

Relationship between IL-10 and PD-L1 in liver hepatocellular carcinoma tissue and cell lines

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Research

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Abstract

Background Programmed death-ligand1 (PD-L1) plays a critical role in host immunity in the setting of cancer progression. Interleukin 10 (IL-10) is a multi-cellular, multi-functional cytokine that regulates cell growth and differentiation and participates in inflammatory and immune responses. The purpose of this study was to clarify the relationship between PD-L1 and IL-10 and their clinical importance in liver hepatocellular carcinoma(LIHC).

Methods LIHC patients (n=100) who underwent surgery with preoperative therapy were included in the study. By immuno-histochemical staining, PD-L1, IL-10 and CD8 positive cells were examined in resected specimens. The gene expression levels of PD-L1, IL-10 and Met were detected by qRT-PCR and Western blots, and differentially compared in cancer, adjacent and normal tissues. In cell experiments, the Bel7405 and MHCC97-H cell-lines were incubated with IL-10 or anti-IL-10 antibody, and then PD-L1 and Met expression levels were compared by ELISA and Western blots. The effect of crizotinib and/or IL-10 on the proliferation, invasion and migration of LIHC cell-lines was estimated by CCK8 and transwell assay.

Results In tumor tissues, the mRNA and protein levels of PD-L1, IL-10 and Met were higher than those in adjacent tissues. The high expression levels of PD-L1 and IL-10 indicated a poor prognosis. IL-10 reduced the expression of PD-L1 in LIHC cell-lines via Met signaling pathway. Over-expression of PD-L1 increased the levels of IL-10, and Met in in LIHC tissue and cell lines. The combination of crizotinib and IL-10 were more effective in inhibiting the proliferation, migration and invasion of LIHC cell lines.

Conclusions The combination of IL-10 and PD-L1 monoclonal antibody may have therapeutic promise in treating LIHC.

Introduction

Primary liver cancer (referred to as liver cancer) is the second leading cause of death worldwide, with histological types of hepatocellular carcinoma, intrahepatic cholangiocarcinoma, hepatocellular carcinoma, and fibrolamellar liver cancer. Liver hepatocellular carcinoma (LIHC) accounts for about 90% of primary liver cancer. The prevalence of liver cancer varies among regions, with Central and South Asia, Northern Europe and East China, Europe having the lowest incidence, followed by Western Europe and South Africa while East Asia, Southeast Asia and West Africa have the highest incidence. Worldwide, more than 782,000 cases of new primary liver cancer arose in 2012, accounting for 5.6% of the world's total cancers, taking the 6th place among all cancers (the top five were lung cancer, female breast cancer, gastrointestinal cancer, prostate and esophageal cancer). In 2012, 746,000 people were killed by liver cancer, accounting for 9.1% of all deaths during the same period, ranking the second. A majority of patients with liver cancer were from China, accounting for almost half of all liver cancer cases worldwide, with a high incidence rate and mortality rate. There were 316,000 cases of deaths, accounting for 14.2% of all deaths (ranking the 2nd), with an incidence and mortality rate far higher than the average level of the world. Wide infection of chronic HBV in China along with long-term intake of aflatoxin(1 -

5) contributed to this phenomenon. The application of surgical resection in the treatment of liver cancer has proved highly efficient. In addition, radiofrequency ablation, chemotherapy, and biotherapy have also contributed to improved life quality of patients. However, studies on prolonging the survival of advanced LIHC patients requires more efforts, indicating the urgent need for novel treatments.

Programmed death-ligand 1 (PD-L1, also called B7-H1 or CD274) is expressed on the surface of many kinds of tumor cells and tumor-infiltrating immune cells (ICs). PD-L1 over-expression in cancer cells inhibits the activity of T lymphocytes (6, 7), which results in the blocking of anti-tumor immunity and immune escape (8). In 2013, the first PD-L1 inhibitor was used in the treatment of lung cancer and achieved amazing clinical results.

In the next few years of drug clinical trials, PD-L1 inhibitors have also achieved good results in the treatment of cancer (9–11). However, it also has some setbacks during treatment, which predominantly involve side-effects and a low clinical response rate (12–14). Therefore, to improve the clinical efficacy (15, 16), the PD-L1 inhibitor is usually combined with chemotherapy (17, 18) and/or radiotherapy (17, 19, 20). Some recent studies have focused on the efficacy of PD-L1 inhibitors combined with immune factors (21–23).

Interleukin 10 (IL-10) is a multifunctional cellular immunosuppressive factor produced by Th2 cells, and plays an important inhibitory role in inflammatory and immunological responses. By inhibiting the activation of natural killer (NK) and T cells, IL-10 enables tumor cells to escape immune surveillance (24, 25). However, IL-10 also functions in immune activation, which promotes tumor-specific immune surveillance and reduces the occurrence of pathogenic inflammatory reactions by activating T and NK cells. Therefore, IL-10 plays a key role in this two-way immune regulatory system (26–28). Some articles indicate that the expression level of PD-L1 is correlated with the expression level of IL-10 in the tumor micro-environment (29–33). However, the relationship between both factors remains unclear.

Mesenchymal-epithelial transition factor (Met) is a tyrosine kinase (TK) receptor for hepatocyte growth factor (HGF) (34, 35) (Fig. 1A). Activation of the Met gene leads to multiple downstream pathways that promote a tumorigenic phenotype.

In our study, we found that in LIHC tissue, PD-L1 expression levels were positively correlated with IL-10 expression levels. Over-expression of PD-L1 upregulates IL-10 expression in LIHC tissue and cells lines by positive feedback regulation. IL-10 down-regulates the expression levels of PD-L1 in LIHC cells-lines by negative feedback regulation. We have also discovered that PD-L1 and IL-10 are linked by the Met signaling pathway (Fig. 1B and C).

Thus, our study provides new insights into the functional role of IL-10. The efficacy of combined IL-10 and PD-L1 inhibitor therapy was shown to be better than use of the PD-L1 inhibitor alone.

Material And Methods

Bioinformatics Analysis

A bioinformatics analysis was conducted by the TCGA database (<https://www.cancer.gov/>) and the TCGA-based visualization website GEPIA (<http://gepia.cancer-pku.cn/>). The expression correlation of PD-L1 and IL-10 genes of LIHC was analyzed. The analyses also included the relationship between IL-10 and overall survival (OS) or disease-free survival (DFS) .

Clinical samples

One hundred patients that comprised 55 males and 45 females, with liver hepatocellular carcinoma (LIHC) in the Third Affiliated Hospital of Soochow University from September 2013 to September 2018 were included in the study, which was approved by the Hospital Ethical Review Committee. No patients were treated with any relevant chemotherapy, radiotherapy, targeted therapy, or immunotherapy before surgery. Among 100 LIHC surgical resections, 50 cases were highly differentiated and 50 cases moderately or poorly differentiated. From each patient, three specimens were collected from liver hepatocellular carcinoma tissues, adjacent tissues to the tumor, and normal tissues. The specimens were confirmed by pathological diagnosis as LIHC. A portion of the tissues was embedded in paraffin and another part was freshly resected surgical tissues, and stored in liquid nitrogen for further experiments.

Patient follow-up

Patients were instructed to return for follow-up, including a clinical examination, thoracic computed tomography, and abdominal ultrasonography, at 3-6 month intervals for one year, and then every 6-12 months thereafter.

Immunohistochemistry

The paraffin-embedded tumor samples were sectioned to 5 μm slices. The tissue section slides were deparaffinized and rehydrated. For PD-L1, IL-10 and CD8 were stained by corresponding antibodies. Detailed experimental processes can be seen in the supplementary materials. The cell intensity and the rate of positive cells were recorded (Table S1). For data analysis, the final scores of less than eight were defined as low expression and scores of eight or more, were defined as high expression.

Quantitative RT-PCR

The tissue pieces were first cut into small sections, and then a homogenizer was used to extract the total RNA using the Trizol method. We compared the differences in IL-10, PD-L1, and Met mRNA expression among cancerous tissues, adjacent tissues, and normal tissues by quantitative RT-PCR using the described primers (please see Table S2). Detailed experimental processes can be seen in the supplementary materials.

Cell culture

Human liver hepatocellular carcinoma Bel7405 and MHCC-97-H cell-lines were obtained from the Cell Research Center, Third Affiliated Hospital of Soochow University for in vitro studies. Detailed culture conditions can be found in the supplementary materials.

SiRNA construction of liver hepatocellular carcinoma cell-lines

We used products of obtained from the Gene Pharma Company to build siMET and siPD-L1. Detailed experimental process can be seen in the supplementary materials. SiRNA sequences are listed in Table S3.

Over-expression of PD-L1

The hepatocellular carcinoma cell lines over-expressing PD-L1(Lentivirus-mediated) is from Cell Research Center ,the Third Affiliated Hospital of Soochow University.In the article, it is abbreviated as LV-PD-L1.

ELISA

One day before drug treatment, cells in logarithmic growth phase were removed and seeded on a six-well plate at a density of 3×10^4 - 10^5 per well.When the cells reach 70% to 80% confluency, add different concentrations of IL10(Creative BioMart,New York,USA) or anti-IL10 antibody (Creative Biolabs,New York,USA)to the cells.When the cells were cultured for 24 to 48 hours, the cells were counted by a hemocytometer to ensure that the number of cells per well was approximately the same.PD-L1 quantikine elisa kit(R&D systems,Minneapolis,MN,USA) was used to test the PD-L1 expression level in cell culture supernate or cell lynate of after 24 hours and 48h.

At the same time, After successful transfection of si PD-L1 Bel7405, si PD-L1 MHCC-97H, and LV PD-L1 Bel-7405, LV PD-L1 MHCC-97H cell culture supernatant was taken to test the IL-10 expression level after 24,48 hours.Human IL-10 high sensitivity Elisa kit(Multi Sciences, Hangzhou, Zhejiang, China) was used to test the IL-10 expression level.

CCK8

We used the CCK8 assay (Dojindo, Tokyo, Japan) to detect the proliferation of cells after treatment with Crizotinib (Cell Signaling Technology, Danvers, MA, USA) and Crizotinib combined with IL-10. Lastly, we added Crizotinib or Crizotinib combined with IL-10 to the constructed siMet Bel7405 and siMet MHCC-97-H. The proliferation of cells was detected by CCK8. Detailed experimental processes can be seen in the supplementary materials.

Transwell Test

Analysis of tumor cell migration and invasion was performed by a transwell chamber assay (Corning, New York, USA). About $2-5 \times 10^3$ cells, Bel7405 or siMet-Bel7405 cells and MHCC-97H or siMet-MHCC-97H

cells, were seeded onto the upper chamber in serum-free medium, and then 500 μ l of medium containing 1% FBS was added to the lower chamber. Crizotinib or crizotinib combined with IL-10 were added to the lower chamber. After 24 or 48 h incubation, the cells were fixed by incubating in 4% formaldehyde at room temperature for 5 min. After staining with crystal violet, the number of cells that migrated or invaded were observed under the light microscope.

Western blots

PD-L1 expression levels, Met signaling pathway-related molecules (Met and phospho-Met) and their downstream MAPK signaling pathway-related molecules (akt, phospho-akt, Mek, phospho-Mek, Erk, phospho-Erk) were analyzed by Western blotting. Meanwhile, we performed Western immunoblotting on LIHC cells that over-expressed or knocked-down PD-L1 gene expression to detect differences in IL-10 expression levels.

After that, Crizotinib or Crizotinib combined with IL-10 were added to the cells. Met signaling pathway-related molecules (Met and phospho-Met) were analyzed. In our tissue experiments, the tissue pieces were first cut into small sections, and then a homogenizer was used to extract tissue protein using the protein extraction agent. We compared the differences in IL-10, PD-L1, and Met expression among cancerous tissues, adjacent tissues, and normal tissues. Detailed experimental processes can be seen in the supplementary materials.

Statistical Methodology

Quantitative data were presented as mean \pm SD. Comparison of the differences between the two groups of data used the X^2 test. Kaplan-Meier curves were generated for OS and DFS. SPSS version 25.0 statistical software was used to assess Cox regression analyses. Alpha values of $P < 0.05$ were considered statistically significant. All data was analyzed by SPSS version 25.0 or Graphpad 5.0 software. Western blotting gray values were analyzed by Image J software.

Results

Patient characteristics and immunohistochemistry.

The demographic and clinicopathologic characteristics of 100 LIHC patients are summarized in Tables 1 and 2. By the immunohistochemistry (IHC) evaluation, PD-L1 was mainly expressed in the membrane and cytoplasm (Fig. 2A, and B) and IL-10 in the cytoplasm of cancer cells (Fig. 2C, and D). CD8 was mainly located on the surface of the T lymphocyte membrane (Fig. 2E, and F). Among the 100 LIHC cases, 75 cases (75.0%) exhibited high PD-L1 expression levels and 67 cases (67.0%) displayed high IL-10 levels. A comparison of the clinicopathological characteristics according to the PD-L1 expression levels is listed in Table 1. Higher PD-L1 expression levels tended to be related to patients of higher age ($P = 0.048$), poor tumor differentiation ($P = 0.001$), advanced T stage (0.000), higher IL-10 expression levels (0.000) and

higher CD8 expression levels (0.023). Higher IL-10 expression levels were associated with higher age (P = 0.000), advanced T stage (P = 0.000), N stage (P = 0.000), tumor size (P = 0.000) and higher PD-L1 expression (P = 0.000) of LIHC (Table 2).

Table 1
Demographic and clinical characteristics deatd-ligand1 expression in
100 patients with LIHC

Characteristic	n	PD-L1		χ^2	P
		Low	High		
		25	75		
Sex				0.337	0.562
Male	55	15	40		
Female	45	10	35		
Age (years)				3.922	0.048
< 60	32	12	20		
≥ 60	68	13	55		
Tumor differentiation				12.000	0.001
High	50	20	30		
Moderate/Low	50	5	45		
T stage				59.524	0.000
T1/T2	28	22	6		
T3/4	72	3	69		
N stage				0.000	1.000
N0	52	20	32		
N1	48	5	43		
Tumor location				2.772	0.096
Left half liver	62	12	50		
Rjght half liver	38	13	25		
Tumor Size				0.152	0.696
< 5 cm	73	19	54		
≥ 5 cm	27	6	21		
IL10 status				50.52	0.000

P < 0.05 was considered the difference has statistical significance .

Characteristic	n	PD-L1	χ^2	P	
Low expression	26	20	6		
High expression	74	5	69		
CD8					
Low expression	30	3	27	5.143	0.023
High expression	70	22	48		
P < 0.05 was considered the difference has statistical significance .					

Table 2
Demographic and clinical characteristics and IL-10 in 100 patients
with LIHC

Characteristic	n	IL10		χ^2	P
		Low	High		
		33	67		
Sex				1.813	0.178
Male	55	15	40		
Female	45	18	27		
Age (years)				95.528	0.000
< 60	32	8	24		
≥ 60	68	25	43		
Tumor differentiation				2.216	0.137
High	50	13	37		
Moderate/Low	50	20	30		
T stage				42.477	0.000
T1/T2	28	23	5		
T3/T4	72	10	62		
N stage				100.00	0.000
N0	52	16	36		
N1	48	17	31		
Tumor location				0.455	0.500
Left half liver	62	22	40		
Rjght half liver	38	11	27		
Tumor Size				100.00	0.000
< 5 cm	73	19	54		
≥ 5 cm	27	14	13		
PD-L1 status				27.876	0.000

P < 0.05 was considered the difference has statistical significance .

Characteristic	n	IL10	χ^2	P
Low expression	25	19	6	
High expression	75	14	61	
CD8			1.811	0.178
Low expression	30	7	23	
High expression	70	26	44	
P < 0.05 was considered the difference has statistical significance .				

Positive correlation between IL-10 and PD-L1 expression levels in tissues.

Among the 100 LIHC cases, the high expression level of PD-L1 was observed in 72 tumor tissues (72.0%) and in 28 adjacent tissues (28.0%). In tumor tissues, the expression of PD-L1 was significantly higher than that of adjacent tissues (P = 0.000; Table 3). Meanwhile, the high expression levels of IL-10 were observed in 67 tumor tissues (67.0%) and in 35 adjacent tissues (35.0%). In tumor tissues, the expression of IL-10 was significantly higher than that of adjacent tissues (P = 0.000; Table 4). According to TCGA database, there was a positive correlation between PD-L1 and IL-10 expression (Fig. 3A), Furthermore, there was also positive correlation between PD-L1 and CD8 expression (Fig. 3B). In our study, by Pearson correlation analysis, there was a positive correlation between the expression levels of PD-L1 and IL-10 in tumor and adjacent tissues (Fig. 3C and D), and a positive correlation was also found between the expression levels of PD-L1 and CD8 in tumor and adjacent tissues (Fig. 3E, and 3F). Moreover, when analyzing both mRNA by PCR and protein expression by Western blots, we found expression levels of PD-L1 and IL-10 in tumor tissues were higher than those found in adjacent and normal tissues, and the expression levels of Met in the tumor tissues were higher than those found in adjacent tissues (Fig. 3G-J).

Table 3
Comparison of PD-L1 expression levels in LIHC tissues and adjacent tissues

	n	PD-L1 high expression	PD-L1 low expression	χ^2	P
LIHC tissue	100	75	25	44.220	0.000
adjacent tissue	100	28	72		
P < 0.05 was considered the difference has statistical significance .					

Table 4

Comparison of IL10 expression levels in LIHC tissues and adjacent tissues

	n	IL high expression	IL low expression	χ^2	P
LIHC issue	100	67	33	20.488	0.000
adjacent tissue	100	35	65		
P < 0.05 was considered the difference has statistical significance .					

Relationship between the IL-10 expression level and survival periods

According to TCGA database, the expression level of the IL-10 gene did not affect overall survival (OS) or disease free survival (DFS) of patients with LIHC (Fig. 4A and 4B). However, in our study, the high IL-10 expression level group tended to have shorter survival periods than those with low IL-10 expression levels. The 5-year overall survival (OS) rate ($p=0.01$) in LIHC patients with the IL-10 high expression level was poor as compared those with low expression levels, but disease free survival (DFS) was of no statistical significance (Fig. 4C and 4D). The 5-year DFS ($p=0.02$) and OS ($p=0.04$) in LIHC patients with low IL-10/PD-L1 expression levels were longer than those with high IL-10/PD-L1 expression levels (Fig. 4E and 4F).

Correlation between IL-10 and PD-L1 expression levels in LIHC cell-lines

ELISA studies showed that in both Bel7405 and MHCC 97-H cells, the over-expression of PD-L1 expressing cells increased the IL-10 expression levels. The IL-10 expression levels in siPD-L1 Bel7405 and MHCC 97-H cells was lower than that found in the LV-PD-L1 and control group (Fig 5A and 5C). The IL-10 expression level was time-dependent. Detection by Western blots showed that the knockdown of PD-L1 (siPD-L1) decreased IL-10 levels, and yet the over-expression of PD-L1 (LV-PD-L1) increased the levels and appearance of IL-10, Met and phospho-Met cells, when compared with control cells (Fig. 5B, and D).

Detecting by ELISA, PD-L1 was expressed in both cell culture supernatant and cell lysates of Bel7405 and MHCC 97-H. The PD-L1 expression level was significantly higher in cell lysates than that found in the supernatant (Fig. 6A-D). At the protein level, IL-10 inhibited PD-L1 expression in both cell-lines, and anti-IL-10 antibody promoted the expression of PD-L1 in both cell-lines (Fig. 6A-F). IL-10 acted by inhibiting the Met signaling pathway and the downstream MAPK signaling pathway (Fig. 6E, and F). Knockdown of Met gene expression in both cell-lines had no significant effect on PD-L1 expression levels after different treatments (Fig. 6G). The above phenomena were concentration and time-dependent.

Effect of IL-10 and Crizotinib on proliferation, migration and invasion of LIHC cells.

When compared with crizotinib treatment alone, the combination of IL-10 and Crizotinib more potently inhibited proliferation, migration and invasion of LIHC cells (Fig. 7A and B; Fig. 8A and B). After knockdown of the Met gene, there was no significant difference found in terms of the proliferation,

migration and invasion of LIHC cells when comparing any of the treatments with combination therapy of both drugs and crizotinib alone (Fig. 7C and D; Fig. 8C and D). Moreover, Western blot assay showed that the combination of crizotinib and IL-10 was more potent at inhibiting PD-L1 expression than use of crizotinib alone. This effect worked via the Met signaling pathway (Fig. 7E and F).

Discussion

Bioinformatics is a new subject and plays an important role in current cancer research for studying the collection, processing, storage, dissemination, analysis and interpretation of biological information. TCGA is a cancer gene database developed by the National Cancer Institute and the National Human Genome Research Institute in the United States. Using the relevant database, we have found a correlation between the expression levels of PD-L1 and IL-10 in patients with LIHC, which provides novel ideas and a basis for our experimental research.

At the transcriptional and translational levels, the higher expression of IL-10 and PD-L1 were observed in cancer tissues, when compared with those in normal tissues. In tumor and adjacent tissues, the PD-L1 expression level was positively correlated with IL-10 expression levels, and was also positively correlated with the CD8 + T cells. These results are consistent with previous studies(36,37).Moreover, in cancer tissues, the expression levels of Met at both the transcriptional and translational levels was significantly higher than those in adjacent tissues.

We also discovered that higher IL-10 expression levels were associated with higher age, advanced T stage, N pathological stage and higher PD-L1 expression. On the other hand, higher PD-L1 expression levels tended to be related to patients of higher age group, poor tumor differentiation, advanced T stage, higher IL-10 expression levels and higher CD8 expression levels. In addition, PD-L1 combined with IL-10 might also be an independent prognostic factor in patients with LIHC. Patients in the low IL-10/low PD-L1 group had an improved prognosis than those in the high IL-10/high PD-L1 group. Results were consistent with prior studies(38–40).LIHC patients with high IL-10 expression levels usually had a shorter overall survival (OS), and with low IL-10 expression levels had longer OS. As a result, IL-10 might be an independent prognostic factor in patients with LIHC(41–44).

Some of our experimental results are consistent with bioinformatics analysis and have shown that PD-L1 and IL-10 expression levels were positively correlated, furthermore, PD-L1 and CD8 expression levels were also positively correlated. However, some results are inconsistent with bioinformatics analysis. The TCGA survival analysis has shown that the expression level of the IL-10 gene did not affect overall survival (OS) or disease free survival (DFS) of patients with LIHC. However, in our study, the high IL-10 expression level group tended to have shorter survival periods than those with low IL-10 expression levels. These different reasons might be best described as follows. First, the TCGA database generally collects tumor information from European and American research studies. Differences in expression levels of related factors were due to ethnic differences. Second, the TCGA database generally uses high-throughput sequencing to detect the expression at the gene level, which might not be different at the protein level.

Third, due to the difference in sample numbers, data reported in the current study was different from the result of TCGA.

In tissue experiments, we found that the expression levels of PD-L1 and IL-10 were higher in cancer tissues than in adjacent tissues. There was also a positive correlation between the two factors. Similarly, in cellular experiments, we found that over-expression of PD-L1 can over-activate the Met signaling pathway, thereby up-regulating IL-10 expression. Thus, we infer that whether in cancer tissues or cells, over-expressed PD-L1 can activate the Met signaling pathway via positive feedback regulation, thereby up-regulating IL-10 expression. Instead, we also found that IL-10 down-regulates the expression of PD-L1, and anti-IL-10 up-regulates the expression of PD-L1 through the Met signal pathway. Thus we infer that IL-10 can down-regulate PD-L1 expression by inhibiting the Met signaling pathway and the down-stream MAPK signaling pathway via negative feedback regulation (Fig. 1B and C).

Some previously published studies suggest that IL-10 is an immunological negative regulator and inhibits the function of T, B and dendritic cells and up-regulates PD-L1 expression (29–31, 45). Recent data have shown that IL-10 release might be mediated following PD-L1 blockade, which sustains immunosuppression in ovarian cancer (30). HGF/MET signaling is essential for cancer survival (46) and important in the interaction between IL-10 and PD-L1, which is involved in the immune response, including an increase in IL-10 (47–49) and PD-L1 which co-localizes with MET (50). Thus, in our results, IL-10 down-regulates PD-L1 expression, and does so probably by blocking the Met signaling pathway and the downstream MAPK signaling pathway. When the Met gene was knocked down, we observed no significant differences in the PD-L1 expression levels in different intervention groups. The results demonstrated that the Met signaling pathway plays an important role between IL-10 and PD-L1. The role of IL-10 is complex and multifaceted. IL-10 promotes immune activation, increases tumor-specific immune surveillance and reduces the occurrence of pathogenic inflammatory reactions by activating T and NK cells.

Reportedly, IL-10 inhibits immune cell function and up-regulates PD-L1 expression (29–31, 45). However, our study suggests that IL-10 activates immune cell function, inhibits the Met signaling pathway, and down-regulates PD-L1 expression in LIHC cells. The results provide new mechanisms concerning the interaction between IL-10 and PD-L1, which might benefit clinical applications in the near future. Therefore, more in vivo and in vitro experiments are arranged to determine the involved modes and mechanisms of action. Our research of course, has still much follow-up work with a deeper exploration of the molecular mechanism of this phenomenon being required.

Declarations

Data availability

All the data were available upon appropriate request .

Disclosure

Qian Qian and Jianping Chen contributed equally to this work.

Authors' Contributions

Qian Qian wrote the manuscript. Changping Wu revised the manuscript. Qian Qian, Jianping Chen and Weibing Wang did the data analysis. Qian Qian and Jianping Chen did the data collection.

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Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work. The work was performed in accordance with the local Ethics Committee of the Third Affiliated Hospital of Soochow University. Written informed consent was obtained from each patient, including signed consent for tissue analysis and consent to be recorded for potential medical research at the time of sample acquisition.

Conflict of Interest

The author declare that there are no conflicts of interest regarding the publication of this paper.

Consent for publication

All presentations of LIHC cases have agreed for publication.

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Figures

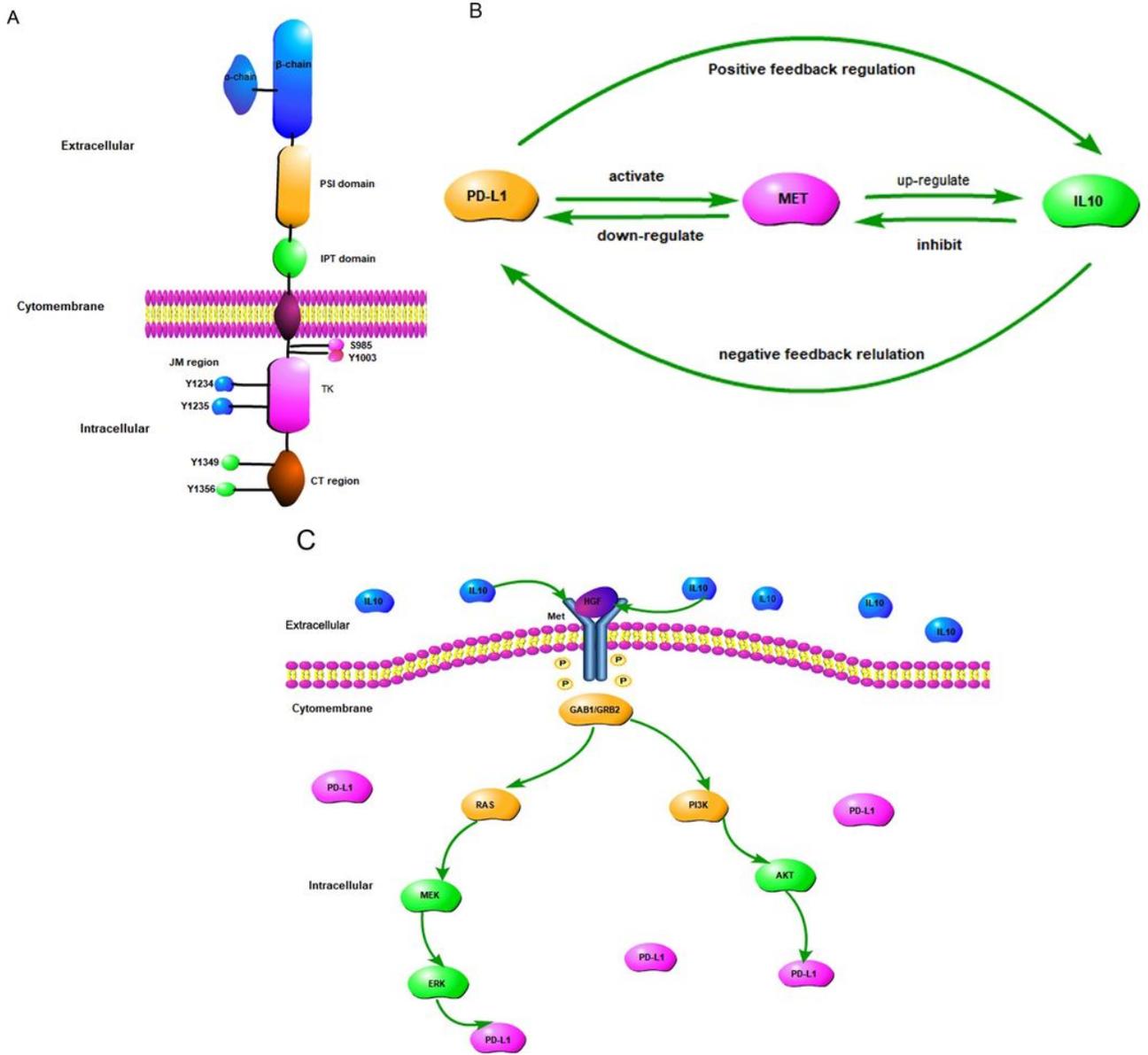


Figure 1

Hand-drawn picture of interactions between PD-L1, Met, and IL10. A, Met protein structure: Met is a heterodimer consisting of an extracellular alpha chain, and a beta chain that spans the membrane. The Met receptor has three functionally distinct domains, including the extracellular, transmembrane, and

intracellular domain. The intracellular domain functions in three channels, including: a region adjacent to the membrane-proximal intracellular domain, a tyrosine kinase catalytic structure with tyrosine kinase activity domain, a C-terminal domain that interacts with a variety of downstream signaling molecules. B, dynamic interactions between PD-L1, Met, and IL10. The over-expression of PD-L1 upregulates the expression of IL-10 in LIHC via a positive feedback loop While IL-10 down-regulates the expression of PD-L1 in LIHC via a negative feedback loop. C, IL10 acts on the HGF (hepatocyte growth factor)/Met signaling pathway, thereby affecting its downstream akt, MAPK signaling pathway before down-regulating the PD-L1 expression.

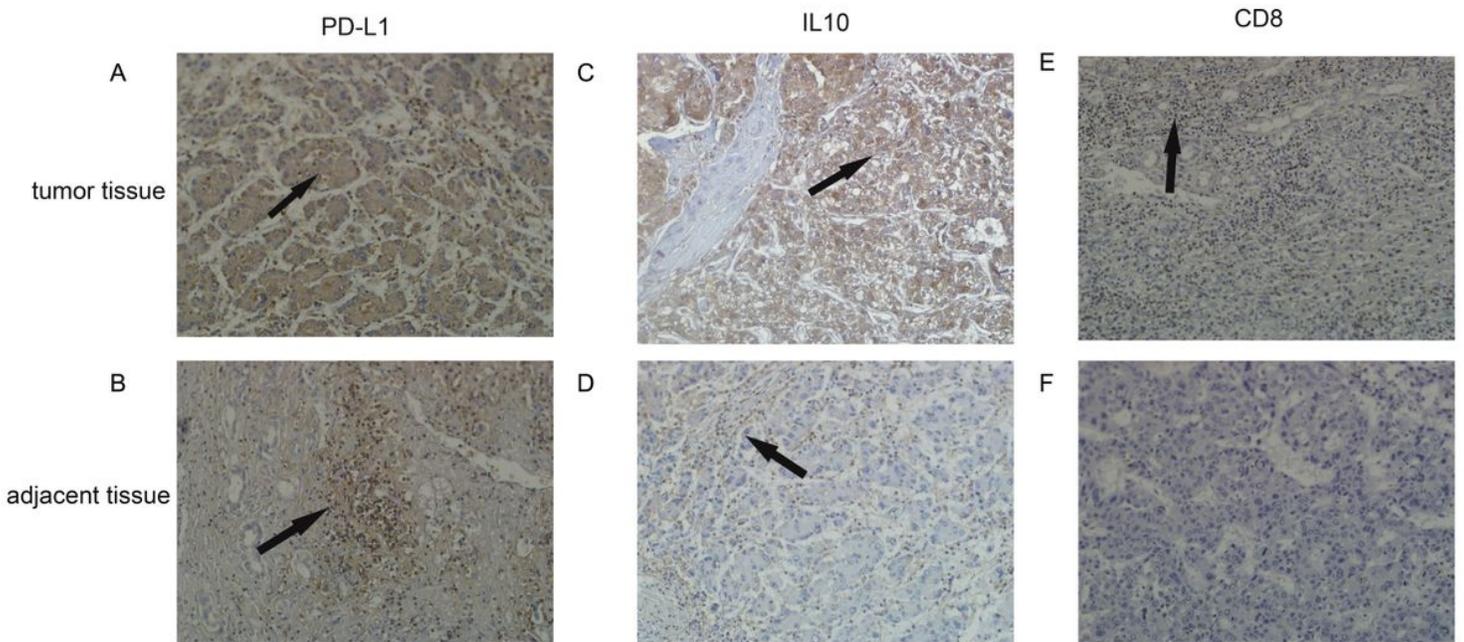


Figure 2

Expression of PD-L1, IL10 and CD8 in LIHC tissues. A, B. Representative images of immunohistochemical staining with anti-PD-L1 in LIHC tissues (A) and adjacent tissues (B). C, D. Representative images of anti-IL10 in LIHC tissues (C) and adjacent tissues (D). E, F. Representative images of anti-CD8 in LIHC tissues (E) and adjacent tissues (F). The arrows are for positive regions. Magnification: $\times 200$.

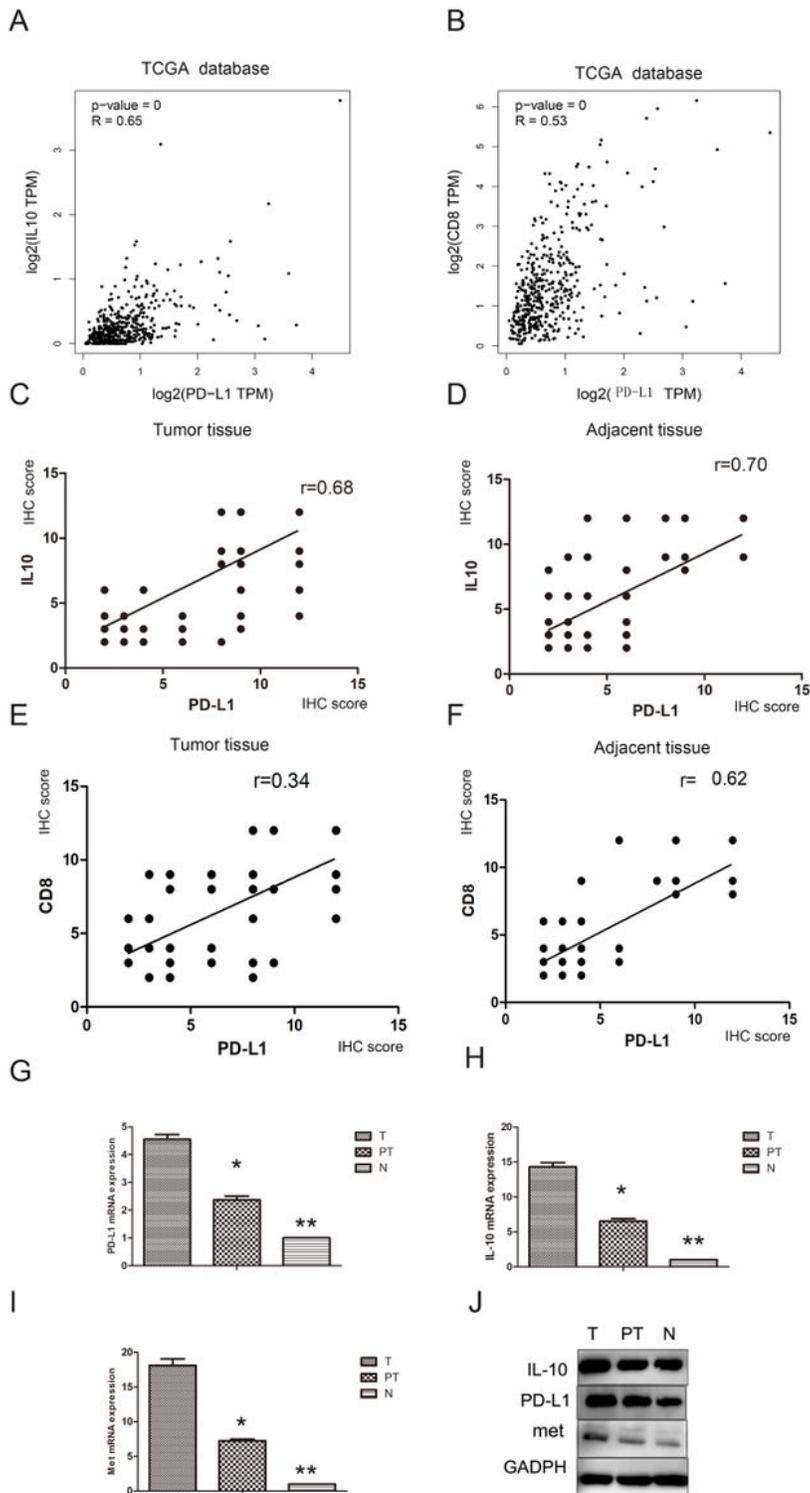


Figure 3

Relationship between the expression levels of PD-L1 IL10 and CD8, and comparison of PD-L1, IL10 and Met in LIHC. A. Correlation analysis of PD-L1 and IL10 gene expression in LIHC according to TCGA database. B. Correlation analysis of PD-L1 and CD8 gene expression in LIHC according to TCGA database. C, D. Pearson correlation analysis of expression level of PD-L1 and IL10 in LIHC tissues (C) and adjacent tissues (D). E, F. Pearson correlation analysis of expression level of PD-L1 and CD8 in LIHC

tissues (C) and adjacent tissues (D). E-H. Comparison of IL-10, PD-L1 and Met mRNA expression levels in LIHC tissues, adjacent tissues and normal tissues by quantitative real-time PCR and Western blots. $P \leq 0.05$ was considered statistically significant. T: tumor tissues; PT: adjacent tissues; N: normal tissues. * $P < 0.05$ compared with tumor tissues; ** $P < 0.05$ compared with adjacent tissues

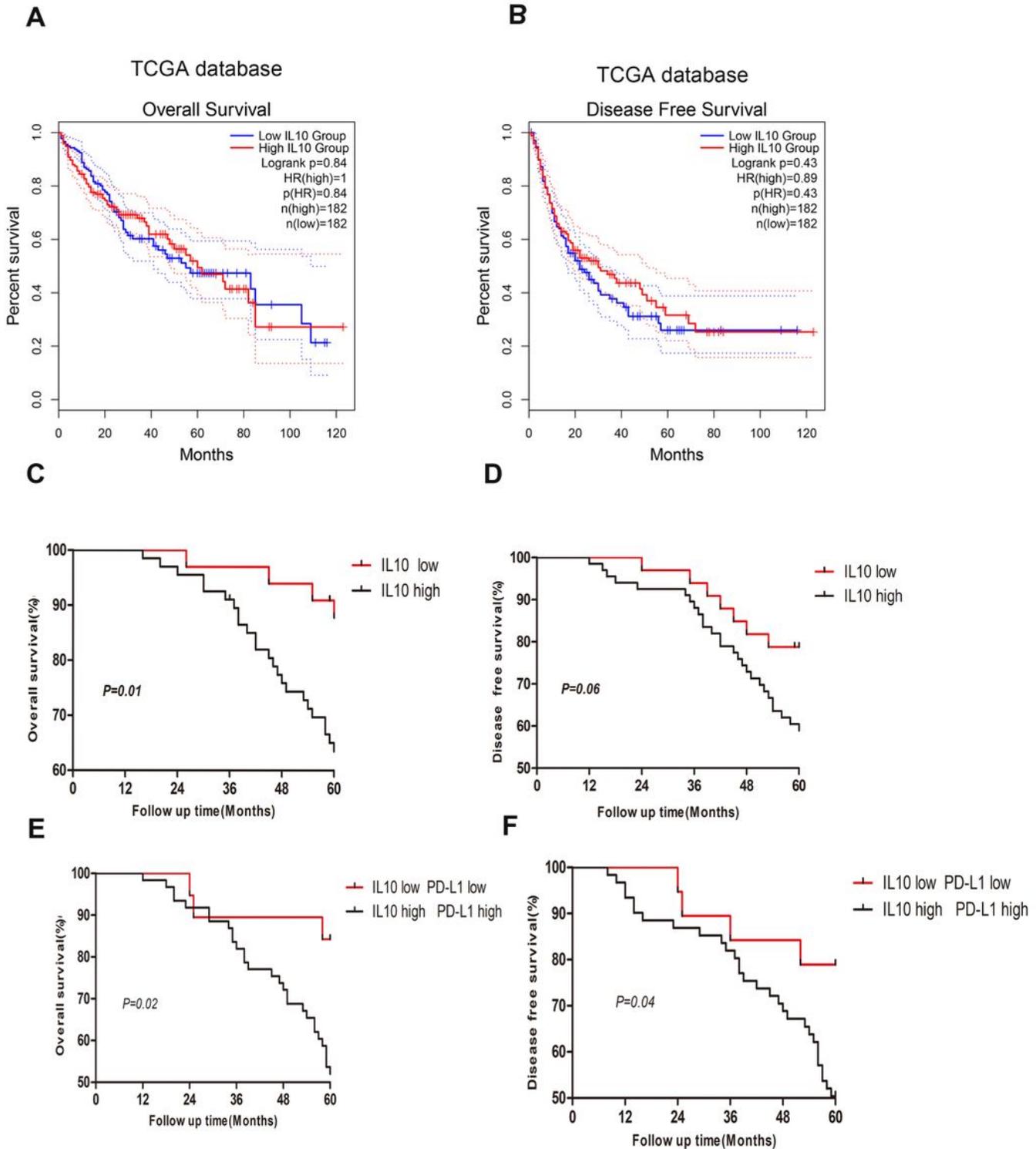


Figure 4

Prognosis according to the PD-L1 and IL-10 expression levels in patients with LIHC. A, B. Overall survival (OS) and disease-free survival (DFS) according to IL-10 expression of patients with LIHC according to TCGA database. C, D. Overall survival (OS) and disease-free survival (DFS) of patients with LIHC in relation to IL-10 expression status. E, F. OS and DFS rates of patients in relation to the PD-L1 and IL-10 expression status.

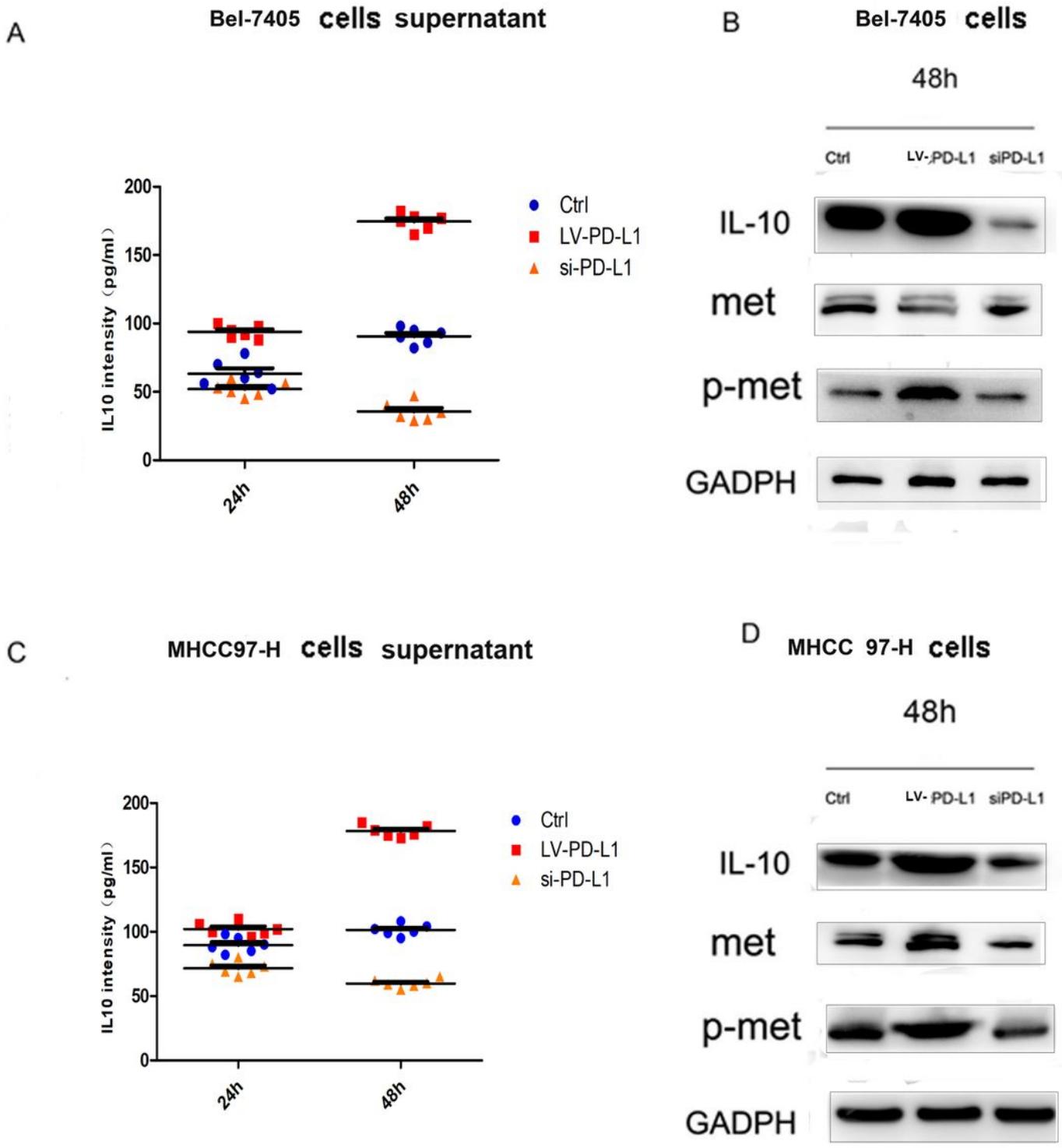


Figure 5

Effects of PD-L1 knockdown and over-expression on the expression levels of IL-10, Met and phosphor-Met in LIHC cell lines. Bel7405 and MHCC 97-H cells (C, D) were transfected with PD-L1 siRNA to knock down the expression of PD-L1 (siPD-L1), and with lentivirus to over-express PD-L1 (LV-PD-L1). Cells without any transfection were as the control. The IL-10 levels were detected by ELISA (A, C). The levels of IL-10, Met, p-Met were measured by Western blots. GADPH was used as the loading control (B, D).

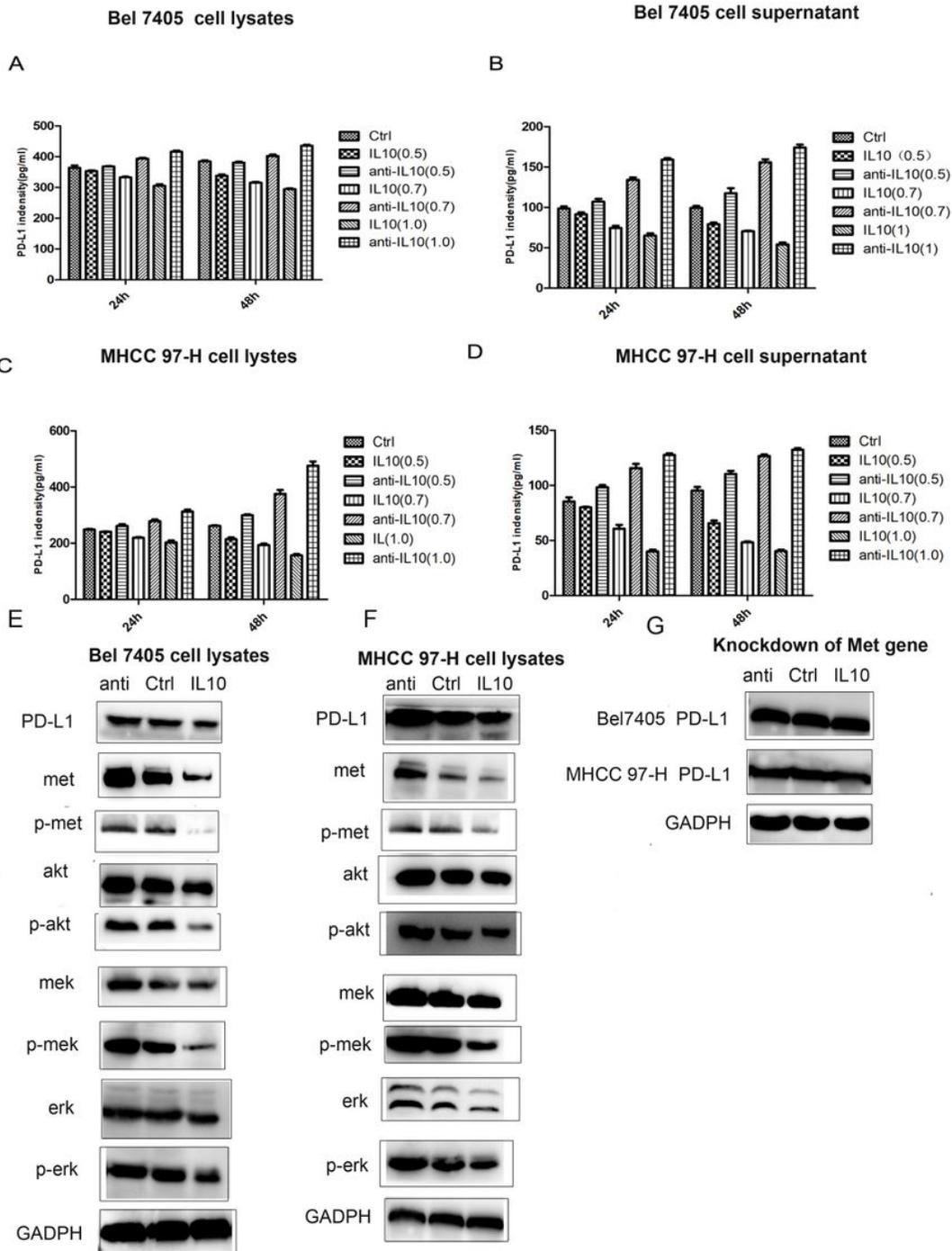


Figure 6

Figure 7

Effects of IL-10 and crizotinib on migration and invasion on LIHC cell lines. A, B. Effects of crizotinib and combination of crizotinib and IL-10 on invasion and migration on LIHC cell lines. C, D. Effects of crizotinib and combination of crizotinib and IL-10 on invasion and migration of LIHC cell lines after Met gene was knocked down in two cell lines. E, F. Western blot experiments for the effects of crizotinib and combination of IL10 and crizotinib on expression of PD-L1, Met signal pathway in two cell lines. *, $p < 0.05$ compared with control group. ** $p < 0.05$ compared with crizotinib group. Δ $p \geq 0.05$ compared with crizotinib group.

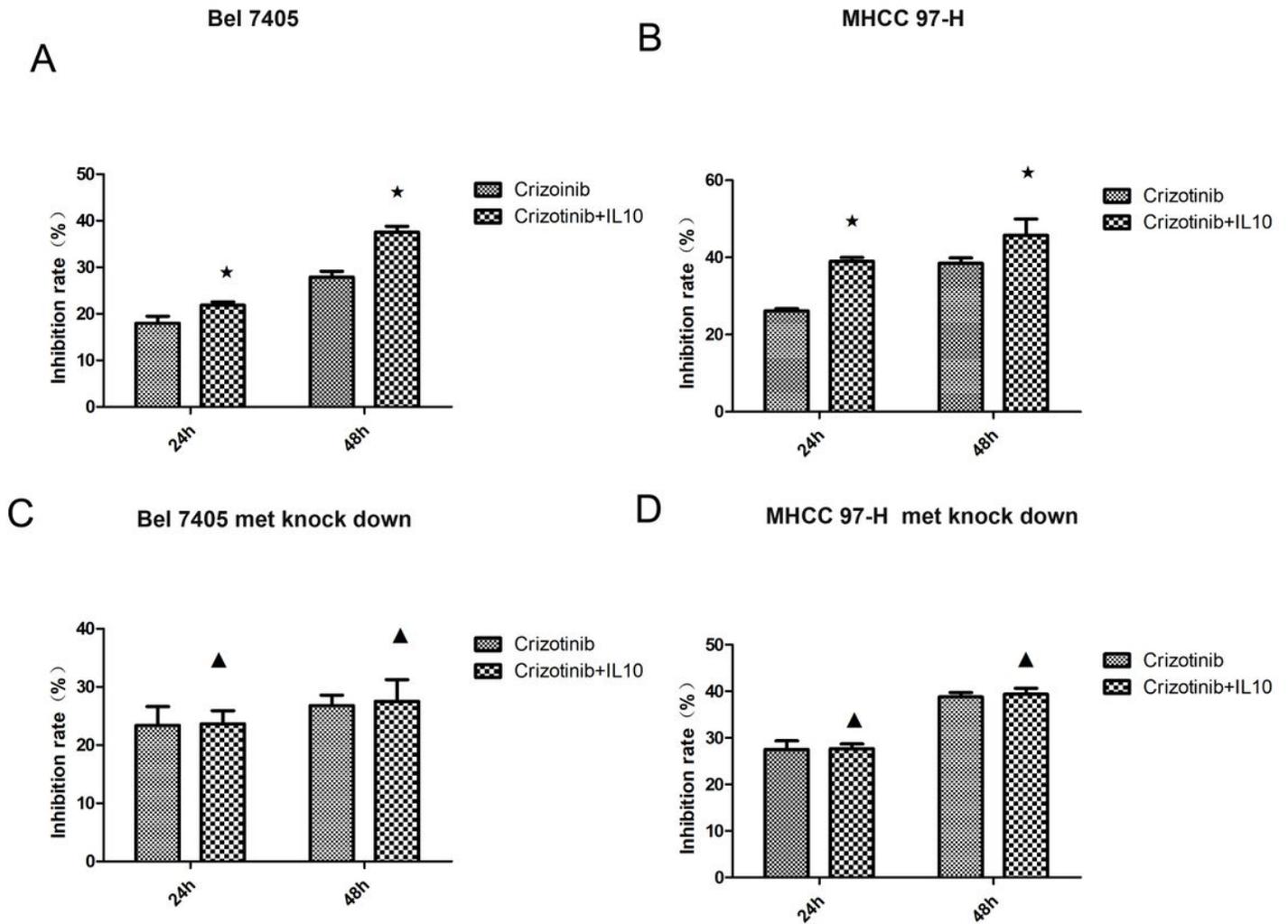


Figure 8

Effects of IL10 and Crizotinib on proliferation of LIHC cell lines. A, B Effect of crizotinib combined with crizotinib and IL10 on proliferation. C, D Effect of crizotinib combined with crizotinib and IL10 on proliferation of Bel7405 and MHCC 97-H after Met gene was knocked down. \square $P < 0.05$ compared with Crizotinib group; Δ $P \geq 0.05$ compared with Crizotinib group.

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