Journal of Hepatocellular Carcinoma

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ORIGINAL RESEARCH

Lenvatinib Induces Immunogenic Cell Death and Triggers Toll-Like Receptor-3/4 Ligands in Hepatocellular Carcinoma

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Purpose: Immunogenic cell death (ICD) is a cell death modality that plays a vital role in anticancer therapy. In this study, we investigated whether lenvatinib induces ICD in hepatocellular carcinoma and how it affects cancer cell behavior.

Patients and Methods: Hepatoma cells were treated with 0.5 μM lenvatinib for two weeks, and damage-associated molecular patterns were assessed using the expression of calreticulin, high mobility group box 1, and ATP secretion. Transcriptome sequencing was performed to investigate the effects of lenvatinib on hepatocellular carcinoma. Additionally, CU CPT 4A and TAK-242 were used to inhibit *TLR3* and *TLR4* expressions, respectively. Flow cytometry was used to assess PD-L1 expression. Kaplan–Meier and Cox regression models were applied for prognosis assessment.

Results: After treatment with lenvatinib, there was a significant increase in ICD-associated damage-associated molecular patterns, such as calreticulin on the cell membrane, extracellular ATP, and high mobility group box 1, in hepatoma cells. Following treatment with lenvatinib, there was a significant increase in the downstream immunogenic cell death receptors, including TLR3 and TLR4. Furthermore, lenvatinib increased the expression of PD-L1, which was later inhibited by TLR4. Interestingly, inhibiting *TLR3* in MHCC-97H and Huh7 cells strengthened their proliferative capacity. Moreover, TLR3 inhibition was identified as an independent risk factor for overall survival and recurrence-free survival in patients with hepatocellular carcinoma.

Conclusion: Our study revealed that lenvatinib induced ICD in hepatocellular carcinoma and upregulated *PD-L1* expression through *TLR4* while promoting cell apoptosis through *TLR3*. Antibodies against PD-1/PD-L1 can enhance the efficacy of lenvatinib in the management of hepatocellular carcinoma.

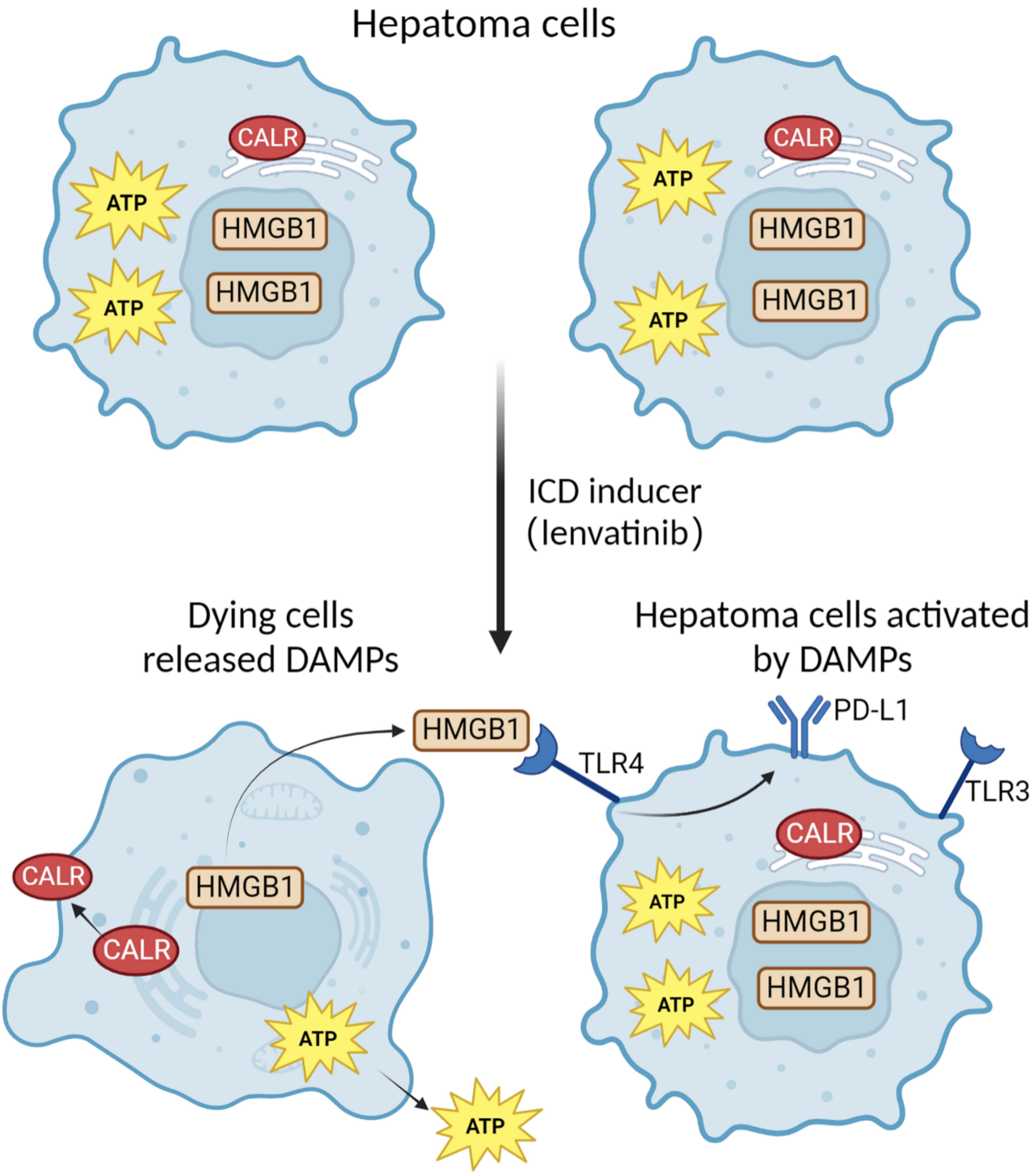
Keywords: hepatocellular carcinoma, immunogenic cell death, lenvatinib, toll-like receptor

Introduction

Hepatocellular carcinoma (HCC) ranks sixth in cancer incidence and fourth in cancer-related deaths.[1] Unfortunately, many patients with HCC are diagnosed at a moderate-to-advanced stage, where treatment is limited to local or systemic therapies. The current first-line systemic treatments for HCC, such as sorafenib, lenvatinib, donafenib, and anti-PD1 antibodies, have limited efficacy, extending patients' median survival by less than six months.[2–5] Given these shortcomings, there is an urgent need to explore new medication regimens that can effectively treat advanced HCC and improve patient outcomes. Lenvatinib is an emerging multiple kinase inhibitor targeting *VEGFR1-3, FGFR1*-4, *PDGFRα, RET*, and c-Kit,[6] which is an alternative first-line treatment option for advanced HCC. Lenvatinib has been shown to suppress tumor growth by inhibiting angiogenesis and tumor proliferation and is characterized by

Received: 18 December 2022
Accepted: 20 April 2023
Published: 24 April 2023

Graphical Abstract



immunomodulatory activity compared to other treatment regimens.[7,8] Earlier research has indicated that that lenvatinib treatment increases the $CD8^+$ T cell population[7] and decreases the Treg cell population in HCC.[9] Furthermore, it has been shown to decrease immune checkpoints like Tim-3 and PD-1 expression while increasing the expression of IFNG and

GZMB in $CD8^+$ T cells and natural killer cells.[8] In addition, lenvatinib augments the expression of natural cytotoxicity receptors by tumor-infiltrating NK cells and the expression of cytotoxic cytokines in tumor tissues.[10] To explore the immunomodulatory mechanism and improve the efficacy of lenvatinib, we hypothesized that lenvatinib could serve as an inducer of immunogenic cell death (ICD) in HCC.

ICD is a unique cell death modality triggered by selected anticancer therapies,[11,12] stimulating the innate and adaptive immune system against cell death-associated antigens and subsequently inducing tumor cell immunogenicity. Various anticancer treatments, such as chemotherapy, phototherapy, and radiotherapy, are known to induce ICD by releasing damage-associated molecular patterns (DAMPs) from dying cells. The released DAMPs activate innate immune responses and establish immunological memory. ICD-characterized DAMPs include exposure to endoplasmic reticulum chaperones, secretion of ATP, high mobility group box 1 (HMGB1), activation of the cancer cell-intrinsic type I IFN response, and the consequent secretion of the chemokine ligand 10.[13] DAMPs-triggered immune responses transform immune profile from "cold" to "hot" triggering synergies with immune checkpoint inhibitors and ultimately benefiting long-term tumor management.[14] This is because ICD induction stimulates an effective antitumor immune response. However, it is important to note that not all anticancer therapies are capable of inducing ICD. For instance, while radiofrequency ablation,[15] radiotherapy,[16] FOLFOX4 regimen[17] and doxorubicin[18] have been shown to induce in HCC, small-molecule agents have yet to be detected.

In this study, we investigated the induction of ICD by lenvatinib and its effects on the behavior of HCC cancer cells. Our findings suggest that lenvatinib promoted ICD in HCC cells and increased PD-L1 expression via TLR4. PD-1 or PD-L1 antibodies can enhance the efficacy of lenvatinib in HCC treatment.

Methods

Cell Culture

Hepatoma cell lines, including Huh7, PLC, Hep1-6, and MHCC-97H, were obtained from the liver cancer institution in Zhongshan Hospital and maintained in DMEM (hyClone) containing 10% FBS (hyClone) at 37°C with 5% CO2. The use of all cell lines was approved by the Institutional Ethics Committee of Zhongshan Hospital. Agents including lenvatinib (0.5 μM, Selleck), CU CPT 4a (27 μM, TOCRIS),[19] Poly I:C (10 μg/mL, Selleck)[20] and TAK-242 (10 μM, Selleck)[21] were solubilized in DMSO.

Patients

This study was approved by The Institutional Ethics Committee of Zhongshan Hospital, Fudan University and was conducted in accordance with the Helsinki Declaration (WMA Declaration of Helsinki, 2013). All the patients involved were pathologically diagnosed with HCC at Zhongshan Hospital. Written informed consent was obtained from all the patients before the surgery or pharmacological treatment. 27 patients who had received lenvatinib or lenvatinib plus anti-PD-1 antibodies and 343 patients who had not received any treatment before the surgery were collected were involved in this study. Only patients who met the following inclusion criteria were enrolled: (I) >18 years and ≤75 years of age; (II) diagnosed by cytologic/histologic evidence or by noninvasive diagnostic measurements recommended by the EASL; (III) preserved liver function was classified as Child-Pugh class A without any ascites but patients were intolerant to complete resection. Patients were excluded if they met any one of the following criteria: (I) a history of other malignancies; (II) liver functional status of Child-Pugh B/C; (III) cardiac, pulmonary, cerebral, or renal dysfunction; (IV) with extrahepatic metastasis or macroscopic vascular invasion.

The dose of lenvatinib was either 12 mg/day (weight; ≥ 60 kg) or 8 mg/day (weight; < 60 kg). The anti-PD-1 antibody administration protocol was applied according to the manufacturer's instructions (1 time/21 days). Lenvatinib and the Anti-PD1 antibody were discontinued a week and a month before surgery, respectively.

HCC Xenograft Model

Animal experiments were approved by the Ethics Committee of the Zhongshan Hospital Biomedical Research Department and followed the Guideline for ethical review of animal welfare in China (GB/T 35892–2018). A total of

12 eight weeks old male BALB/c mice were obtained from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). Huh7 cells (1*10^6) were mixed with Matrigel (Corning) at 3:1 and injected into the armpit. Mice were randomized into lenvatinib-treated group and control group when the tumor grew up to 100mm^3. Mice were fed up with lenvatinib (2mg/kg/day) or equivalent volume of saline by gastric gavage for four weeks in lenvatinib-treated group and control group, respectively. Neck breaking method was applied in mice to cause immediate death. All mice were raised in a specific pathogen-free environment in Zhongshan hospital.

RNA Extraction and Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol (Thermo), phase-separated with chloroform, precipitated using isopropyl alcohol, washed with 75% ethanol, and redissolved in water. RNA reverse transcription reactions and RT-qPCR were conducted using Goldenstar™ RT6 cDNA Synthesis Mix and 2×TSINGKE® Master qPCR Mix from Tsingke Biotechnology. qRT-PCR primer pairs for *TLR3, TLR4*, and *PIK3R1* were obtained from PrimerBank. Primer sequences for *TLR3*: (f)5′-CAAACACAAGCATTCGGAATCTG (r)5′-AGGAATCGTTACCAACCACATT; TLR4:(f)5′-AGACCTGTCCCT GAACCC (r)5′-CGATGGACTTCTAAACCAGCCA; pik3r1: (f) 5′-TGGACGGCGAAGTAAATT (r) 5′-AGTGTGAC ATTGAGGGAGTCG; gapdh: (f) 5′CTGGGCTACACTGAGCACC- (r) 5′-AAGTGGTCGTTGAGGGCAATG. RT-qPCR was performed using a 7500 Fast Real-Time PCR System. Fold change values were calculated using the $2^{-\Delta\Delta}$Ct method.

Protein Extraction and Western Blot

Cells or tissues were homogenized and lysed in RIPA buffer (Biyuntian) with protease inhibitors (Sigma). Proteins were separated by SDS-PAGE, electroblotted onto Trans-Blot Turbo Transfer Pack 0.2 μm PVDF membrane (BioRad), and incubated overnight with antibodies, followed by corresponding secondary antibodies (Abclone). The primary antibodies were used for Western blot were listed in Table 1. Secondary antibodies conjugated to horseradish peroxidase were then used as also listed in Table 1.

Immunofluorescence

The cells were cultured in six-well plates, fixed using 3% paraformaldehyde, and permeabilized using 0.5% Triton X-100 (Tx100). Fixed cells were incubated in 10% goat serum for 30 min and stained with primary antibodies overnight at 4°C. The samples were washed three times using PBST, incubated with fluorescent secondary antibodies for 1 h in a wet box, and stained using 0.1 ug/mL DAPI for 5 min in the dark.

Table 1 Antibodies Applied in Western Blot, Immunohistochemistry, Immunofluorescence and Flow Cytometry

Antibody	Sources	Article Number	Working Concentration
TLR3	ABclonal	A11778	1:1000 (WB)
TLR4	ABclonal	A5258	1:2000(WB)
PI3K	Cell Signaling Technology	17,366	1:1000(WB)
AKT	Cell Signaling Technology	4691	1:1000(WB)
pAKT	Cell Signaling Technology	4060	1:1000(WB)
Beta-actin	Cell Signaling Technology	8457	1:1000(WB)
Gapdh	abcam	ab8245	1:1000(WB)
Mouse IgG HRP	Cell Signaling Technology	7076	1:1000(WB)
Rabbit IgG HRP	Cell Signaling Technology	58,802	1:1000 (WB)
TLR3	ABclonal	A11778	1:1000 (IF)
Alexa Fluor Plus 594	ThermoFisher	A21203	1:500 (IF)
Calreticulin	ABclonal	A18013	1:1000(FC)
PD-L1	Biolegend	124,312	1:50

Flow Cytometry

Flow cytometry was used to determine cell apoptosis rate and PD-L1 expression. Single-cell suspensions were resuspended in 2% BSA for flow cytometry, and the cells were subsequently incubated with antibodies for 30 min at 4°C. The antibodies used for flow cytometric analysis are listed in Table 1. Flow cytometric analysis was performed using a BD Accuri C6 Plus flow cytometer (BD Biosciences). Data analysis was conducted using FlowJo V10, and the relative protein expression was analyzed using the mean fluorescence intensity.

IHC

Tissues were fixed using 4% paraformaldehyde at 4°C overnight after washing with PBS. After dehydration, permeabilization, wax-dip, and embedding, 5 μM sections were cut. The slices were processed in the following order: dewaxing, hydration, antigen repair, and BSA blocking. Afterward, slices were incubated with primary antibodies overnight, and secondary antibodies were added for 1 h. Finally, the chromogenic substrate, tetramethylbenzidine, was added for color development. The antibodies used for IHC are listed in Table 1.

RNA Sequencing

Transcriptome sequencing was performed by Genergy Biotechnology Co. (Shanghai, China). RNA-Seq libraries were generated according to the manufacturer's protocols using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (Illumina, CA, USA). All the samples were sequenced on the MGISEQ-2000 platform. 27.71Gb raw data was generated from 4 samples. The raw data were normalized. Clean reads were obtained from the raw data by discarding adapter and poly-N sequences and reads of low quality. Differential expression analysis was based on edgeR package in R software (v.4.1.2). A p value < 0.05 and a fold change > 2 were set as the threshold for significant differential expression.

Statistics

As appropriate, patients' baseline characteristics were reported as mean (± standard deviation), median (range), or percentage. The Mann–Whitney U and Student's t-tests were used in comparing continuous variables. The $\chi 2$ and Fisher exact tests were used to compare categorical variables. Overall survival was examined using the Kaplan–Meier and Log rank tests. Factors with a p-value <0.10 in univariate analyses were introduced into the multivariate Cox proportional hazards model to determine the independent impact on overall survival (OS). Hazard ratios (HR) and 95% confidence intervals (CIs) were estimated using a nonparametric Log rank test with the Cox proportional hazards model. All statistical analyses were performed using SPSS for Windows (version 24.0; IBM, NY). Correlation analyses were performed using Spearman's rank correlation coefficient. Statistical significance was set at a two-tailed p-value <0.05. The graph abstract was created with biorender.

Results

Lenvatinib Induced ICD in Hepatoma Cells

We investigated whether lenvatinib induces ICD in hepatoma cells by examining the presence of classical DAMPs in human hepatoma-derived cell lines, including Huh7, MHCC-97H, and PLC, as well as in mouse hepatoma-derived Hepa1-6 cells. In line with pharmacokinetic analyses, which revealed plateaued lenvatinib concentrations (8–12 mg once daily) at 1.23–2.11 ng/mL in HCC patients,[22] we treated the cells with a lenvatinib concentration of 5 μM for two weeks to simulate the effects of lenvatinib on hepatoma tumor cells. Flow cytometry demonstrated that lenvatinib treatment resulted in a higher proportion of apoptotic cells in comparison to the control group (Figure 1A).

There was an increase in extracellular ATP in lenvatinib-treated hepatoma cells, whereas intracellular ATP levels decreased or remained constant (Figure 1B). Furthermore, we examined the surface expression of calreticulin on hepatoma cells using flow cytometry, which demonstrated a significant increase in calreticulin expression on the surface of all hepatoma cells following lenvatinib treatment (Figure 1C). In addition, ELISA and Western blot analyses showed higher HMGB1 levels in the culture medium of lenvatinib-treated hepatoma cells (Figure 1D). Summarily, these results demonstrated that ICD markers, including calreticulin expression, ATP release, and HMGB1 release, were significantly increased in hepatoma cells upon treatment with lenvatinib.

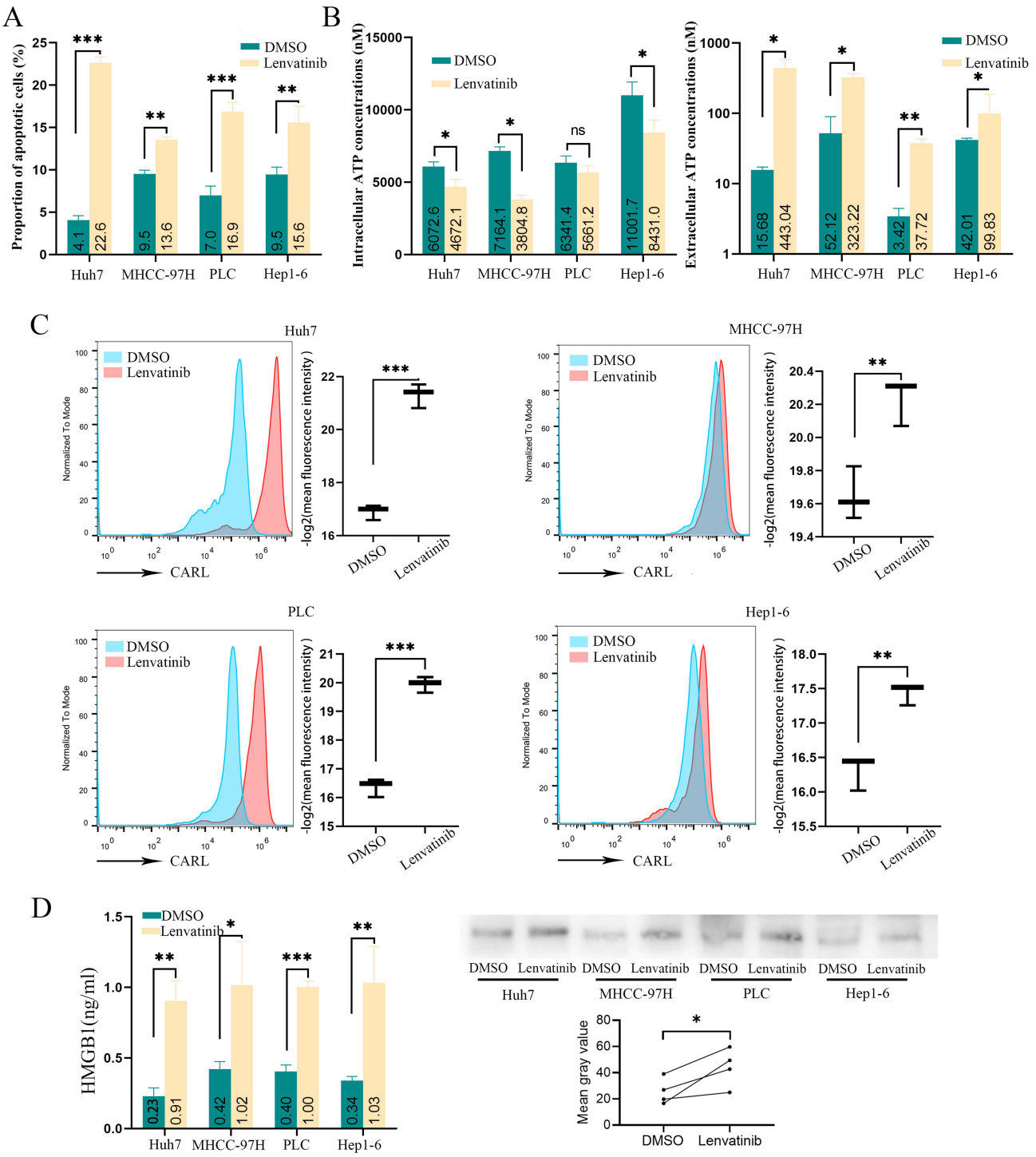


Figure 1 (**A**) The proportion of apoptotic cells in lenvatinib-treated cells was higher than that in the control group. (**B**) Extracellular ATP was significantly increased after treatment of lenvatinib and intracellular ATP was decreased or remained constant. (**C**) Lenvatinib significantly increased calreticulin expression on the surface of all hepatoma cells. (**D**) Elisa and Western blot analyses revealed higher HMGB1 in the culture medium of lenvatinib-treated hepatoma cells than that in untreated cells. *p < 0.05, **p < 0.01, *** p <0.001.

Transcriptome Sequencing Revealed the Increased Expression of TLR3 and TLR4 in Hepatoma Cells

To further understand the effects of lenvatinib treatment on hepatoma cells, we conducted transcriptome sequencing of both lenvatinib-treated and untreated Huh7 and MHCC-97H cells. Subsequently, we performed differential gene

expression analysis on both cell types, identifying 48 genes that were significantly upregulated in both groups. To determine the biological context of these genes, we conducted GO and KEGG pathway analyses, which revealed enrichment in pathways such as the interferon signaling pathway and primary alcohol metabolic process, among others (Figure 2A). Notably activation of the interferon signaling pathway is one of the characteristics of ICD. The downstream receptors of DAMPs, including *TLR3* and *TLR4*, exhibited 69-fold and 14-fold elevation in lenvatinib-treated Huh7 cells, respectively (Figure 2B). *TLR4* expression exhibited a 4.2-fold increase in lenvatinib-treated MHCC-97H cells (Figure 2C). Based on the results of transcriptome sequencing analysis, we speculated that lenvatinib induces ICD in hepatoma cells and triggers *TLR3* and *TLR4* upregulation.

Lenvatinib Upregulated the Expression of TLR3 and TLR4

In support of our hypothesis, we quantified the expression of *TLR3* and *TLR4* in the hepatoma cells. Following two weeks of lenvatinib treatment, we observed a significant increase in mRNA expression of TLR3 in Huh7, PLC, and MHCC-97H cells (Figure 3A). Western blotting and immunofluorescence revealed enhanced *TLR3* expression in lenvatinib-treated cells (Figures 3B and C). In addition, we observed an increase in mRNA and protein expression of TLR4 in Huh7, PLC, and MHCC-97H cells after lenvatinib treatment (Figures 3D and E).

We examined the expression of *TLR3* and *TLR4* in vivo. Two groups of BALB/c mice with xenograft tumors were administered 5 mg/kg lenvatinib or saline. Mice treated with lenvatinib had significantly smaller tumor sizes (Figures 4A

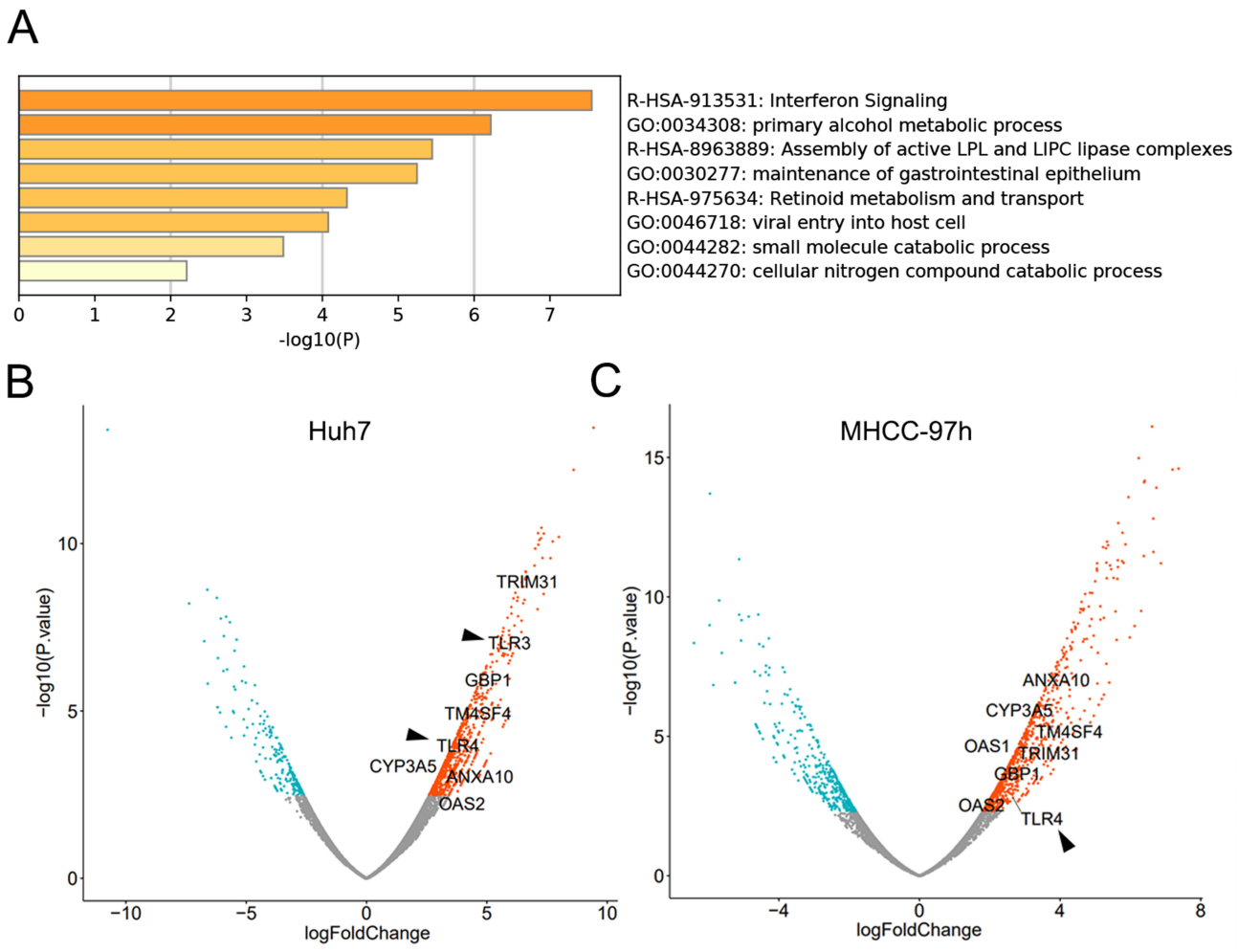


Figure 2 (**A**) GO and KEGG pathway analysis of increased genes after lenvatinib treatment. (**B** and **C**) Volcano plot showing differential expressed genes between lenvatinib-treated and untreated Huh7 cells and MHCC-97h cells. The TLR3 and TLR4 was listed in arrow heads.

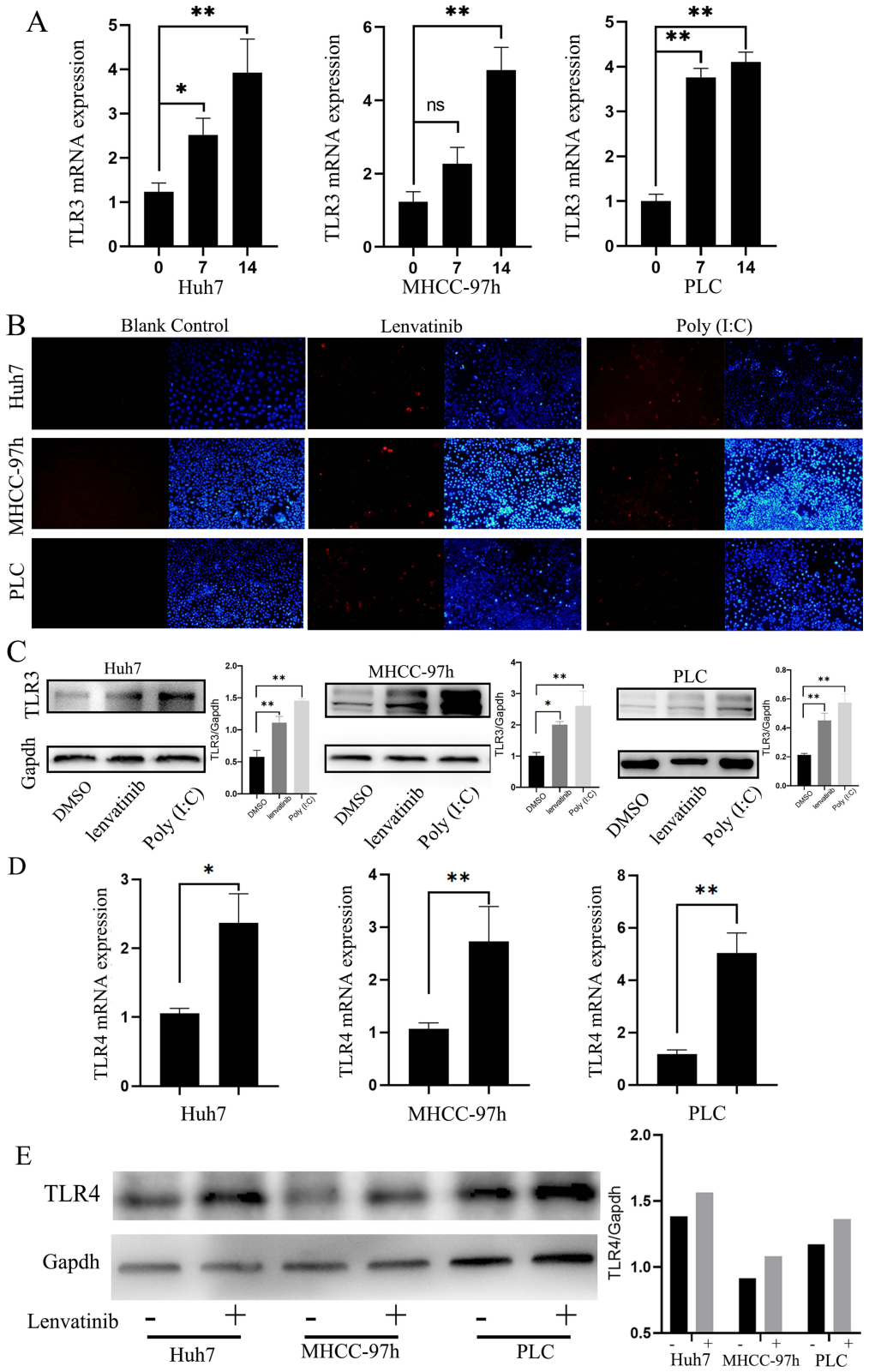


Figure 3 After treatment with lenvatinib for 2 weeks, both the mRNA expression(**A**) and protein expression (**B** and **C**) of TLR3 was significantly increased. Moreover, the mRNA (**D**) and protein (**E**) expression of TLR4 were also upregulated. *$p < 0.05$, **$p < 0.01$.

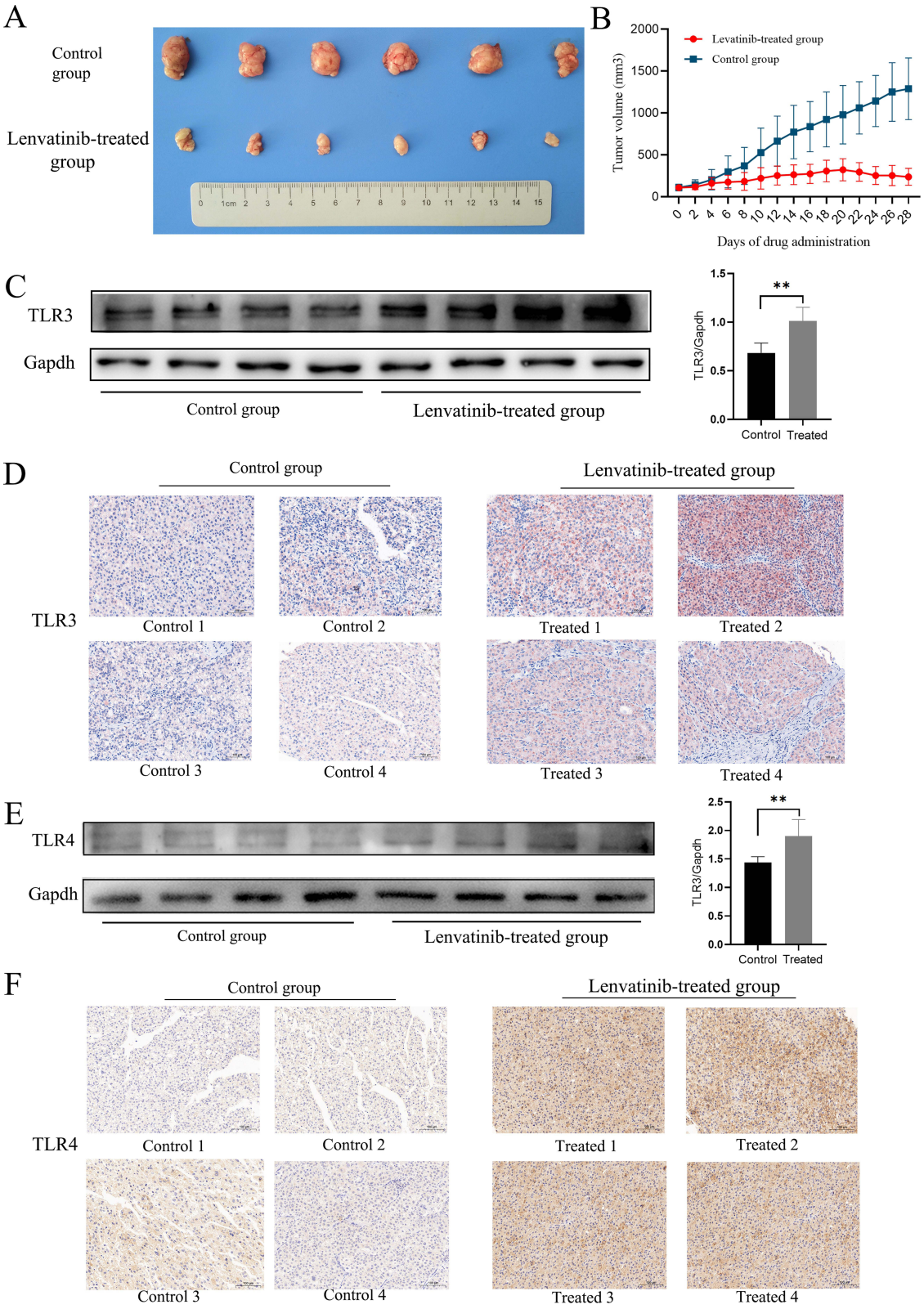


Figure 4 Tumor size was significantly decreased after lenvatinib treatment (**A** and **B**). Western blot and IHC showed that protein expression of *TLR3* (**C** and **D**) and *TLR4* (**E** and **F**) were also increased in vivo. $^{**}p < 0.01$.

https://doi.org/10.2147/JHC.S401639 DovePress

and B). Two tumors from each group were transferred to wild-type mice to evaluate their tumor-formation ability. No tumors transferred from lenvatinib-treated mice formed new tumors, indicating that the tumor formation ability was significantly decreased after lenvatinib treatment. In vivo, the expressions of *TLR3* and *TLR4* also increased in lenvatinib-treated mice (Figures 4C–F).

Lenvatinib Upregulated the Expression of PD-L1 by *TLR4*

Previous studies reported the induction of ICD and the expression of toll-like receptors accompanied by alterations in the expression of immune checkpoints. Correlation analysis between *TLR3/4* and common immune checkpoints in the TCGA database. We checked the gene expression level of TLR3/4 and a series of immune checkpoints including TIM3, CD274 (PD-L1), OX40, CD27, CTLA4 and LAG3. Among all immune checkpoints, *CD274* had the highest correlation with *TLR3* and *TLR4* (*TLR3*: r= 0.461, $p< 0.001$; *TLR4*: r= 0.503, $p< 0.001$; Figure 5A). Flow cytometry analysis of PD-L1 expression indicated an increase in lenvatinib-treated Huh7, MHCC-97H, and PLC cells (Figures 5B and C). We inhibited the expression of *TLR4* using TAK-242 and observed that PD-L1 expression was decreased (Figure 5B); however, there was no significant change in PD-L1 expression after *TLR3* inhibition (CU CPT 4A) (Figure 5C).

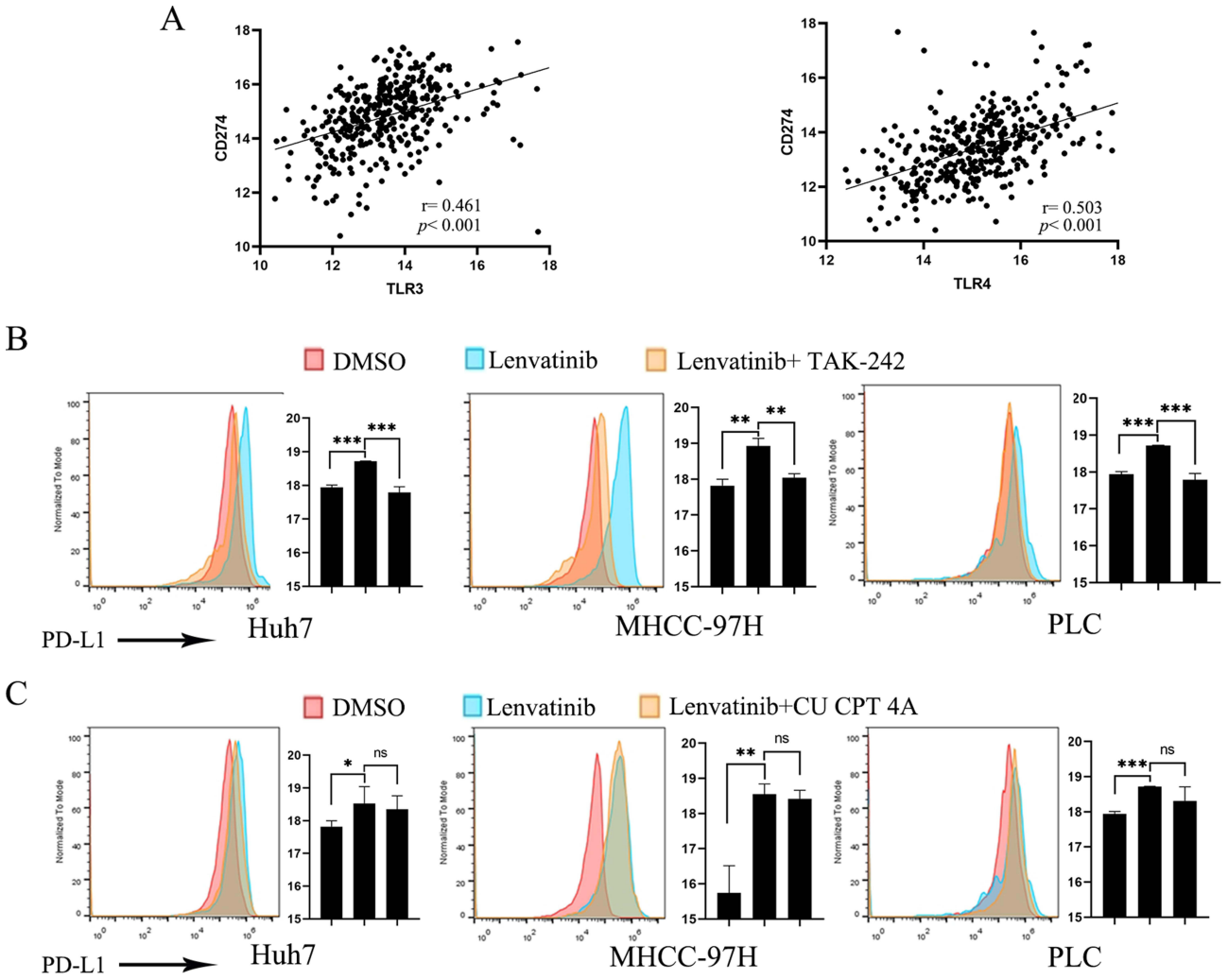


Figure 5 (**A**) TCGA database revealed a strong correlation between *TLR3*/*TLR4* and *CD274*. (**B**) PD-L1 expression was significantly increased after two weeks lenvatinib treatment and decreased after the inhibition of *TLR4*. (**C**) The inhibition of *TLR3* had not significantly affected PD-L1 expression. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

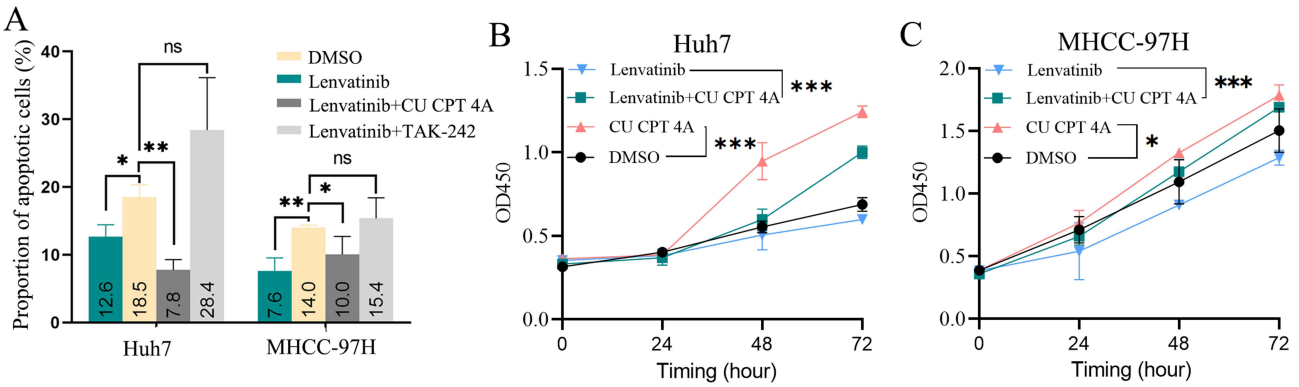


Figure 6 (**A**) The inhibition of *TLR3* decreased the proportions of apoptotic cells. (**B**) CCK8 assay indicated that inhibition of *TLR3* reinforced the proliferative capacity of MHCC-97H and Huh7 cells (**B** and **C**). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

The Upregulation of *TLR3* Promoted Cell Death in Hepatoma Cells

We investigate the effects of TLR3 and TLR4 inhibition on cell apoptosis and proliferation. We inhibited the expression of *TLR3* and *TLR4* using CU CPT 4A and TAK-242 in Huh7 and MHCC-97H cells, respectively. The corresponding proportion of apoptotic cells significantly decreased when *TLR3* expression was inhibited (Figure 6A). Furthermore, the CCK8 assay indicated that *TLR3* inhibition reinforced the proliferative capacity of MHCC-97H and Huh7 cells (Figures 6B and C).

Patients Who Had Received Lenvatinib-Treatment Had a Higher Positivity of *TLR3* and *TLR4*

Most patients who received lenvatinib were in the moderate-to-advanced stages and had lost the chance to undergo surgery. Biopsy was also not recommended for these patients. However, the combination of lenvatinib and anti-PD-1 antibodies resulted in downstaging and subsequent eligibility for resection in some patients. Samples from 27 patients who had received lenvatinib or lenvatinib plus anti-PD-1 antibodies and 343 samples from patients who had not received any treatment before the surgery were collected. Among the 27 patients, 3 received lenvatinib, and 24 received lenvatinib plus anti-PD-1 antibodies. IHC was performed to assess *TLR3* and *TLR4* expression in these patients (Figures 7A and B), which revealed a higher positivity of *TLR3* in patients treated with lenvatinib (70.4% vs 44.4%), and the same was observed in *TLR4* (70.8% vs 14.2%). Among the 343 untreated patients, overall survival and recurrence-free survival were longer for patients with positive *TLR3* than those with negative *TLR3* (all $p< 0.05$, Figure 7C). *TLR3* was also an independent risk factor for overall and recurrence-free survival in the multivariate analyses (Table 2). We also performed survival analysis using the TCGA database, and the results were consistent with IHC (Figure 7D). However, no significant association between *TLR4* expression and overall survival was observed (Figures 7E and F). Correlation analysis revealed that patients with positive *TLR3* and smaller tumor size had a higher proportion of the tumor capsule (Table 3).

Discussion

Through our study, we have found that lenvatinib has the ability to induce ICD in hepatoma cells and activate the *TLR3/4* pathway in HCC, making it the only small-molecule agent with such properties for HCC treatment. We have also observed that upregulation of TLR4 enhances PD-L1 expression in hepatoma cells, while the upregulation of TLR3 promotes cell apoptosis. This is the first study to report on lenvatinib's ability to induce ICD and activate TLR3/4 pathway in HCC cells.Previous studies have reported that Ienvatinib can increase the response rate to PD-1 treatment by upregulating the proportion of infiltrated $CD8^+$ T cells[7] and downregulating the proportion of Treg cells in HCC;[9] however, these studies did not explore the reasons for this phenomenon. Lenvatinib is an angiogenesis inhibitor and a tumor suppressor in HCC.[23] Angiogenesis inhibitors can improve the tumor microenvironment through vascular normalization.[24] In this study, we observed that in addition to the suppression of angiogenesis, lenvatinib also has the ability to induce ICD in hepatoma cells. This additional contribution uncovers a new mechanism through which

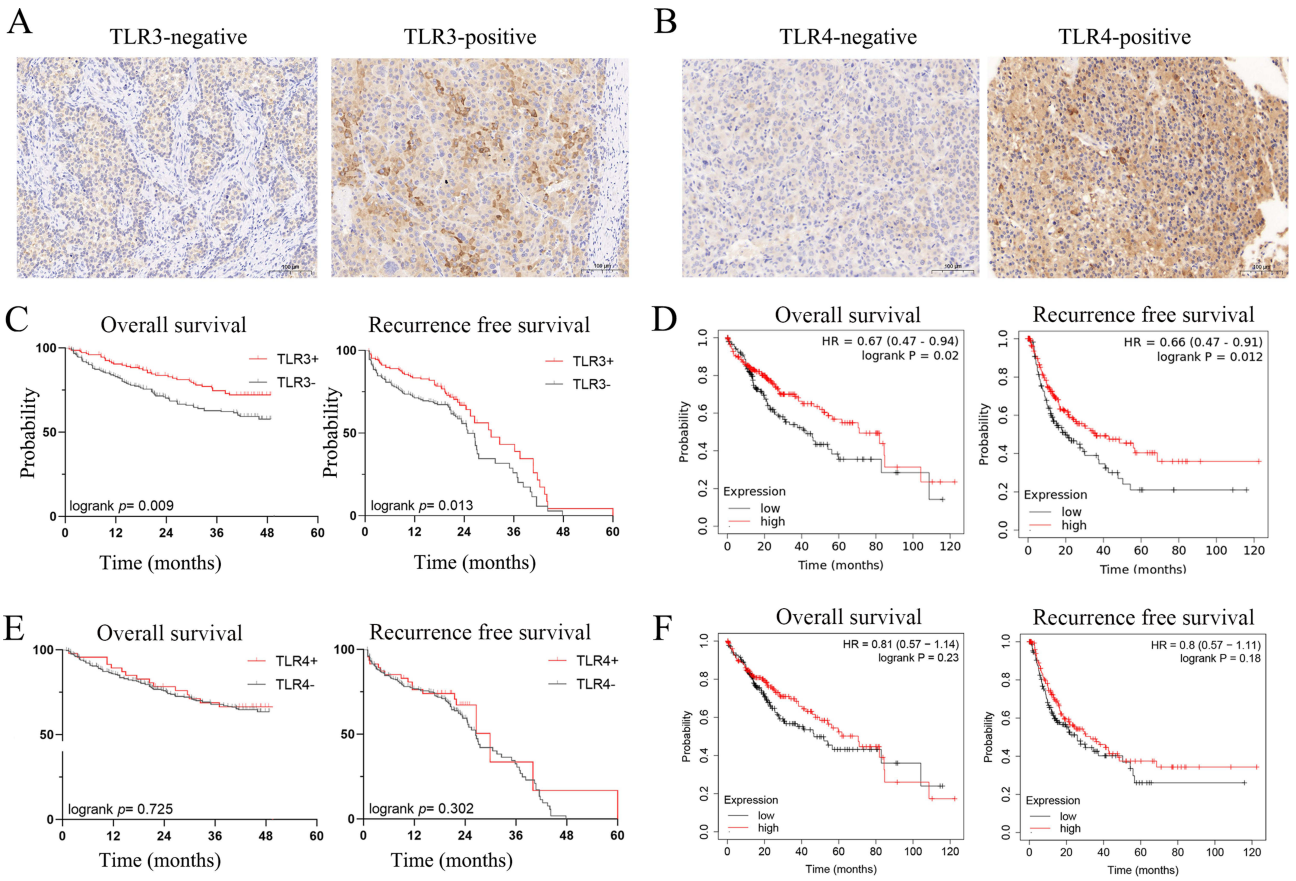


Figure 7 (**A**) Representative images of TLR3 in hepatocellular carcinoma (HCC). (**B**) Representative images of TLR4 in HCC. (**C**) Kaplan–Meier estimates overall survival (OS) and recurrence-free survival (RFS) according to TLR3 expression in HCC patients. TLR3-positive patients had a better prognosis than TLR3-negative patients. (**D**) Data from TCGA database showed higher TLR3 mRNA levels in HCC was positively associated with OS and RFS. (**E**) Kaplan–Meier estimates for OS and RFS according to TLR4 expression in HCC patients. TLR4 expression was not associated with prognosis. (**F**) Data from TCGA database showed higher TLR4 mRNA levels was not associated with prognosis in HCC.

lenvatinib can remodel the tumor microenvironment and transformed "cold tumors", which were insensitive to immunotherapy, into "hot tumors".[25,26] We also observed that lenvatinib promotes the expression of PD-L1 in HCC through *TLR4*. Lenvatinib plus anti-PD1 antibodies has emerged as a hotspot in HCC research. These results indicate synergy between lenvatinib and immune checkpoint therapy, especially PD-1/PD-L1 antibodies.

Additionally, we observed that lenvatinib upregulated the expression of PD-L1 in HCC, which is different from previous studies that have reported decreased expression of PD-L1 by lenvatinib. Previous studies have shown that lenvatinib can decrease the expression of PD-L1 in HCC by blocking the FGFR4-GSK3β axis.;[27] however, FGFR inhibition with lenvatinib activated the IFNγ-pSTAT1-PDL1 axis and increased PD-L1 expression in renal cell carcinoma.[28] In addition, lenvatinib inhibits PD-L1 expression in endothelial cells without affecting PD-L1 expression in cancer cells.[8] One possible reason for this discrepancy is the variable drug concentration and timing. The drug concentrations and timings in the previous three studies were 10 μM for 24 h, 1 or 3 μM for 48 h, and 3 μM at any time. Such high concentrations are not achievable in clinical practice. Pharmacokinetic analyses of lenvatinib demonstrated that the highest plasma concentration (16 mg/day) in HCC patients was 344 ng/mL (<0.7μM), which was lower than the drug concentrations in the in vitro experiments. The most used dose of lenvatinib in patients with advanced HCC is 8 or 12 mg/day. Considering that the overall lenvatinib plasma concentrations were approximately 0.5 uM in the clinic (12 mg/day),[22] we used the same concentration to treat cells for two weeks. A high dose of lenvatinib downregulates PD-L1 expression, whereas a low dose has the opposite effect. Besides, previous studies have found that PD-L1 is primarily expressed in macrophages in HCC[29,30] and TLRs are also highly expressed in macrophages. In this study, we mainly

Table 2 Univariate and Multivariate Analysis of Overall Survival and Recurrence-Free Survival

Variables	Overall Survival			Recurrence-Free Survival		
	Univariate Analysis	Multivariate Analysis		Univariate Analysis	Multivariate Analysis	
	p value	*p* value	(95% CI)	*p* value	*p* value	(95% CI)
Sex (male)	0.227			0.904		
Age (>60 years)	0.198			0.46		
HbsAg(positive)	0.561			0.573		
TB (>20.4 μmol/L)	0.173			0.23		
γ-GT (≥60 U/L)	0.935			0.623		
High differentiation	0.344			0.914		
Albumin (<35 g/L)	0.008	0.011	0.506(0.398–0.899)	0.377		
AFP (≥ 20 ng/mL)	<0.001	<0.001	2.424(1.560–3.764)	0.001	0.002	2.013(1.281–3.161)
Tumor size (>5 cm)	<0.001	<0.001	2.314(1.575–3.401)	0.003	<0.001	2.186(1.467–3.257)
Tumor number (>1)	0.542		NA	0.007	0.027	1.948(1.079–3.517)
Tumor capsule	<0.001	0.013	0.598(0.398–0.899)	0.01	0.077	0.682(0.447–1.042)
TLR3 positivity	0.01	0.009	0.690(0.460–1.033)	0.013	0.062	0.440(0.440–1.021)
TLR4 positivity	0.725			0.302		

Abbreviations: CI, confidence interval; HbsAg, Hepatitis B surface antigen; TB, total bilirubin; AFP, alpha fetoprotein; TLR, toll-like receptor.

Table 3 Clinic Baseline and Correlation Analysis with TLR3 or TLR4

Variables		TLR3 Expression		*p*	TLR4 Expression		*p*
		Negative	Positivity		Negative	Positivity	
Sex	Male	158	129	0.669	242	45	0.103
	Female	32	23		51	4	
Age, year	<55	108	91	0.573	171	28	0.862
	≥55	82	61		98	17	
HbsAg	Negative	22	23	0.334	38	7	0.801
	Positivity	168	129		255	42	
TB, μmol/L	<20.4	169	142	0.152	265	46	0.438
	≥20.4	21	10		28	3	
γ-GT, U/L	<40	121	91	0.47	184	28	0.450
	≥40	69	61		109	21	
Albumin, U/L	<35	8	12	0.149	16	4	0.690
	≥35	182	140		245	77	
AFP, ng/mL	<20	71	57	0.98	107	21	0.396
	≥20	119	95		186	28	
Tumor size, cm	≤5	81	89	0.003	143	27	0.415
	>5	109	63		150	22	
Tumor number	1	172	141	0.461	269	44	0.640
	>1	18	11		24	5	
Tumor capsule	No	116	65	<0.001	158	23	0.365
	Yes	74	87		135	26	
Tumor differentiation	I–II	137	120	0.146	223	34	0.314
	III–IV	53	32		70	15	

Abbreviations: HbsAg, Hepatitis B surface antigen; TB, total bilirubin; AFP, alpha fetoprotein.

focus on the regulation of TLR on PD-L1 expression in tumor cells, but tumor-associated macrophage also plays a critical role in the development of HCC.[31,32]

ICD-associated DAMPs activate antitumor immune responses by activating the innate immunity represented by *TLR3* and *TLR4*.[33,34] Interestingly, the expressions of *TLR3* and TLR4 increased in lenvatinib-treated cells and played

contrasting roles in tumors. High expression of *TLR3* accelerates apoptosis of tumor cells, whereas *TLR4* upregulates PD-L1 to enhance immune escape capacity. These results were consistent with previous studies. *TLR3* mediates NK cell activation in HCC and directly induces apoptosis of liver cancer cells,[35,36] whereas *TLR4* promotes HCC initiation and development through the FGF21-IL17A[37] or BCL6-PD1[38] axis. In addition to HCC, *TLR4* activation induces PD-L1 expression in non-small cell lung cancer[39] and melanoma.[40] Studies have indicated that *TLR3* or *TLR4* agonists cooperate with immune checkpoint blockade treatments in cancer.[41–44] A combination of lenvatinib and immune checkpoints can resolve the deficiencies of monotherapies and improve the efficacy of drug therapy in advanced HCC.

This study had several limitations. First, we did not determine how ICD-associated DAMPs activated *TLR3* or *TLR4*. Future studies can investigate the mechanisms underlying this interaction. Second, we were unable to access HCC samples from patients treated with only lenvatinib. Therefore, we collected tumor samples from patients receiving lenvatinib plus anti-PD1 antibodies. No studies have investigated the effect of anti-PD1 treatment on *TLR3* or *TLR4* expression. Third, the hypothesis that PD-L1 expression varies with lenvatinib concentration requires further validation using cell models, animal models and clinical trials.

In conclusion, our study provides new insights into the mechanisms of lenvatinib-induced remodeling of the tumor microenvironment in HCC. We found that lenvatinib induces ICD in HCC and upregulates PD-L1 expression via *TLR4* while promoting cell apoptosis via *TLR3*. Remodeling of the immune microenvironment by lenvatinib is mediated in part by lenvatinib-induced ICD. The combination of lenvatinib and antibodies against PD-1 or PD-L1 may act synergistically in HCC treatment. PD-1 or PD-L1 antibodies can enhance the therapeutic effects of lenvatinib in HCC patients by targeting different aspects of the tumor microenvironment. Further studies are needed to validate these findings and explore the potential clinical implications of combining lenvatinib with immune checkpoint inhibitors in HCC treatment.

Abbreviations

ICD, immunogenic cell death; HCC, hepatocellular carcinoma; DAMPs, damage-associated molecular patterns; HMGB1, high mobility group box 1; HR, hazard ratios; CIs, confidence intervals; OS, overall survival; RFS, recurrence-free survival; TLR, toll-like receptor; IHC, immunohistochemistry.

Data Sharing Statement

The data used to support this research are included within this article and raw data are available from the corresponding authors.

Funding

This work was supported by National Natural Science Foundation of China (No. 82072672; No. 82072677), National Natural Science Youth Foundation of China (No. 82103417) and Beijing Igandan Foundation (GDXZ-08-22).

Disclosure

We declare no conflict of interest.

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