Original Article

PPARα Suppresses PD-L1-Mediated Immune Escape by Down-regulating SPP1 in Human Hepatocellular Carcinoma

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Abstract

Purpose

The purpose of this study was to investigate the role of peroxisome proliferator-activated receptor α (PPARα) in hepatocellular carcinoma (HCC) and illuminate the potential molecular mechanisms involved.

Materials and Methods

Quantitative real-time polymerase chain reaction, Western blotting and immunohistochemistry were conducted to characterize PPARα expression in HCC tissues. Kaplan-Meier survival analysis was performed to evaluate the relationship between PPARα expression and overall survival. Gain- and loss-of-function approaches were adopted to investigate the effects of PPARα on programmed cell death ligand 1 (PD-L1) - mediated immune escape of HCC cells. Enzyme-linked immunosorbent assay was used to determine interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2) released by T lymphocytes co-cultured with HCC cells. Luciferase reporter assay was carried out to validate whether PPARα affected secreted phosphoprotein 1 (SPP1) promoter activity. Rescue experiments were conducted to further verify the presumed molecular mechanism involved.

Results

Here we found that PPARα was significantly down-regulated in HCC tissues compared with para-cancerous tissues and that patients with low PPARα expression experienced a shorter overall survival. PPARα over-expression significantly inhibited PD-L1 expression in HCC cells, whereas PPARα depletion promoted PD-L1 expression in HCC cells. Furthermore, PPARα over-expression promoted the release of IFNγ, TNFα and IL-2 by T lymphocytes co-cultured with HCC cells, whereas PPARα knockdown suppressed the production of these cytokines. Mechanistic studies revealed that PPARα repressed PD-L1 expression in HCC cells by down-regulating SPP1.
Conclusion

Our data indicate that PPARα suppresses PD-L1-mediated immune escape in HCC through down-regulating SPP1.

Key words

PPARα, PD-L1, Immune escape, SPP1, Hepatocellular carcinoma
Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-associated morbidity and mortality around the world, resulting in approximately 700,000 deaths annually [1,2]. It was estimated by the American Cancer Society that around 42,000 HCC cases were newly diagnosed and nearly 30,000 patients died of this malignancy in the United States in 2018 [3]. It is well acknowledged that there are many factors accounting for the high mortality in HCC patients, such as drug resistance, distant metastasis, stem cell-like properties and immune escape [4-6]. Furthermore, evidence is accumulating that immune escape has emerged as a crucial factor contributing to the initiation and progression of HCC [7, 8]. In spite of great advances in diagnosis and treatment of HCC, the long-term prognosis of patients remains rather satisfactory. Therefore, it is imperative to better understand the molecular mechanisms underlying HCC development and develop novel therapeutic strategies.

Peroxisome proliferator-activated receptor α (PPARα) is a member of the super-family of nuclear hormone receptors [9]. PPARα can be activated by a structurally diverse class of synthetic chemicals known as peroxisome proliferators and plays key roles in inflammation and lipid homeostasis [10,11]. Previous studies have reported that PPARα is implicated in multiple types of human malignancies, such as oral cancer [12], prostate cancer [13], medulloblastoma [14], glioma [15] and ovarian cancer [16]; nonetheless, its biological role in HCC remains poorly understood.

Secreted phosphoprotein 1 (SPP1), also known as osteopontin, is an extracellular matrix protein composed of about 300 amino acids and play vital roles in diverse types of biological processes [17,18]. A recent study has demonstrated that SPP1 could positively regulated programmed cell death ligand 1 (PD-L1) expression to promote immune escape in human lung adenocarcinoma [19]. Besides, PPARα has been confirmed to negatively modulate SPP1
expression through suppressing its promoter activity in human macrophages [20].

In the current study, we aimed to characterize the biological role of PPARα in HCC and elucidate the potential molecular mechanisms. Here we found that PPARα was significantly down-regulated in HCC tissues compared with matched adjacent tissues. Besides, our data revealed that PPARα suppressed (PD-L1-mediated immune escape of HCC cells through negative regulation of SPP1.

Materials and Methods

1. Patients and tissue sample collection

Clinical specimens of tumorous tissues and adjacent normal tissues were obtained from 60 HCC patients who received surgical resection at West China Hospital of Sichuan University (Chengdu, China). The clinicopathological characteristics of HCC patients were listed in Table 1. Overall survival was defined as the time interval between the date of surgery and the date of death or last follow-up.

2. Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. Reverse transcription was conducted using PrimeScript RT Regent Kit (Takara, Dalian, China) following the manufacturer’s guidelines. Real time PCR was carried out on the ABI 7000 Taqman System (Applied Biosystems, Foster, CA) using the SYBR Green Real-Time PCR Master Mix Kit and specific primers. Specific primers were designed and synthesized by Takara and their sequences were as followed: PPARα, forward 5′-GTGTGAGGGGTAAAGCAA-3′ and reverse 5′-GCTAATGCAGAGGGGTAGG-3′;
SPP1, forward 5’-TCACCTGTGCCATACCAGTT-3’ and reverse 5’-TGTGGTCAT GGCTTTTCGTTG-3’; PD-L1, forward 5’-GTGCCGACTACAAGCGAATT-3’ and reverse 5’-CTTGGAAATTGGTG GGTTG-3’; GAPDH, forward 5’-CCATGTTCGTCATGGGTGTGACCA-3’ and reverse 5’-GCCAGTAGAGGCAGGGATGATGTTC-3’. GAPDH was used as an internal control to normalize the expression of PPARα, SPP1, and PD-L1. The relative mRNA expression was determined using 2^(-ΔΔCT) method.

3. Western blotting analysis

Cell protein was extracted using RIP A lysis buffer (Takara) containing 1 nM PMSF (Invitrogen) according to the manufacturer’s instructions. Subsequently, 20 μg protein per well was electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated with primary antibodies at 4°C overnight. The membrane was then incubated with horseradish peroxidase (HRP)-labeled secondary at temperature for 2 hours. The bands were detected using an ECL Western Blotting Kit (Invitrogen) and analyzed on a Gel Doc XR System (Bio-Rad Laboratories, Hercules, CA).

4. Immunohistochemical analysis

Paraffin-embedded tissues were sectioned at 4.5-μm thickness. After being dewaxed and hydrated, sections were incubated with 3% H₂O₂ for 30 minutes to block the endogenous peroxidase activity. Following antigen recovery by repeated cooling and heating, 5% bovine serum albumin was applied to block non-specific binding. The sections were then incubated
with primary antibodies overnight at 4°C. After being rinsed with phosphate buffered saline three times for 5 minutes each, slices were treated with biotinylated secondary antibody for 1 hour, followed by incubation with streptavidin-HRP for 20 minutes. Diaminobenzidine (DAB) substrate was used to visualize the staining of target proteins. Slides were then observed and photographed under a microscope (Olympus, Tokyo, Japan).

5. Cell culture

A normal human liver L02 cell line and four human HCC cell lines, HepG2, Huh6, Huh7, and SK-Hep1, were purchased from Chinese Academy of Sciences (Shanghai, China). All the cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum in 5% CO₂ humidified atmosphere at 37°C.

6. Cell transfection

Cell transfection was conducted using Lipofectamine 2000 following the manufacturer’s protocols (Invitrogen). PPARα over-expression and knockdown studies were conducted using pcDNA3.1 and pLKO.1 (GenePharma Co. Ltd., Suzhou, China), respectively. Short hair RNA (shRNA) specially targeting PPARα or SPP1 was designed by GenePharma Co. Ltd. Transfection efficiency was determined using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting after 48 hours’ incubation.

7. PD-L1 expression analysis on HCC cell surface by flow cytometry

PD-L1 expression on the surface of HCC cells was analyzed by fluorescence-activated cell sorting (FACS). In brief, HCC cells were incubated with phycoerythrin-conjugated anti-PD-L1 antibody (Abcam, Abcam, Cambridge, MA) for 15 minutes in the dark at room
temperature. An isotype control was used to set up the separation gate. Afterwards, cell samples were analyzed by a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA).

8. Enzyme-linked immunosorbent assay

The levels of interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2) released by T lymphocytes were measured using the human ELISA Kit (Takara) following the manufacturer’s instructions. Briefly, 96-well plates were coated with cytokine capture antibodies overnight at 4℃. After three times’ wash, the wells were then blocked using blocking buffer for 1 hours at room temperature. Subsequently, 100 μL of co-culture supernatant was added into the wells and incubated for 2 hours at room temperature. Afterwards, 100 μL of detection antibody was added into the wells followed by 100 μL of diluted streptavidin peroxidase. Finally, addition of TMB substrate and sulphuric acid was used to stop the reaction and the absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

9. Luciferase reporter assay

Luciferase reporter assay was conducted to validate whether PPARα could affect SPP1 promoter activity. Briefly, SPP1 promoter fragments were cloned into the pGL3-basic dual luciferase reporter vectors (Promega, Madison, WI). Luciferase activity of the reporter vectors carrying SPP1 promoter fragments was measured 48 h after transfection using a dual luciferase reporter system (BD Bioscience). The Firefly luciferase activity was normalized by the Renilla luciferase activity.
10. HCC cell/ T cell co-culture

Peripheral blood mononuclear cells obtained from healthy donors were isolated by Lymphoprep density gradient centrifugation. T-cell activation was conducted in accordance with a protocol provided by eBioscience (San Diego, CA). Subsequently, activated T cells were co-cultured with HCC cells at a ratio of 10:1 for 16 hours. Afterwards, co-culture media were collected and used for cytokine analysis.

11. Statistical analysis

The data were expressed as the mean±standard deviation. SPSS ver. 20.0 software (IBM Corp., Armonk, NY) was used to performed data analysis. Differences between two groups were analyzed using Student’s t-test. Differences among three or more groups were analyzed by one-way ANOVA followed by Turkey’s multiple comparison. Kaplan-Meier survival analysis was conducted to assess the relationship between PPARα expression and overall survival. Spearman’s correlation analysis was carried out to evaluate the relationship between PPARα expression and SPP1 expression in HCC tissues. Significance was presented as a p-value of < 0.05 and < 0.01.

12. Ethical statement

This study was approved by the Medical Ethics Committee of West China Hospital of Sichuan University. All the participants gave their written informed consent.
Results

1. Decreased expression of PPARα predicts poor prognosis of HCC patients

Previous studies have shown that PPARα is implicated in multiple types of human malignant tumors, nonetheless, its biological role in HCC remains poorly understood. With a view to investigating the functional role of PPARα in HCC, we firstly examined its expression pattern in 60 paired tumorous tissues and adjacent normal tissues. As evident from qRT-PCR analysis, the majority of HCC tissues exhibited significantly lower PPARα mRNA expression levels than matched para-cancerous tissues (Fig. 1A). Consistently, western blotting analysis and IHC staining showed that PPARα protein expression was remarkably down-regulated in HCC tissues compared with corresponding non-cancerous tissues (Fig. 1B and C). To evaluate the relationship between PPARα expression and overall survival of 60 HCC patients, tumorous tissues were assigned into high PPARα expression group and low PPARα expression group according to the mean value of its mRNA expression levels. The log-rank test and Kaplan-Meier survival analysis manifested that patients with low PPARα expression experienced a much shorter overall survival than those with high PPARα expression (Fig. 1D). In addition, Fisher’s exact test demonstrated that PPARα down-regulation was associated with higher TNM stages and lymph node metastasis, but there was no significant correlation between PPARα expression and other clinicopathological parameters, such gender, age and tumor size (Table 1). Furthermore, qRT-PCR and western blotting analyses were used to evaluate PPARα endogenous expression in four HCC cell lines (HepG2, Huh-6, Huh-7 and SK-Hep1). As displayed in Fig. 1E and F, PPARα was significantly down-regulated in HCC cell lines compared with normal human liver cell line L02. Collectively, these findings indicate that decreased PPARα expression predicts poor prognosis of HCC patients.
2. SPP1 is significantly up-regulated and correlates negatively with PPARα expression in HCC tissues

A previous study identified that PPARα negatively regulated SPP1 expression through suppressing its promoter activity. Here we performed qRT-PCR analysis to examine PPARα mRNA expression in 60 pairs of tumorous tissues and matched adjacent normal tissues. As shown in Fig. 2A, HCC tissues displayed higher PPARα mRNA expression levels than corresponding non-tumorous tissues. In consistent with the findings of qRT-PCR analysis, western blotting and IHC staining demonstrated that SPP1 protein expression was significantly increased in tumorous tissues compared with matched para-cancerous tissues (Fig. 2B and C). To evaluate the correlation between SPP1 expression and overall survival of 60 HCC patients, tumorous tissues were classified into high SPP1 expression group and low SPP1 expression group on the basis of the average value of its mRNA expression levels. Kaplan-Meier survival curve analysis showed that HCC patients with high SPP1 expression had a significantly shorter overall survival (Fig. 2D). In addition, Spearman’s correlation analysis revealed a significant negative correlation between SPP1 mRNA expression and PPARα mRNA expression in HCC tissues (Fig. 2E). Taken together, these data suggest that SPP1 expression is significantly up-regulated and correlates negatively with PPARα expression in HCC tissues.

3. PPARα inhibits PD-L1 expression in HCC cells

Growing evidence has confirmed that PD-L1 mediates escape of tumor cells from immune surveillance of T lymphocytes. Immune escape is a critical hallmark of human tumorigenesis and tumor progression. To investigate the effects of PPARα on PD-L1 expression, gain- and loss-of-function approaches were used to manipulate its expression in
HCC cells. Over-expression and knockdown investigations were conducted in SK-Hep1 cells (lowest endogenous PPARα expression) and HepG2 cells (highest endogenous PPARα expression), respectively. Transfection efficiency was assessed by qRT-PCR and western blotting analyses (Fig. 3A and B). As exhibited in Fig. 3C and D, PPARα overexpression significantly inhibited PD-L1 mRNA and protein expression in SK-Hep1 cells compared with empty control group, while PPARα ablation dramatically promoted PD-L1 mRNA and protein expression in HepG2 cells compared with negative control treatment. Additionally, FACS analysis demonstrated that PPARα over-expression significantly decreased PD-L1 protein expression on the surface of SK-Hep1 cells in comparison with empty control group, whereas PPARα depletion markedly increased PD-L1 protein expression on the surface of HepG2 cells in comparison with negative control group (Fig. 3E). Furthermore, qRT-PCR analysis showed that HCC tissues displayed higher PD-L1 expression levels than corresponding normal tissues (Fig. 3F). Notably, Spearman’s correlation analysis revealed that a negative correlation existed between PPARα mRNA expression and PD-L1 mRNA expression in cancerous tissues (Fig. 3G). To sum up, these results suggest that PPARα suppresses PD-L1 expression in HCC cells.

4. PPARα facilitates release of immunoregulatory cytokines by T lymphocytes co-cultured with HCC cells

To evaluate the effects of PPARα on immune surveillance of T lymphocytes, we determined the levels of immuno-regulatory cytokines released by T lymphocytes co-cultured with HCC cells. As evident from enzyme-linked immunosorbent assay, PPARα over-expression significantly elevated the levels of TNF-α, IFN-γ and IL-2 released by T lymphocytes co-cultured with SK-Hep1 cells compared with empty control group, while PPARα knockdown reduced the levels of TNF-α, IFN-γ and IL-2 produced by T lymphocytes.
co-cultured with HepG2 cells compared with negative control group (Fig. 4A and B). Taken together, these findings indicate that PPARα strengthens immune surveillance of T lymphocytes on HCC cells.

5. SPP1 mediates the inhibitory effect of PPARα on PD-L1 expression in HCC cells

To illuminate the potential molecular mechanism underlying human hepatocellular carcinogenesis and progression, we conducted subsequent mechanistic studies. As presented in Fig. 5A and B, PPARα over-expression significantly repressed SPP1 expression compared with empty control group, while PPARα ablation notably promoted SPP1 expression. To validate whether PPARα down-regulates SPP1 expression through inhibiting its promoter activity, we constructed reporter vectors carrying SPP1 promoter fragments and performed luciferase reporter assays. As exhibited in Fig. 5C, PPARα over-expression dramatically reduced the luciferase activity of the reporter vectors, while PPARα depletion remarkably enhanced the luciferase activity of the reporter vectors. As evident from qRT-PCR analysis and western blotting, shRNA-mediated SPP1 ablation significantly decreased PD-L1 mRNA and protein expression levels compared with negative control group (Fig. 5D and E). In addition, we transfected shRNA-PPARα-treated HepG2 cells with shRNA-SPP1 and found that SPP1 ablation partially reversed the promoting effects of shRNA-PPARα on PD-L1 mRNA and protein expression (Fig. 5D and E). In sum, our data suggest that SPP1 mediates the inhibitory effect of PPARα on PD-L1 expression in HCC cells.

6. PPARα suppresses immune escape through SPP1/ PD-L1 axis in HCC

To validate the potential molecular mechanism by which PPARα exerts its suppressing effect on immune escape of HCC cells, we transfected shRNA-PPARα-treated HepG2 cells
with shRNA-SPP1. As exhibited Fig. 6A, SPP1 depletion partially reversed the inhibitory effect of shRNA-PPARα on the release of IFN-γ by T lymphocytes co-cultured with HepG2 cells. In addition, we found that SPP1 knockdown also alleviated the suppressing effects of shRNA-PPARα on the production of TNF-α and IL-2 by T lymphocytes co-cultured with HepG2 cells (Fig. 6B and C). Collectively, our findings indicate that PPARα inhibits immune escape of HCC cells through SPP1/PD-L1 axis.

Discussion

HCC ranks as the fifth most common tumor and the third leading cause of cancer-related mortality worldwide, which has posed a great threat to public health. Till now, chemotherapy, radiotherapy and surgical resection are still frequently-used approaches for HCC treatment [2,5]. It is widely acknowledged that great advances in the treatment of HCC have also been achieved over the past several decades. Nonetheless, the long-term prognosis of patients who suffer from HCC remains rather disappointing. Therefore, there is an urgent need to develop novel and effective therapeutic alternatives for HCC patients. Nowadays, immunomodulatory therapy is considered as a potential and promising choice in cancer treatment. It is well documented that escape of tumor cells from the surveillance of T lymphocytes is a critical hallmark of human carcinogenesis and malignant progression [21, 22]. Moreover, a growing number of evidence has revealed that the initiation and development of HCC partially attributes to the inhibitory anticancer immunity [23, 24]. Thus, it is meanwhile to identify the key genes which are implicated in tumor evasion from immunosurveillance.

PPARα belongs to the super-family of nuclear hormone receptors and plays important roles in many biological processes. Past studies have identified PPARα as a crucial regulator
in several kinds of human malignant neoplasms, such as oral cancer, prostate cancer, glioma and ovarian cancer [12,13,15,16]. However, the biological role of PPARα in HCC has not been fully characterized. In the current study, we found that PPARα was significantly down-regulated in cancerous tissues in comparison with para-cancerous tissues and that its decreased expression was associated with a shorter overall survival of patients. Additionally, a previous study has identified that PPARα negatively regulates SPP1 gene expression by inhibiting its promoter activity[20]. Furthermore, Zhang et al. reported that SPP1 positively promoted immune escape in lung adenocarcinoma cancer via positive regulation of PD-L1 expression [19]. Mounting studies have demonstrated that increased PD-L1 expression contributes to immune escape of cancer cells and that blocking PD-L1/PD-1 axis may be a potential immunotherapeutic approach for human malignant tumors [25-27].

In the present study, we noticed that SPP1 expression was significantly increased in tumorous tissues compared with adjacent normal tissues and that high expression of SPP1 was linked to poor prognosis. In addition, a negative correlation was observed between PPARα expression and SPP1 expression in cancerous tissues. Besides, we found that PPARα over-expression promoted PD-L1 expression in HCC cells, while PPARα inhibited PD-L1 expression in HCC cells. Hence, we speculated that PPARα may be involved in immune escape of tumors cells in hepatocellular carcinogenesis and malignant progression. Functional studies have displayed that PPARα over-expression promoted the release of IFN-γ, TNF-α and IL-2 by T lymphocytes co-cultured with HCC cells, whereas PPARα depletion suppressed the production of these immunoregulatory cytokines by T lymphocytes co-cultured with HCC cells. It is well documented that decreased IFN-γ, TNF-α and IL-2 production is closely related to weakened T cell immunity [28,29]. Afterwards, mechanistic investigations were conducted to elucidate the potential molecular mechanisms by which PPARα exerts its
suppressing effect on immune escape of HCC cells. Furthermore, mechanistic studies revealed that PPARα repressed PD-L1-mediated immune escape of HCC cells through down-regulating SPP1.

In conclusion, our study demonstrated for the first time that PPARα was significantly down-regulated in HCC tissues and that decreased expression of PPARα correlated with poor prognosis. Furthermore, our findings revealed that PPARα inhibited immune escape of HCC cells through SPP1/PD-L1 axis. Thus, this work may provide new insights into understanding the molecular mechanism underlying HCC development and imply targeting SPP1/PD-L1 axis as a potential therapeutic choice for HCC.

**Conflicts of Interest**

Conflict of interest relevant to this article was not reported.
References


Fig. 1. Decreased expression of PPARα predicts poor prognosis of HCC patients. (A) Relative mRNA expression levels of PPARα in 60 paired HCC tissues and adjacent non-cancerous tissues were determined by qRT-PCR analysis. (B) PPARα protein expression levels in HCC tissues and matched para-cancerous tissues were examined by western blotting analysis. (C) The expression patterns of PPARα in HCC tissues and corresponding normal tissues were visualized by IHC staining. (D) HCC patients were classified into high PPARα expression group and low PPARα expression group according to the mean value of its mRNA expression levels. The log-rank test and Kaplan Meier survival curves were used to analyze the relationship between PPARα expression and overall survival. (E) Relative mRNA expression levels of PPARα in normal human liver cell line L02 and four HCC cell lines (HepG2, Huh-6, Huh-7 and SK-Hep1) were detected by qRT-PCR analysis. (F) PPARα protein expression in normal human liver cell line L02 and four HCC cell lines (HepG2, Huh-6, Huh-7 and SK-Hep1) was examined by western blotting analysis. **p<0.01. PPARα, peroxisome proliferator-activated receptor α; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry.
**Fig. 2.** SPP1 is significantly up-regulated and negatively correlates with PPARα expression in HCC tissues. (A) Relative mRNA expression levels of SPP1 in 60 pairs of HCC tissues and corresponding non-tumorous tissues were examined by qRT-PCR analysis. (B) SPP1 protein expression levels in tumorous tissues and adjacent normal tissue were determined by western blotting analysis. (C) The expression patterns of SPP1 in HCC tissues and para-cancerous tissues were visualized by IHC staining. (D) HCC patients were divided into high SPP1 group and low SPP1 group based on the mean value of its mRNA expression levels. The log-rank test and Kaplan-Meier survival curves were applied to determine the correlation between SPP1 expression and overall survival. (E) Spearman’s correlation analysis was used to analyze the relationship between PPARα mRNA expression and SPP1 mRNA expression in tumorous tissues. **p<0.01. SPP1, secreted phosphoprotein 1; PPARα, peroxisome proliferator-activated receptor α; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry.
Fig. 3. PPARα inhibits PD-L1 expression in HCC cells. (A) Relative PPARα mRNA expression level was examined by qRT-PCR analysis after transfection with PPARα expression vectors in SK-Hep1 cells or shRNA-PPARα in HepG2 cells. (B) PPARα protein expression level was determined by western blotting after transfection with PPARα expression vectors in SK-Hep1 cells or shRNA-PPARα in HepG2 cells. (C) Relative PD-L1 mRNA expression level was detected by qRT-PCR analysis after transfection with PPARα expression vectors in SK-Hep1 cells or shRNA-PPARα in HepG2 cells. (D) PD-L1 protein expression level was examined by western blotting after transfection with PPARα expression vectors in SK-Hep1 cells or shRNA-PPARα in HepG2 cells. (E) FACS analysis was used to detect the PD-L1 protein expression on the surface of SK-Hep1 and HepG2 cells. **p<0.01. (F) qRT-PCR analysis was carried out to determine PD-L1 mRNA expression levels in 60 pairs of HCC tissues and adjacent normal tissues. (G) Spearman’s correlation analysis was performed to evaluate the relationship between PPARα mRNA expression and PD-L1 mRNA expression in HCC tissues. PPARα, peroxisome proliferator-activated receptor α; PD-L1, programmed cell death ligand 1; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; HCC, hepatocellular carcinoma; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity.
Fig. 4. PPARα facilitates release of immunoregulatory cytokines by T lymphocytes co-cultured with HCC cells. (A) The levels of TNFα, IFNγ and IL-2 released by T lymphocytes co-cultured with SK-Hep1 cells were measured by ELISA. (B) The levels of TNFα, IFNγ and IL-2 produced by T lymphocytes co-cultured with HepG2 cells were detected by ELISA. **P<0.01. PPARα, peroxisome proliferator-activated receptor α; HCC, hepatocellular carcinoma; TNF-α, tumor necrosis factor α; IFN-γ, interferon γ; IL-2, interleukin 2. ELISA, enzyme linked immunosorbent assay.
Fig. 5. SPP1 mediates the inhibitory effect of PPARα on PD-L1 expression in HCC cells. (A) SPP1 mRNA expression levels were detected by qRT-PCR analysis after transfection with PPARα expression vectors in SK-Hep1 cells or shRNA-PPARα in HepG2 cells. (B) SPP1 protein expression levels were examined by western blotting after transfection with PPARα.
expression vectors in SK-Hep1 cells or shRNA-PPARα in HepG2 cells. (C) Relative luciferase activity of the reporter vector carrying SPP1 promoter fragments was determined after transfection with PPARα expression vectors or shRNA-PPARα. (D) Relative mRNA expression levels of SPP1 and PD-L1 were examined by qRT-PCR analysis after transfection with shRNA-SPP1 in shRNA-PPARα-treated HepG2 cells. (E) Protein expression levels of SPP1 and PD-L1 were determined by western blotting after transfection with shRNA-SPP1 in shRNA-PPARα-treated HepG2 cells. **p<0.01. PPARα, peroxisome proliferator-activated receptor α; SPP1, secreted phosphoprotein 1; PD-L1, programmed cell death ligand 1; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA.
Fig. 6. PPARα suppresses immune escape through SPP1/PD-L1 axis in HCC. (A) The levels of IFN-γ released by T lymphocytes co-cultured with shRNA-PPARα and shRNA-SPP1-treated HepG2 cells were detected by ELISA. (B) The levels of TNFα released by T lymphocytes co-cultured with shRNA-PPARα and shRNA-SPP1-treated HepG2 cells were determined by ELISA. (C) The levels of IL-2 released by T lymphocytes co-cultured with shRNA-PPARα and shRNA-SPP1-treated HepG2 cells were examined by ELISA. **p<0.01. PPARα, peroxisome proliferator-activated receptor α; SPP1, secreted phosphoprotein 1; PD-L1, programmed cell death ligand 1; HCC, hepatocellular carcinoma; TNFα, tumor necrosis factor α; IFNγ, interferon γ; IL-2, interleukin 2; shRNA, short hairpin RNA.
Table 1. Correlation between PPARα expression and clinicopathological characteristics of 60 HCC patients

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