

NLRP3 Promotes Immune Escape by Regulating Immune Checkpoints: A Pan-cancer Analysis

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Abstract

NLRP3 plays a pathogenic role in tumorigenesis by regulating innate and acquired immunity, apoptosis, differentiation, and intestinal microbes in tumors. In different tumors, NLRP3 plays different roles, and its mechanism is complex. Our research aimed to comprehensively investigate the role of NLRP3 in pan-cancers based on multi-omics data in the TCGA database. We found the expression of NLRP3 was changed in tumors compared with paired non-tumor specimens. Most types of tumors showed increased expression of NLRP3. Among them, the overexpressed NLRP3 in liver hepatocellular carcinoma (LIHC) and ovarian cancer (OV) indicated worse overall survival (OS). Further analysis also confirmed overexpressed NLRP3 in colon cancer (COAD) indicated a high probability of microsatellite instability (MSI) and low tumor mutational burden (TMB), which indicated a better response to immune checkpoint inhibitors (ICIs). We also analyzed the association between NLRP3, immune infiltration, and immune checkpoints. Interestingly, overexpression of NLRP3 was closely related to high infiltration of immune cells (T cells, B cells, etc.) and overexpressed immune checkpoints (PD-1, PD-L1, LAG3, etc.). These results demonstrated NLRP3 promoted immune escape in cancers. Finally, we investigated the expression of various immune checkpoints by treating NLRP3 inhibitor MCC950 during the co-culture of peripheral blood mononuclear cells (PBMC) and LIHC cell line Hep3B. We found MCC950 significantly repressed the expression of PD-L1 and LAG3, and promoted the apoptosis rate of Hep3B. In conclusion, our research comprehensive demonstrated the role of NLRP3 in pan-cancer, especially in LIHC. We confirmed inhibition of NLRP3 promoting the immune killing effect to cancer cells by repressing the expression of immune checkpoints.

1. Introduction

Inflammation and persistent infection may contribute to various types of cancers¹. It has been confirmed that inflammation plays key role in cancer initiation, development, progression, angiogenesis, and invasion². Inflammation may be associated with a series of cancer regulatory pathways, including hypoxia, chemotherapy, radiotherapy, or immune attack. As an important component of innate immunity, NLRP3 inflammasome plays an important role in the body's immune response. A large amount of evidence shows that inflammasomes play an important role in pathogen infection and autoimmune diseases. However, their role in tumor progression remains unclear.

Inflammasome induces pro-caspase-1 activation and inflammatory cytokine maturation in the innate immune system. In addition to being related to autoimmune diseases, the overexpression of IL-1 β may also lead to the occurrence of tumors. Several inflammasomes, including NLRP3, NLRP6, NLRC4, NLRP1 and AIM2, may play a pathogenic role in tumorigenesis by regulating innate and acquired immunity, apoptosis, differentiation and intestinal microbes. The role of NLRP3 in tumor progression is very complex. Studies have shown that in various cancers, NLRP3 has both cancer-promoting and anti-tumor effects^{3,4}. For example, NLRP3 inflammasome protects against colon cancer attributed to the effector function of caspase-1 to mediate secretion of IL18 and inhibit liver metastatic growth by enhancing NK

cell tumoricidal action^{5,6}. However, the NLRP3-dependent release of IL-1 β induces immune cells, primarily CD4 + T cells, to express and release IL-22, which has been associated with the aggressive growth of multiple cancers including breast, gastric, lung, and skin cancers⁷.

Although NLRP3 has been reported in certain cancers, these studies focused on a single or a few types of cancer; there have been no studies comparing multiple types of cancer. We carried out a pan-cancer analysis of the NLRP3 inflammasome across various cancer types based on the TCGA datasets. We also investigated the association between NLRP3 and immune infiltration, immune escape, immune checkpoints, DNA methyltransferase (DNMT) levels, and mismatch repair (MMR). These results provide important insights into the roles of NLRP3 in cancer immunology.

2. Materials And Methods

2.1 Data Acquisition

We identified and downloaded the Processed level 3 RNA sequencing data and corresponding clinical annotations from the TCGA database which comprises over 20,000 samples from 33 types of cancer and corresponding non-carcinoma samples.

2.2 The expression and survival analysis of NLRP3 in pan-cancer

The data of NLRP3 transcript for 33 cancer types and adjacent non-carcinoma tissues were extracted from TCGA and used to compare the expression of NLRP3 in pan-cancer and normal tissues. Kaplan-Meier analysis was used to carry out the association of NLRP3 and OS in pan-cancer. Cox analysis was used to calculate correlations between gene expression and OS, progression-free interval (PFS), disease-specific survival (DSS), and disease-free survival (DFS).

2.3 Analysis of MMR and DNA methylation

Association of NLRP3 and immune checkpoints was investigated in pan-cancer patients and LIHC patients. MMR, is a DNA repair mechanism in normal cells that corrects DNA replication errors. Gene mutation frequency may be increased in cancer cells as a result of downregulation of MMR genes or defective MMR⁸. We analyzed the correlation between MMR gene (MutL homolog [MLH]1, MutS homolog [MSH]2, MSH6, postmeiotic segregation increased [PMS]2, and epithelial cell adhesion molecule [EPCAM]) and NLRP3 expression levels. DNA methylation has been implicated in tumorigenesis and cancer progression. As DNMT1, DNMT2, DNMT3A, and DNMT3B are the major enzymes involved in DNA methylation⁹, we analyzed the correlation between their expression levels and those of NLRP3.

2.4 Analysis of immune infiltration

Tumor Immune Estimation Resource (TIMER; <https://cistrome.shinyapps.io/timer/>) allows systemic analysis of immune infiltrates in different cancer types using a deconvolution statistical approach to infer

tumor-infiltrating lymphocyte counts based on gene expression data¹⁰. Using the TIMER algorithm, the associations between NLRP3 levels and the lymphocytes in the tumor microenvironment were examined. Estimation of Stromal and Immune Cells in Malignant Tumor Tissues Using Expression Data (ESTIMATE) uses gene expression profiles to predict the purity of a tumor based on the infiltration of stromal cells/immunocytes¹¹. The ESTIMATE algorithm yields 3 scores based on GSEA of single samples, including (1) stromal score, which indicates the infiltration of stromal cells ; (2) immune score, which describes the degree of immune infiltration; and (3) estimate score, which reflects impurity degree of tumors. We used the algorithm to estimate both immune and stromal scores for a variety of tumor tissues and evaluated the associations between the scores and NLRP3 levels.

2.5 Cell Culture and cell proliferation assay

Human hepatocellular cell line Hep3B and Huh7 were both acquired from the University of Colorado Cancer Center Cell Bank and cultured in DMEM medium, supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ atmosphere.

For cell proliferation assay, 2000 cells per well were seeded into 96 well plates (5 multiple wells were set up). 10 µl CCK-8 solution was added into each well at 1, 2 and 3 days, and incubated for 2 hours. The absorbance values of each well were measured at 450 nm (OD450).

2.6 Cell invasion assays

10⁴ cells suspended in 100µl culture medium (without FBS) were seeded in the upper chamber of transwell plates (BD Biosciences, Bedford, MA, USA). 600µl culture medium (with 10%FBS) were added in the bottom chamber. The filters (Corning Inc., USA) were coated with 50 µL Matrigel (1:8 dilution; BD Biosciences). After 24h, the chambers were fixed by 4% paraformaldehyde for 30 min and then stained by 0.1% crystal violet for additionally 30 min. Finally, at least five fields of chambers were captured and cell number was counted.

2.7 Co-culture of PBMC and Hep3B

Peripheral blood mononuclear cells (PBMC) were extracted from healthy volunteers by density gradient centrifugation in a lymphocyte separation medium (MP Biomedicals, Irvine, CA, USA). T cells in PBMC were activated and expanded with CD3, CD28 antibody, and 10 ng/ml IL-2 (Thermo Fisher Scientific, Waltham, MA, USA), and then co-cultured with Hep3B cells at a ratio of 10:1 in the presence of a fluorescent caspase 3 substrate (BD Biosciences, San Jose, CA, USA).

After 48h, PBMC and Hep3B were collected separately. The expression of immune checkpoints and apoptosis rate were measured by flow cytometry (BD FACSCelesta). All antibodies were purchased from BD Biosciences, including CD45-APC-CY7, CD3-PERCP-CY5.5, CD8-PE-CY7, PD1-APC, TIM3-BV650, LAG3-BV421, CTLA4-BV786, Caspase3-PE, and PDL1-APC antibodies.

2.8 Western Blotting Analysis

The cells of each group were digested and lysed by 100ul RIPA lysate. After completely lysed, the lysate were centrifuged at 4 °C for 15 minutes. The supernatants were collected as the total protein extract. Then, the BCA assay was performed to quantify the proteins (Thermo Fisher Scientific, Waltham, MA, USA). Then, 20µg proteins were loaded and separated by 10% SDS-PAGE gels. The proteins were then transferred to PVDF membranes (0.45 mm, Merck Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% bovine albumin for 1 h at room temperature and then incubated with NLRP3 and GAPDH rabbit polyclonal antibodies (1:1000, Abcam, UK) at 4°C overnight. The secondary antibodies were used at a 1:4000 dilution and were incubated for 1 h at room temperature. Finally, the bands were visualized by ECL reagents (Merck Millipore).

2.9 Statistical Analysis

IBM SPSS 19.0 software was used for statistical analysis of all experimental data, and all data were expressed as mean ± sd. R software and Graphpad Prism version 7.0 software was used to visualize the statistical results. The Wilcox log-rank test was used to assess changes in the sum of gene expression z-scores of cancer tissues compared to adjacent normal tissues. Survival data was analyzed with Kaplan-Meier curves, the log-rank test, and Cox proportional hazards regression model. Spearman's or Pearson's test was performed for correlation analysis. T-test was used for comparison between the two groups. One-way ANOVA analysis was used for comparison between the multiple groups, and LSD-t test was used for pairwise comparison within the group. $P < 0.05$ for the difference was considered as statistically significant.

3. Results

3.1 The expression and survival analysis of NLRP3 in pan-cancer

First of all, we extracted the expression of NLRP3 in pan-cancer based on TCGA-database. We found NLRP3 was overexpressed in CHOL, COAD, ESCA, GBM, HNSC, KIRC, KIRP, LAML, LGG, LIHC, OV, PAAD, SKCM, STAD, TGCT, THCA, UCS, but decreased in ACC, BLCA, LUAD, LUSC, READ, UCEC compared with paired normal specimens (Fig. 1A). Subsequently, we investigated the association of NLRP3 and survival in pan-cancer via Kaplan-Meier analysis. Higher expression of NLRP3 indicated worser overall survival (OS) in LIHC, OV, and TGCT patients (Fig. 1B). Meanwhile, we also investigated the association of NLRP3 and OS, progression-free interval (PFS), disease-specific survival (DSS), and disease-free survival (DFS) via Cox analysis respectively (Fig. 1C). Higher expression of NLRP3 indicated worser PFS in DLBC and GBM patients, worser DSS in DLBC, KICH, and TGCT patients, worser DFS in HNSC patients. In general, the overexpression of NLRP3 indicated worser survival in various cancers.

3.2 The association between NLRP3 and TMB, MMR.

Tumor mutational burden (TMB) reflects the number of mutations contained in tumor cells and is a quantifiable biomarker. Previous report had demonstrated patients with high TMB showed satisfied

response to treatment of ICIs¹². We analyzed the relationship between gene expression and TMB as follows, using Spearman's rank correlation coefficient. The expression of NLRP3 showed positive correlation with TMB in THYM, SARC, and COAD; whereas showed negative correlation with TMB in UVM, THCA, STAD, PRAD, LUSC, LUAD, LIHC, HNSC, and DLBC (Fig. 2A). It indicated high expression of NLRP3 was associated with high TMB in most cancers, thus showed better response to treatment of ICIs. Microsatellite instability (MSI) refers to any change in the length of a microsatellite caused by the insertion or deletion of a repeat unit in a tumor compared with normal tissues, and the appearance of new microsatellite alleles genetic phenomenon. Previous studies also confirmed MSI patients showed stronger resistance to chemotherapy¹³. We determined higher expression of NLRP3 showed negative correlation with MSI in UCEC, TGCT, SKCM, LUSC, HNSC and DLBC; whereas showed positive correlation with MSI only in COAD (Fig. 2B). Mutation of mismatch repair system (MMR) caused DNA replication errors to be unable to be repaired, which will lead to higher somatic mutations. We evaluated the correlation of NLRP3 and the mutation of five MMR genes: MLH1, MSH2, MSH6, PMS2, EPCAM. We found high expression of NLRP3 was closely related to the mutation of MMR in various cancers, including KICH, KIRC, LIHC, PAAD, PRAD, SKCM and UVM (Fig. 2C). DNA methylation can cause changes in chromatin structure, DNA conformation, DNA stability and the way that DNA interacts with proteins, thereby controlling gene expression. we analyzed the correlation between NLRP3 and the four methyltransferases (DNMT1: red, DNMT2: blue, DNMT3A: green, DNMT3B: purple). We found a strong co-expression of NLRP3 and methyltransferases in various cancers, including LIHC, LAML, KIRP, KIRC, KICH, BRCA, BLCA, UVM, TGCT, SARC, PRAD and PAAD (Fig. 2D).

3.3 The association between NLRP3 and immune infiltration.

As NLRP3 was closely related to inflammation, we investigated the association of NLRP3 and immune infiltration (Fig. 2A). Interestingly, NLRP3 was strongly associated with the infiltration of the main lymphocytes in the tumor microenvironment (TME), including B cell, CD4 + T cells, CD8 + T cells, neutrophils, and dendritic cells (DCs). Subsequently, we further assessed the association of NLRP3 and TME via the ESTIMATE algorithm (Fig. 2B). Higher expression of NLRP3 showed a strong correlation with stromal score (infiltration of stromal cells), immune score (immune infiltration), and Estimate score (impurity degree of tumors). These results suggested NLRP3 promoted the immune infiltration of tumors.

3.4 The association between NLRP3 and immune checkpoints.

We examined the association between immune checkpoints and NLRP3 expression in pan-cancer and found a significantly positive correlation between NLRP3 and various immune checkpoints (LAG3, ICOS, CTLA4, TIM3, PD-1, PD-L2, PD-L1, and TIGIT, Fig. 4A) in almost all types of cancers. Here we further showed the correlation scatter diagram of NLRP3 and multiple immune checkpoints in LIHC, which has a strong correlation (Fig. 4B).

3.5 Inhibition of NLRP3 showed no effect to the cell proliferation and invasion of LIHC.

Based on all our described above, NLRP3 played key roles in various cancers, especially in LIHC. Firstly, we investigated whether NLRP3 directly affects the proliferation and invasion of LIHC. NLRP3 inhibitor MCC950 was adopted to repress the expression of NLRP3 in two LIHC cell lines Hep3B and Huh7. MCC950 significantly repressed the expression of NLRP3 from 0.02uM to 0.5uM (Fig. 5A). However, the proliferation ability of neither LIHC cells was not repressed by MCC850 (from 0.02uM to 0.5uM). Similarly, a high concentration of MCC950 (0.5uM) also showed no effect on the invasion ability of LIHC cell lines. These results suggested NLRP3 did not directly affect tumor proliferation and invasion ability.

3.6 Inhibition of NLRP3 repressed immune escape by inhibiting LAG-3 and PD-L1.

As described above, NLRP3 showed significantly positive co-expression with various immune checkpoints. We further suggested NLRP3 plays key roles in immune escape by regulating the expression of immune checkpoints. MCC950 was added in the medium during co-culture of PBMC and Hep3B (10:1 ratio). We found MCC950 significantly repressed the expression of LAG-3 in T cells (Fig. 6A). Meanwhile, MCC950 significantly repressed the expression of PD-L1 in Hep3B, and this repression was concentration-dependent (Fig. 6B). Finally, we investigated to apoptosis rate of Hep3B induced by T cells killing effect. As expected, 0.02uM MCC950 significantly promoted the apoptosis rate of Hep3B during co-culture with PBMC (Fig. 6C).

4. Discussion

Inflammasome is a multimeric protein platform that induces pro-caspase-1 activation and inflammatory cytokine maturation in the innate immune system. Several inflammasomes, including NLRP3, NLRP6, NLRC4, NLRP1 and AIM2, may play a pathogenic role in tumorigenesis by regulating innate and acquired immunity, apoptosis, differentiation and intestinal microbes. The role of NLRP3 in tumor progression is very complex¹⁴. Studies have shown that in various cancers, NLRP3 has both a cancer-promoting effect and an anti-tumor effect¹⁵. To comprehensively demonstrated the role of NLRP3 in cancers, our study analyzed multi-omics data derived from 33 types of cancers based on the TCGA database.

Firstly, there was a significant difference in the expression of various types of cancers compared with paired normal tissues. Combined with further survival analysis, we confirmed overexpression of NLRP3 in LIHC and OV showed strong association with OS. Other types of tumors (DLBC, KICH, HNSC and GBM) also showed close relationship with PFS, DFS and DSS. These results tallied with previous studies, and indicated NLRP3 play key roles in tumor progression¹⁶.

Furthermore, we investigated the correlation between NLRP3 and MSI. At present, MSI detection and research are mostly carried out in COAD. Previous research has confirmed COAD-MSI patients have a better prognosis, higher survival rate compared with microsatellite stability (MSS) patients, but they cannot benefit from 5-FU chemotherapy¹⁷. Our research confirmed overexpression of NLRP3 in COAD was closely related to MSI in COAD. TMB was another index for predicting prognosis and drug response. Tumors with highly non-synonymous TMB express a large number of abnormal proteins, which are

recognized by the immune system as neoantigens. Therefore, ICIs are more likely to recognize TMB induced new antigens, achieving the effect of attacking and killing tumors¹⁸. Our research confirmed overexpression of NLRP3 was closely related to high TMB. In general, overexpression of NLRP3 indicated high probability of MSI (resistance to chemotherapy) and high TMB (sensitivity to ICIs). In another word, high expression of NLRP3 in COAD preferred treatment of ICIs to chemotherapy (5-FU, etc.). Furthermore, we also demonstrated high expression of NLRP3 was closely related to mutations of MMR genes and high expression of DNA methyltransferases. This result suggested NLRP3 was associated with the instability of cancer genome.

Due to the close relationship between NLRP3 and immune regulation, we further investigated the role of NLRP3 in tumor immunology. Interestingly, overexpression of NLRP3 was closely related to high infiltration of various types of immune cells, including T cells, B cells, and so on. This result indicated NLRP3 activated tumor immune response. In general, activated tumor immune response indicated killing effect on the tumor, but this contradicted the survival analysis (Overexpression of NLRP3 indicated worse OS in LIHC and OV). We suggested it may be due to immune escape caused by overexpression of immune checkpoints¹⁹. Therefore, we further analyzed the correlation between NLRP3 and different immune checkpoints. Interestingly, NLRP3 was significantly co-expressed of various immune checkpoints. Subsequently, we investigated the expression of several main immune checkpoints with treating NLRP3 inhibitor. As expect, PD-L1 and LAG3 were both significantly repressed by NLRP3 inhibitor, accompanied with obvious apoptosis of tumor cells. This result confirmed NLRP3 regulated the expression of immune checkpoints, especially PD-L1 and LGA3. Previous study had confirmed inhibition of NLRP3 repressed PD-L1 in lymphoma²⁰. However, rare research reported NLRP3 regulated LAG3. LAG-3 negatively regulates the proliferation, activation and homeostasis of T cells. Blocking LAG-3 in human CD4 + cells promoted cell proliferation and increase the expression levels of IL-2, IL-4, IFN- γ and TNF α ²¹.

In conclusion, our research comprehensively investigated the role of NLRP3 in pan-cancer. We determined overexpression of NLRP3 was closely related to immune escape by regulating PD-L1 and LAG3. Our research disclosed the therapy and diagnosis potential of NLRP3 in cancers.

Abbreviations

Liver hepatocellular carcinoma: LIHC; Ovarian cancer: OV; Colon cancer: COAD; Overall survival: OS; Microsatellite instability: MSI; Tumor mutational burden: TMB; Immune checkpoint inhibitors: ICIs; Peripheral blood mononuclear cells: PBMC; DNA methyltransferase: DNMT; Mismatch repair: MMR; Progression-free interval: PFS, Disease-specific survival: DSS; Disease-free survival: DFS

Declarations

Acknowledgements

Not applicable.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YD and ZY contributed to the experiments performing. YY and YD contributed to the statistical analysis of the data. JX and WS contributed to animal model. XY and QZ contributed to the design of the study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures involving human participants were performed in accordance with Shanghai Pudong Hospital ethical committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All patients provided their written informed consent. The study protocol was approved by the Pudong Hospital Committee on human research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

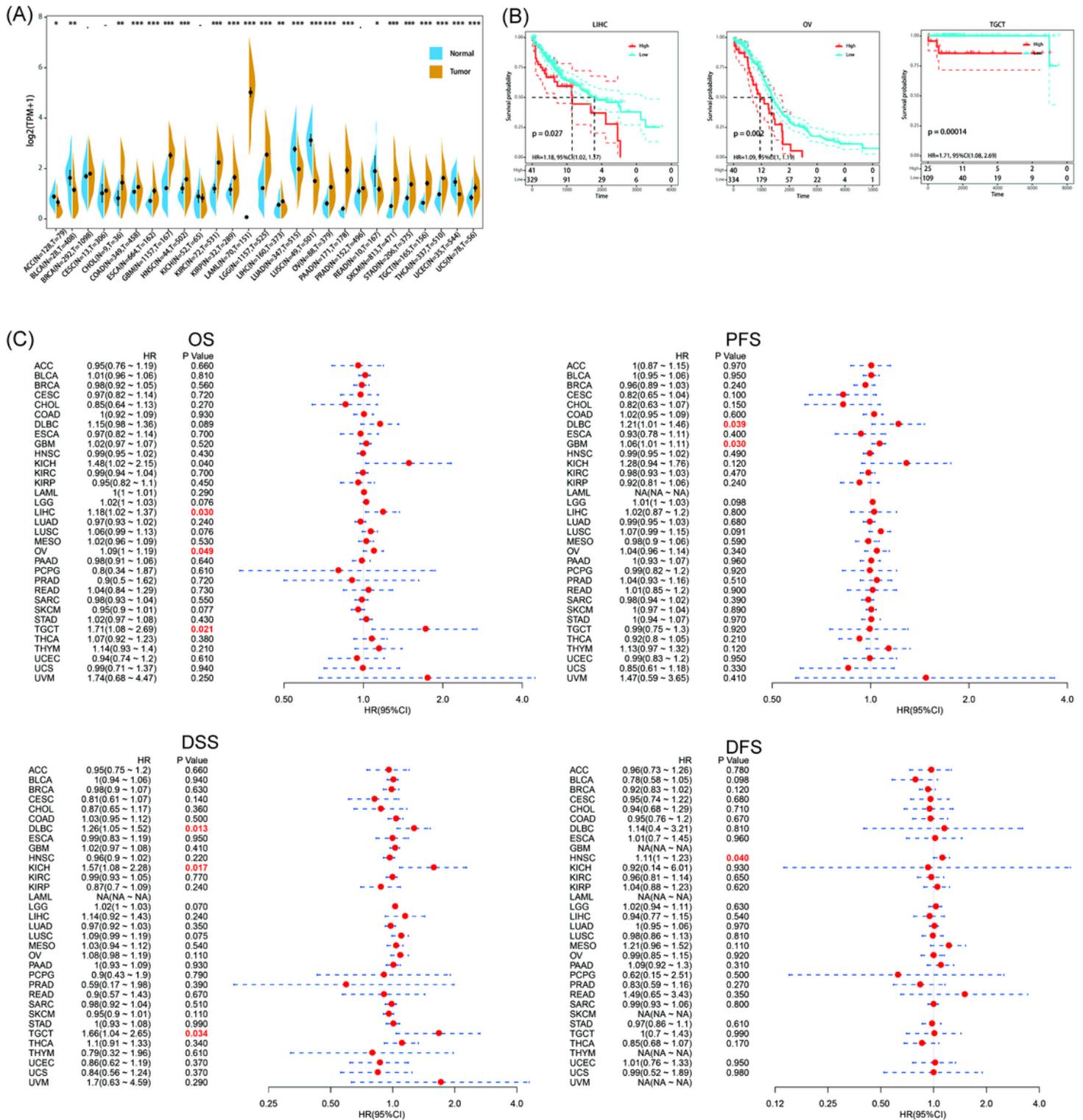


Figure 1

The expression and survival analysis of NLRP3 in pan-cancer. A. The expression of NLRP3 in pan-cancer and normal tissues. B. The association of NLRP3 and overall survival assessed by Kaplan-Meier analysis. C. The association of NLRP3 and overall survival (OS), progression-free interval (PFS), disease-specific survival (DSS), and disease-free survival (DFS) assessed by Cox analysis. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

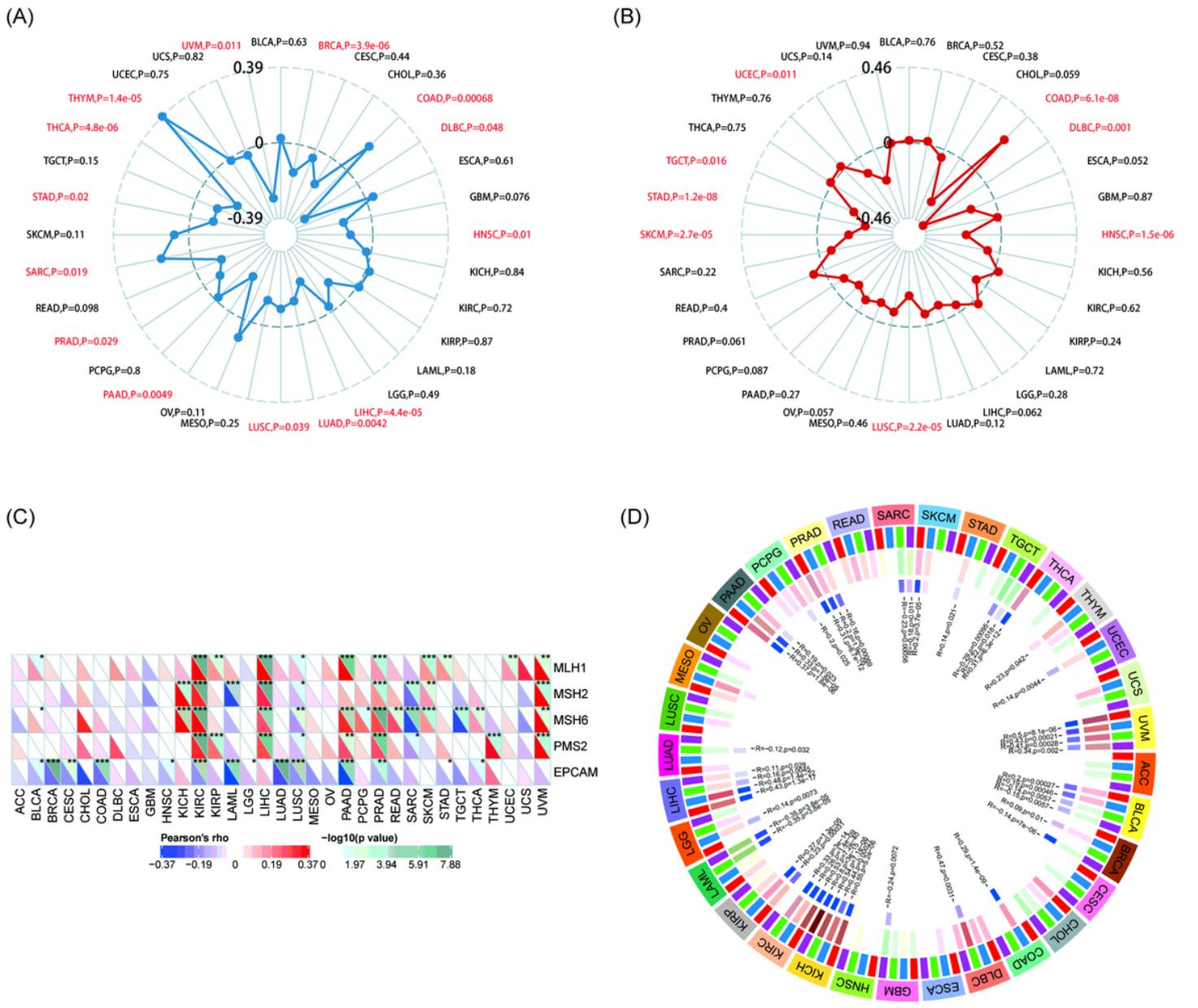


Figure 2

The association between NLRP3 and TMB, MMR. A. The correlation of NLRP3 and TMB analyzed by Spearman rank correlation coefficient. B. The correlation of NLRP3 and MSI analyzed by Spearman rank correlation coefficient. C. The correlation of NLRP3 and mutations of MMR genes analyzed by Pearson's correlation coefficient. D. The correlation of NLRP3 and 4 methyltransferases (DNMT1: red, DNMT2: blue, DNMT3A: green, DNMT3B: purple) analyzed by Pearson's correlation coefficient. (*P<0.05, **P<0.01, ***P<0.001).

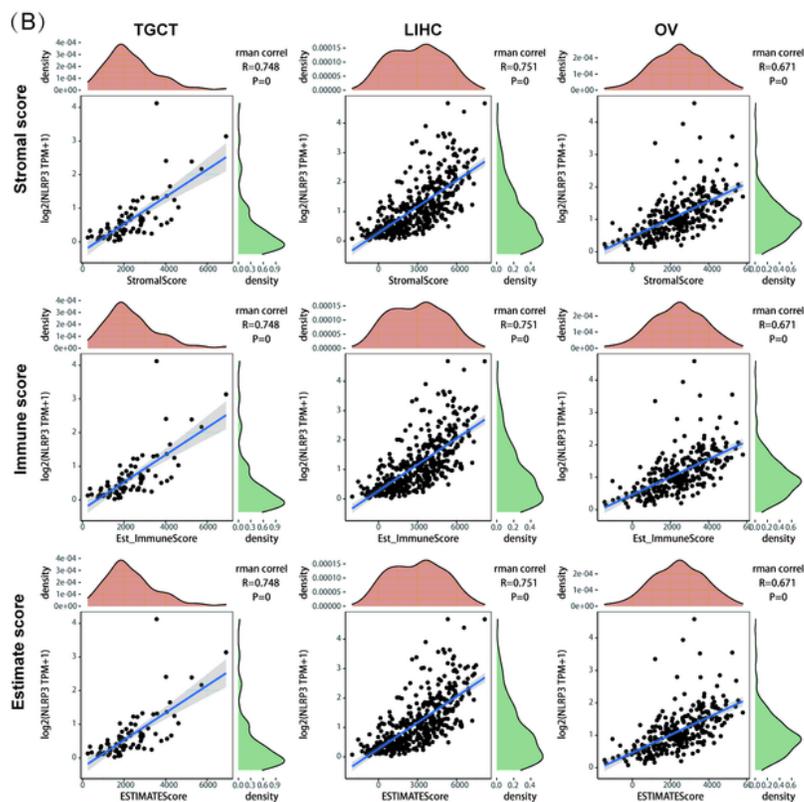
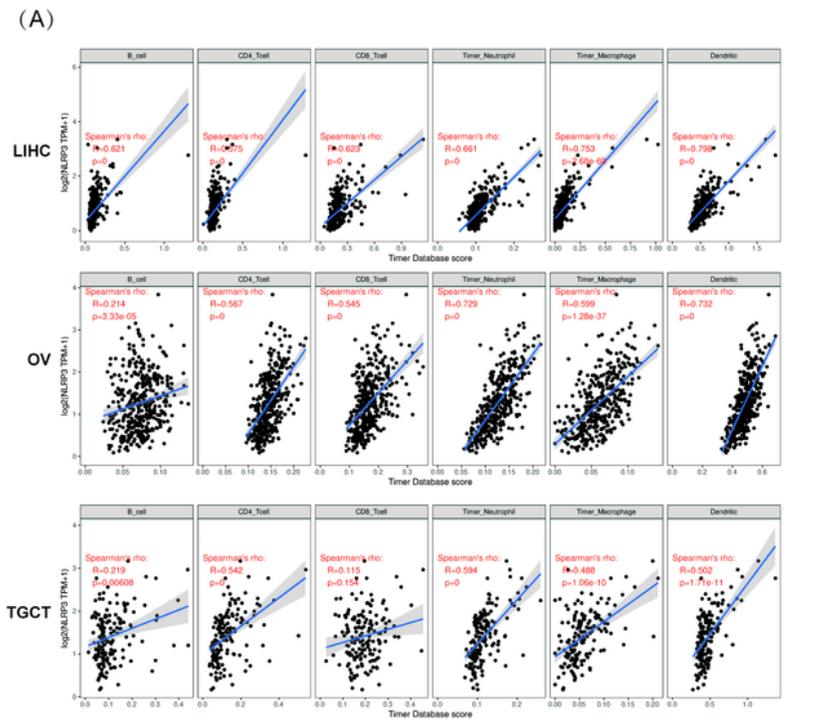


Figure 3

The association between NLRP3 and immune infiltration. A. The association of NLRP3 and lymphocytes analyzed by Spearman's correlation coefficient. B. The association of NLRP3 and stromal score, immune score, and Estimate score analyzed by the ESTIMATE algorithm.

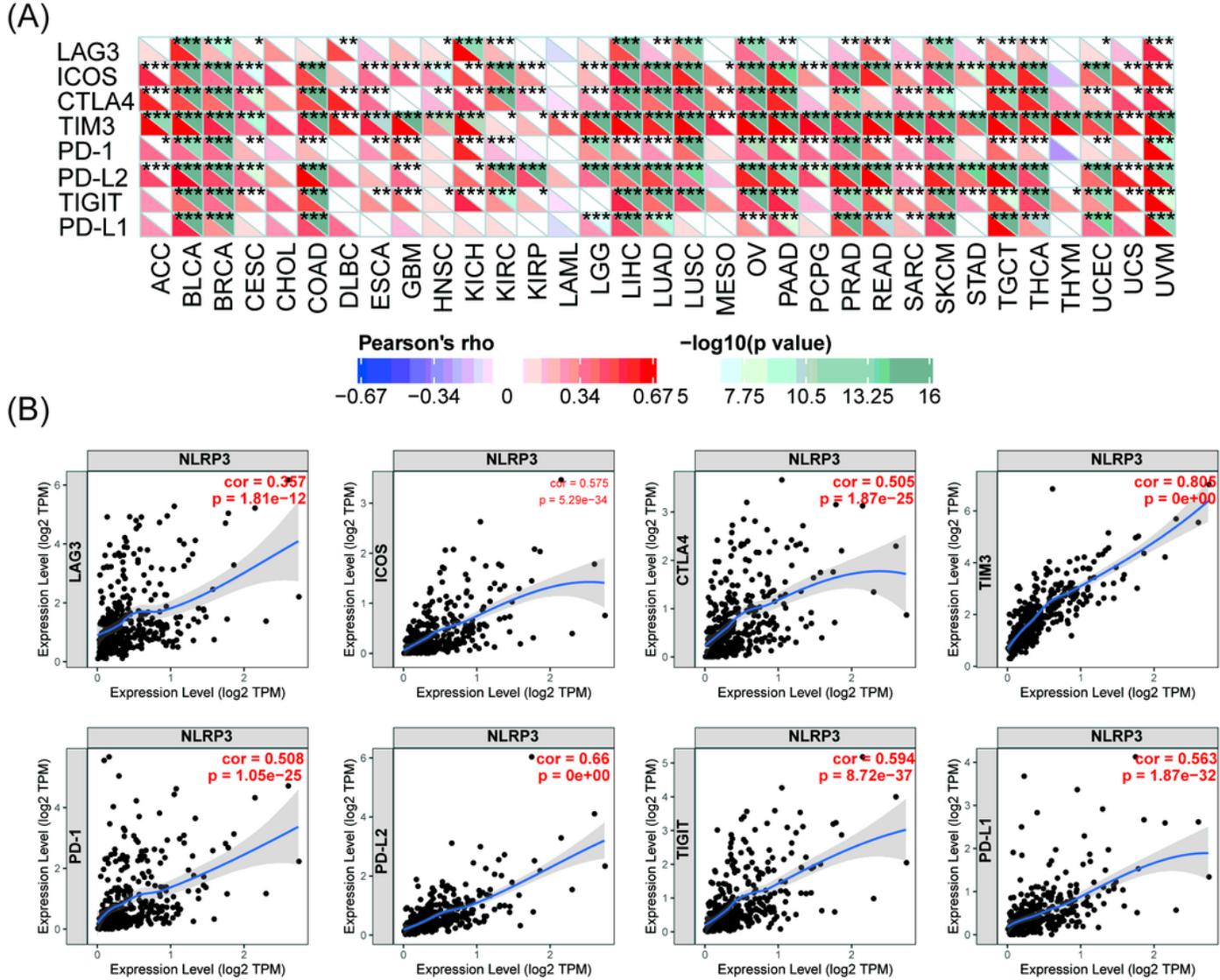


Figure 4

The association between NLRP3 and immune checkpoints. A. The correlation of NLRP3 and different immune checkpoints in pan-cancer analyzed by spearman's correlation coefficient. B. The correlation scatter diagram of NLRP3 and multiple immune checkpoints in LIHC.

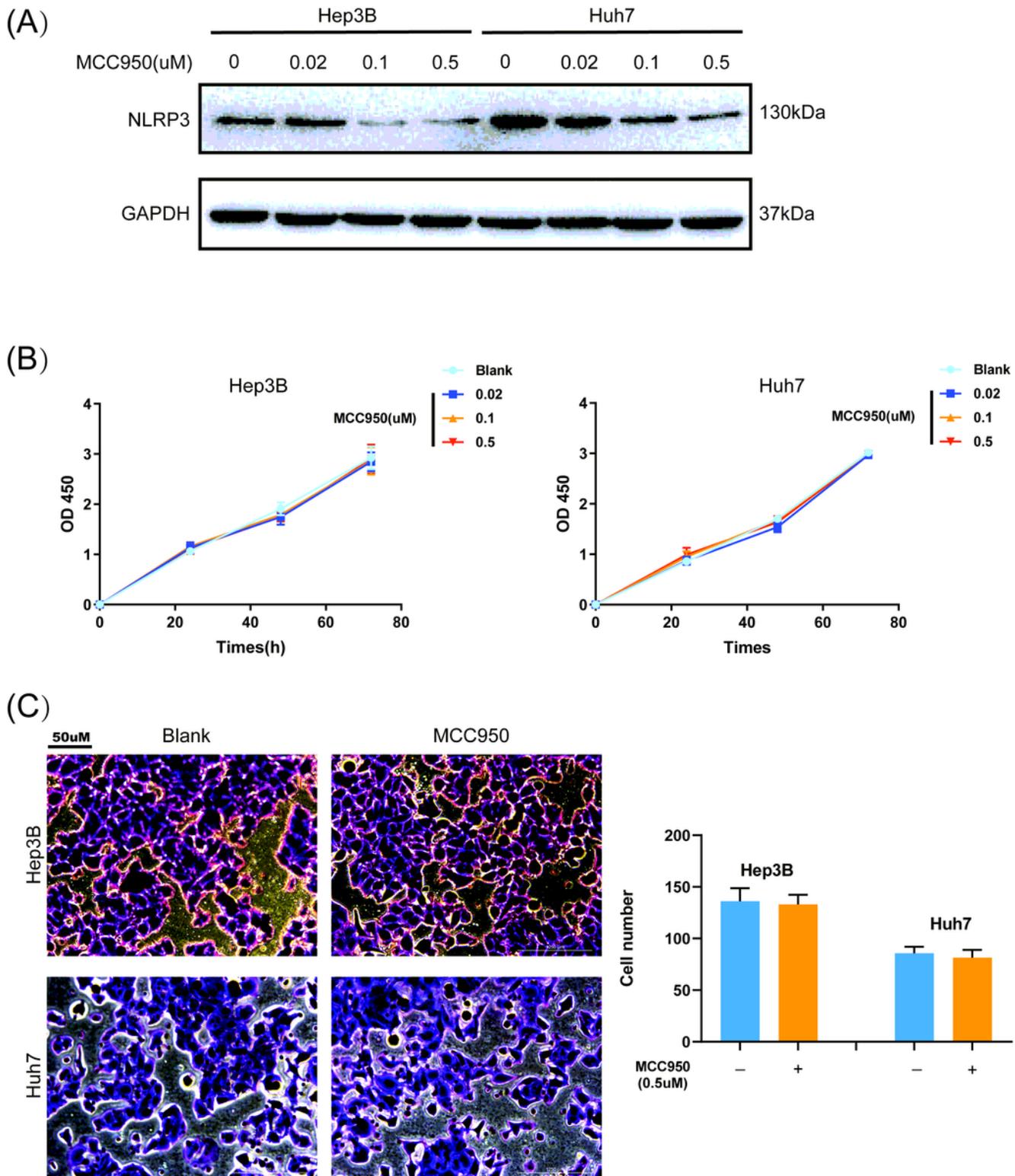


Figure 5

Inhibition of NLRP3 showed no effect to the cell proliferation and invasion of LIHC. A. The expression of NLRP3 in Hep3B and Huh7 after treating with NLRP3 inhibitor MCC950 (0.02 μ M-0.5 μ M for 24h). B. The proliferation ability of Hep3B and Huh7 after treating with MCC950. C. The invasion ability of Hep3B and Huh7 after treating with MCC950 (0.5 μ M for 24h).

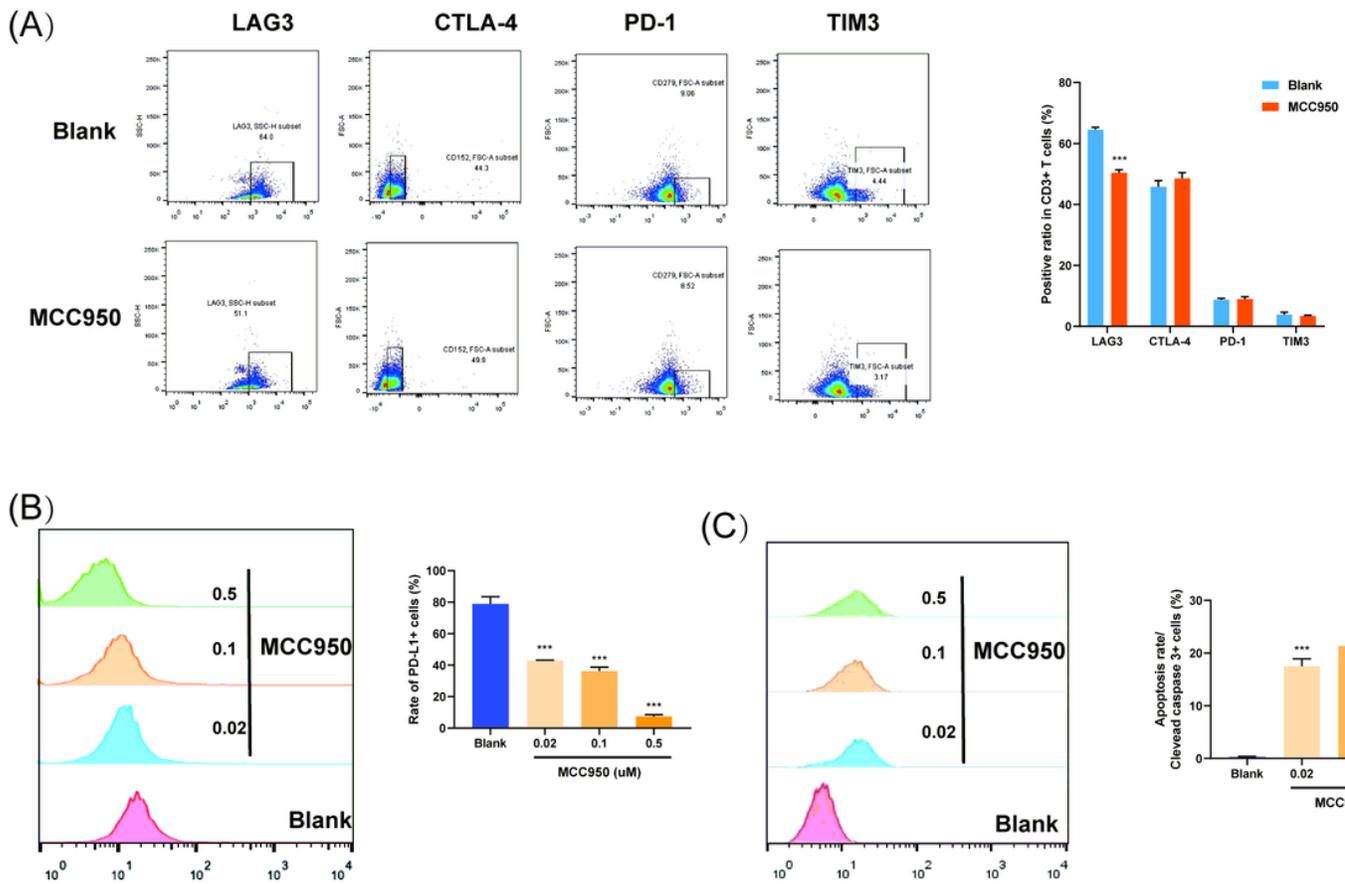


Figure 6

Inhibition of NLRP3 repressed immune escape by inhibiting LAG-3 and PD-L1. Co-culture of PBMC and Hep3B in 10:1 ratio for 48h. A. The expression of immune checkpoints in CD3+ T cells. B. The expression of PD-L1 in Hep3B. C. The apoptosis rate of Hep3B analyzed by expression of cleaved-Caspase 3. (***) $P < 0.001$.

Supplementary Files

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