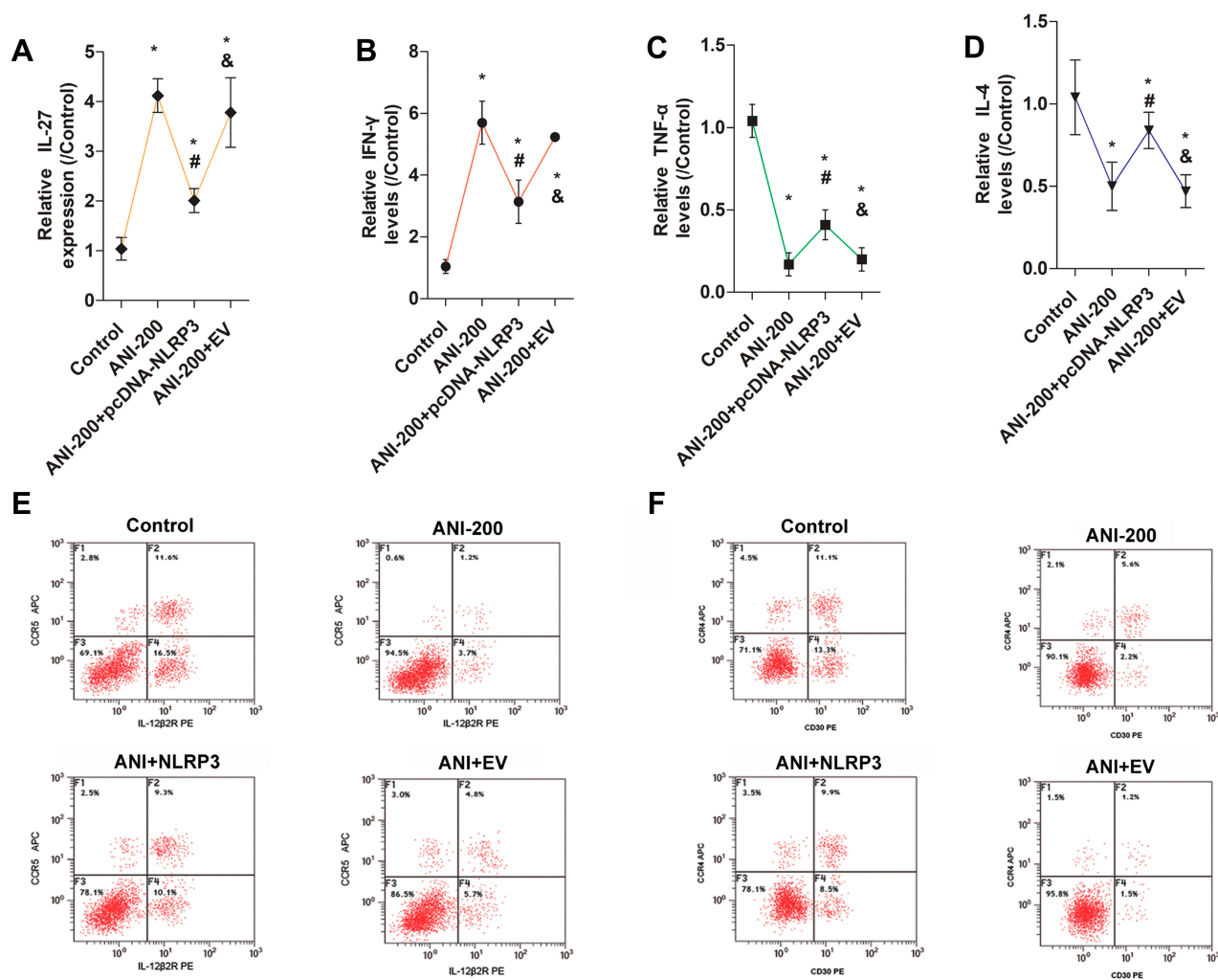


Figure 5 ANI regulates the levels of inflammatory cytokines by inhibiting NLRP3 expression. The mice were randomly divided into four groups ($n = 5$): Control group, ANI-200 group, ANI-200+pcDNA-NLRP3 group and ANI-200+EV group. (A–D) The levels of INF- γ , TNF- α , IL-4 and IL-27 were measured by ELISA. (E–F) Th1 and Th2 cell subsets were sorted by flow cytometry. (* $p < 0.05$ vs control group, # $p < 0.05$ vs ANI-200 group, & $p < 0.05$ vs ANI-200+pcDNA-NLRP3 group).

B and C or with a family history of liver cancer.^{20,21} Chronic alcohol abuse, aflatoxin intake and a long-term high-fat diet can also contribute to HCC.²² Currently, the treatment of HCC includes surgery, radiotherapy and chemotherapy, radiofrequency/microwave ablation and liver transplantation.²³ However, resistance to chemoradiotherapy and the uselessness of liver transplantation still limit the clinical treatment of HCC.²⁴ Medicinal plants have been used to treat liver diseases or to maintain a healthy liver in the last few years.²⁵ In the study of liver cancer, the establishment of animal models is very important. A near-human tumor that studies the biology of human malignancies and screens for anticancer drugs. Therefore, in this study, the anticancer effect of ANI was studied by constructing a xenograft mouse model of HCC. It was found that ANI significantly inhibited the growth of HCC

xenografts and promoted apoptosis. Further mechanistic analysis indicated that the anticancer effect of ANI may be achieved by inhibiting the activation of NLRP3 inflammasome. Overall, ANI may be a promising cancer drug for HCC.

As a belladonna alkaloid, ANI is involved in the treatment of various diseases by regulating oxidative stress, inflammation and apoptosis. It has been found that ANI improved rhabdomyolysis-induced renal insufficiency. The specific mechanism may be by reducing the expression of ER stress markers IRE-1 α , CHOP and ATF4, and inhibiting the activation of TXNIP and NLRP3 inflammasome.^{11,26} Besides, ANI protected against myocardial ischemia/reperfusion (I/R) injury. Further mechanism analysis showed that the role of ANI in myocardial I/R may be by the inhibition of oxidative stress, inflammatory factors and cardiomyocyte apoptosis via

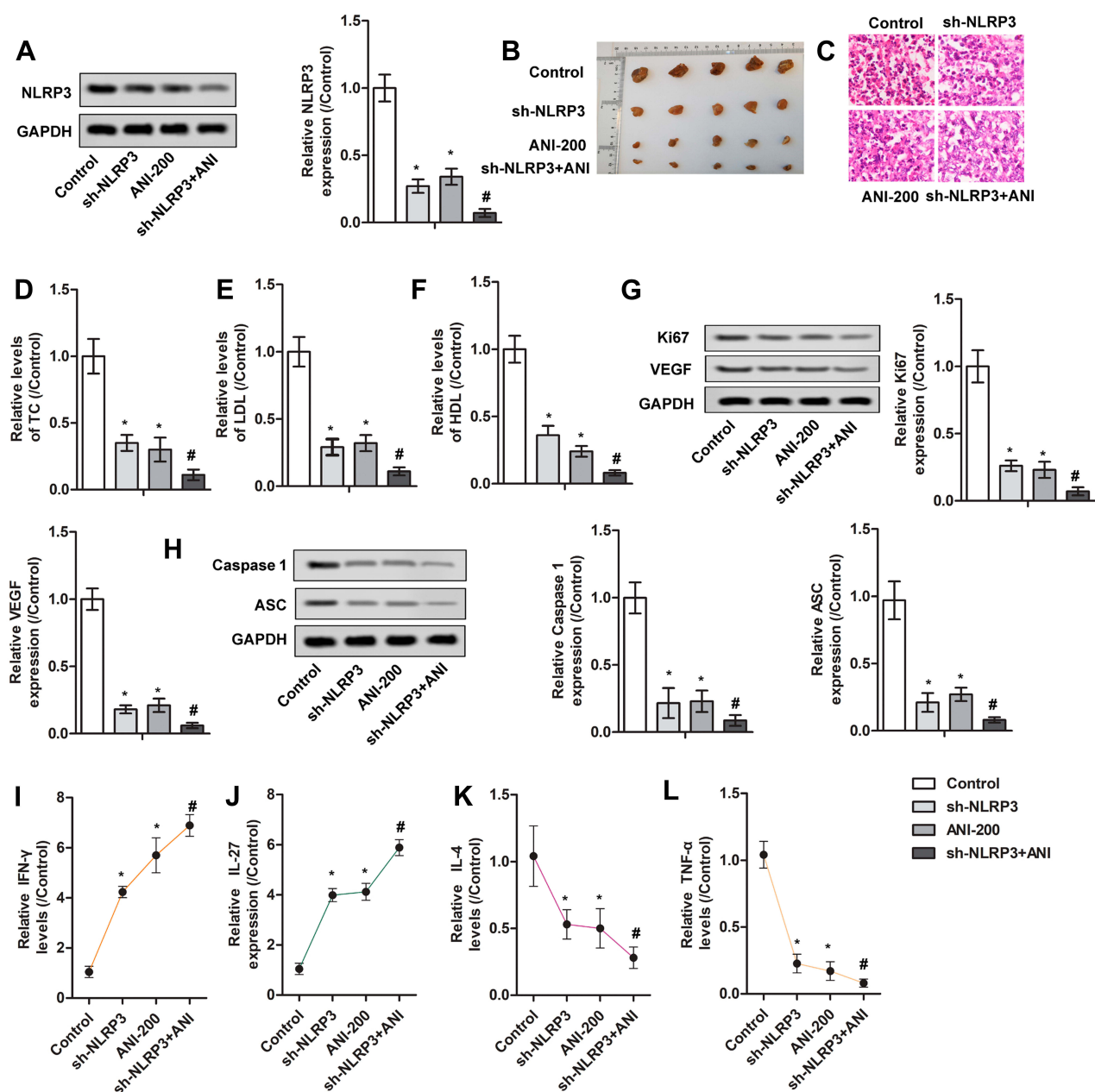


Figure 6 Low expression of NLRP3 enhanced the inhibitory effect of ANI on the development of HCC xenografts in mice. The mice were randomly divided into four groups (n = 5): Control group, sh-NLRP3 group, ANI-200 group and ANI-200+ sh-NLRP3 group. (A) The protein level of NLRP3 was measured by Western blotting. (B) Tumor image. (C) Pathological damage of the liver is detected by H&E staining. (D–F) The levels of TC, HDL and LDL. (G–I) The protein level of Ki67 and VEGF was measured by Western blotting. (J) The protein level of caspase 1 and ASC was measured by Western blotting. (K–L) The levels of INF- γ , TNF- α , IL-4 and IL-27 were measured by ELISA (*p < 0.05 vs control group, #p < 0.05 vs ANI-200 group).

down-regulating the expression of caspase 3 and Bax.^{10,12} In addition, the combination of ANI and neostigmine improved acute fatal crush syndrome by activating α 7 nicotine acetylcholine receptor (α 7nAChR)-dependent JAK2-STAT3 pathway.²⁷ Surprisingly, the anti-inflammatory effect of ANI was also found to be applicable to tumor progression in this study. Specifically, ANI treatment significantly inhibited the activation of NLRP3 inflammasome and prevented the release

of IL-18 and IL-1 β . This anti-inflammatory effect further inhibited the growth of HCC, induced apoptosis and finally inhibited the formation of tumor. Taken together, these results suggest that the anti-inflammatory effect of ANI may further inhibit the occurrence of HCC xenografts.

Activation of NLRP3 inflammasome is closely related to tumorigenesis. As a core part of the inflammatory response, inflammasome plays a dual role in tumorigenesis.

Activation of the NLRP3 inflammasome promoted the release of IL-1 and IL-18 and then accelerated tumor growth and metastasis. Conversely, when the tissue is damaged to a certain extent, the activation of inflammasome suppressed tumorigenesis by promoting the apoptosis of tumor cells. Lee et al found that the inhibition of NLRP3 inflammasome inhibited melanoma cell metastasis by inhibiting the secretion of mature IL-1 β .¹⁶ In HCC, studies have shown that 17 β -estradiol (E2)-induced NLRP3 inflammasome activation inhibited the occurrence of HCC by triggering apoptotic cell death and inhibiting protective autophagy.¹⁷ Studies have shown that NLRP3 can be targeted by miR-223, and the expression of miR-223 was downregulated in HCC tissues, while the expression of NLRP3 was overexpressed. The high expression of NLRP3 promoted the release of IL-1 β and IL-18, thus inducing the progression of HCC.²⁸ On this basis, this study found that ANI-mediated inactivation of NLRP3 inflammasome further inhibited the activation of caspase 1 and the release of IL-1 β and IL-18, thereby inhibiting the occurrence of HCC. These results are consistent with the findings of Wan et al²⁸ and Fan et al.²⁹

Currently, the relationship between Th1/Th2 deviation and malignant tumor has become a research hotspot.³⁰ The reversal of Th2 to Th1 provided a new approach for immunotherapy of tumors. INF- γ and TNF- α are Th1 cell markers, while IL-4 and IL-27 are Th2 cell markers.³¹ Levels of INF- γ , TNF- α , IL-4 and IL-27 can be used to assess the anti-tumor effect of human T cells. Lymph node metastasis was rare in cancer patients with INF- γ and TNF- α levels higher than IL-4 and IL-27.³² Therefore, in this study, ELISA and flow cytometry analysis were used to detect the levels of INF- γ , TNF- α , IL-4 and IL-27 in peripheral blood. The results showed that the levels of INF- γ and IL-27 in peripheral blood of HCC mice treated with ANI were increased compared with the control group. After the overexpression of NLRP3, the levels of INF- γ and IL-27 levels were reduced, while the levels of IL-4 and TNF- α were increased, indicating that ANI regulated the levels of inflammatory cytokines by inhibiting NLRP3 expression. However, this study revealed the regulatory effect of ANI on Th1 and Th2-related inflammatory factors. However, whether ANI maintains the Th1/Th2 balance by inhibiting the activation of NLRP3 inflammasome still needs more evidence to confirm. More evidence on the balance of Th1/Th2 will be explored in more depth in future studies.

Conclusion

In conclusion, current studies have found that ANI can significantly inhibit the growth and metastasis of HCC xenografts and induce apoptosis. Besides, ANI reversed the levels of INF- γ , TNF- α , IL-4 and IL-27. Further mechanism analysis showed that the anticancer effect of ANI on HCC xenografts may be achieved by inhibiting the activation of NLRP3 inflammasome and reducing the production of IL-18 and IL-1 β . Totally, ANI may be a promising potential drug for HCC therapy.

Disclosure

The authors report no conflicts of interest regarding the publication of this article.

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