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ORIGINAL ARTICLE

Role of MAPK activity in PD-L1 expression in hepatocellular carcinoma cells

Shijiang Xing[1], Shengli Chen[1], Xinglong Yang[2], Weiyi Huang[3]

[1]Department of Medical Oncology, Affiliated Xiaolan Hospital, Southern Medical University, Zhongshan 528415, P.R. China. [2]Department of Radiotherapy, Guangzhou First People's Hospital, Guangzhou 510180, P.R. China. [3]Department of Internal Medicine (VI), Guangzhou General Hospital of Guangzhou Military Command, Guangzhou 510016, P.R. China.

Summary

Purpose: *This study was set out to explore the role of MAPK activity in programmed cell death ligand-1 (PD-L1) expression in hepatocellular carcinoma (HCC) cells.*

Methods: *The protein expression of PD-L1 was determined by immunofluorescence and immunohistochemistry, and the expression levels of PD-L1 and MAPK-related proteins were determined by flow cytometry and Western blotting. Meanwhile, the RNA transcription level of CD274 was determined by qRT-PCR.*

Results: *Interferon-γ (IFN-γ), epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling pathways were associated with CD274 gene expression in HCC. Epidermal growth factor (EGF) or IFN-γ stimulation increased CD274 mRNA and PD-L1 protein levels in a representative HCC cell line group, further enhanced by EGF and IFN-γ stimulation. Inhibition of the MAPK pathway by EGFR inhibitors ositinib or MEK 1 and 2 inhibitors selumetinib prevented the up-regulation of CD274 mRNA and PD-L1 proteins and membranes induced by EGF and IFN-γ. IFN-γ increased the transcriptional activity of CD274, while MAPK signaling enhanced the stability of CD274 mRNA.*

Conclusion: *MAPK pathway activity plays a key role in PD-L1 expression in EGF and IFNγ-induced HCC and may provide a target for improving the efficacy of immunotherapy.*

Key words: *hepatocellular carcinoma, programmed cell death ligand-1 (PD-L1), IFN-γ, MAPK pathway*

Introduction

Not until the introduction of immunocheckpoint inhibitor anti-PD-1 has the treatment of advanced HCC made a great leap, enormously improving the survival rate of patients with advanced HCC. According to clinical results, patients with positive PD-L1 expression have a higher response rate against PD-1 therapy. However, even in patients with PD-L1 expression higher than 50%, the response rate to PD-1 is merely 40-60% [1,2]. The possible reason for this result is that the expression of PD-L1 in tumor cells is in a state of high dynamic change and cannot be stabilized at the level that makes the immune checkpoint inhibitor highly responsive [3-6]. Mutations of carcinogenic driver genes such as EGFR, ALK and BRAF are inducers of PD-L1 expression in HCC cells. Among these oncogene-activated cells, the PI3K/mTOR, JAK/STAT, and MAPK pathways are the major drivers of PD-L1 expression [7-10].

Meanwhile, we found that clinically HCC patients with EGFR wild-type presented higher PD-L1 expression levels and better anti-PD-1 efficacy than EGFR gene mutations [11,12]. However, little research has been done on the regulatory mechanism of PD-L1, which limits our ability to provide rationale and guidance for combining immune checkpoint blocking therapy with other therapies. Therefore, in this study, we explored the pathway regulating

Corresponding author: Weiyi Huang, MD. Department of Internal Medicine (VI), Guangzhou General Hospital of Guangzhou Military Command, No.111 Linhua Rd, Guangzhou 510016, P.R. China.
Tel: +86 18924114669,Email: wii3u6@163.com
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CD274 (PD-L1) expression, and specifically studied EGF-dependent MAPK signaling and its role in IFN-γ-induced PD-L1 expression in HCC cells.

Methods

Cell culture

Human HCC cell lines HepG2 and MHCC97 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were isolated until screening for microbial contamination and mycoplasma was performed and confirmed negative. The cells were cultured in RPMI medium containing 10% fetal calf serum (FCS), and then an appropriate amount of glutamine was added. All cells were incubated at 37 °C in a humid environment containing 5% CO_2.

Flow cytometry analysis

Cells were digested with trypsin and kept on ice in phosphate buffered saline (PBS) containing 2% FCS throughout the program. After staining, the cells were maintained in PBS containing 2% FCS until the performance of analysis. The cells were incubated with anti-PD-L1 antibody at 10 μg/ml for 45 min. The binding antibodies were detected by incubating the cells with goat anti-mouse IgG at a dilution of 1:50 for 45 min. Measurements were performed on a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). FlowJo v10 (Tree Star, Ashland, OR, USA) was employed to analyze the data, and the surface receptor expression was expressed as mean fluorescence intensity (MFI). Calibration measurements were conducted for nonspecific binding of background fluorescence and secondary antibodies.

Western blot

Lysates from cells were prepared using a mammalian protein extraction reagent with 1:100 diluted protease and phosphatase inhibitors, and proteins were separated by SDS-PAGE. Target proteins were detected with suitable antibodies, and images were captured using a digital imaging system (Bio-Rad, Hercules, CA, USA). The optical density (OD) quantification of the target protein was calculated using ImageJ relative to the loading control β-actin or GAPDH.

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed according to standard methods.

RNA sample collection and qRT-PCR

The total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and possible DNA contamination was removed using TURBO DNAase ambion (Life Technologies, Carlsbad, CA, USA, AM2238). The RNA was then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, CA, USA, 28025013). Real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA, 1708886) according to the manufacturer's instructions. The following primers were used: CD274 forward: 5'-CAATGTGACCAGCACACTGAGAA-3', reverse: 5'-GGCATAATAAGATGGCTCCCAGAA-3'; GAPDH forward: 5'-CCCACTCCTCCACCTTTGAC-3', reverse: 5'-CCACCACCCTGTTGCTGTAG-3'. Relative gene expression was calculated using the dual delta CT method with GAPDH as a loading control. All qRT-PCR experiments were conducted in triplicate.

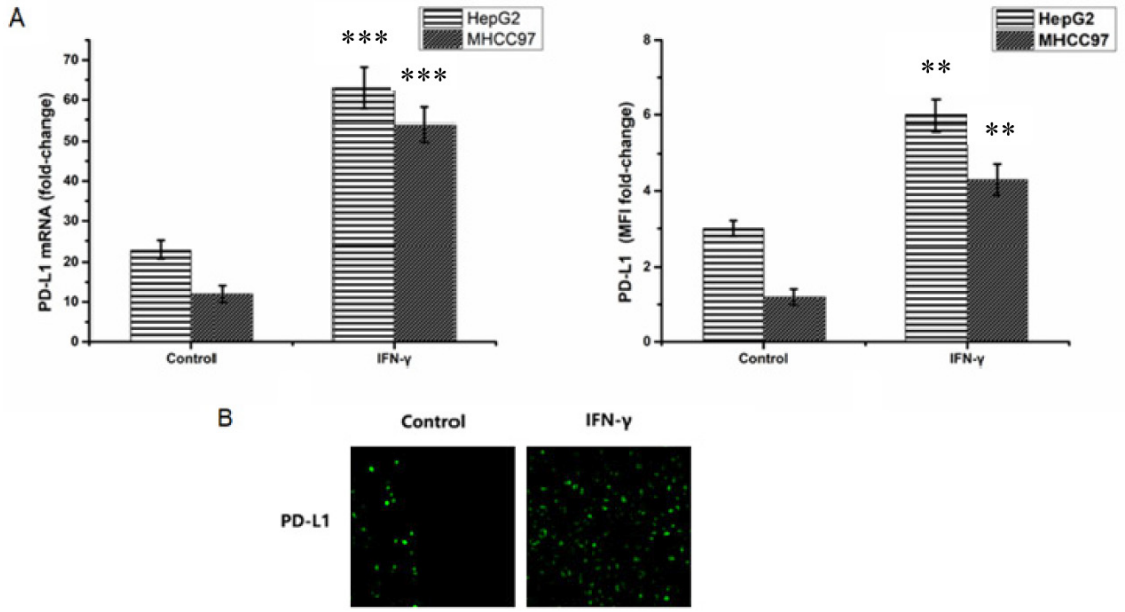

Figure 1. Expression of PD-L1 induced by IFN-γ. **A:** QRT-PCR detection on the gene level of PD-L1 in the HCC cells treated with IFN-γ and in hepatocellular carcinoma cells of the blank group. **B:** The protein levels of PD-L1 on the cell membrane of the HCC cells in hepatocellular carcinoma cells of IFN-γ and blank group were detected by flow cytometry and inverted fluorescence. ** p<0.01, *** p<0.001 when compared with the control group.

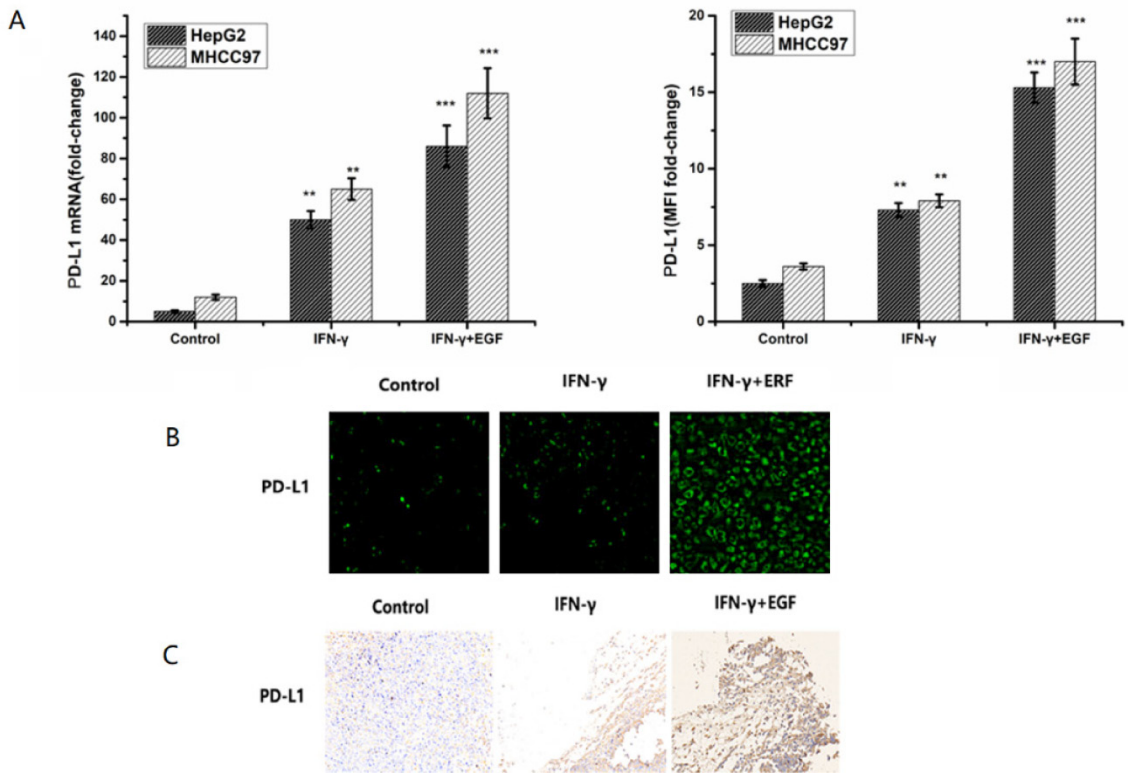

Figure 2. EGF increased IFN-γ-induced PD-L1 expression. **A:** The gene level of PD-L1 of cells in the three groups (blank group, IFN-γ treated group, IFN-γ+EGF treated group) was detected by qRT-PCR assay. **B:** The fluorescence intensity of the inverted fluorescence represents the expression level of PD-L1, and so was the brown area in immunohistochemistry. **C:** In immunohistochemistry, the brown area represents the expression level of PD-L1. ** p<0.01, *** p<0.001 when compared with the control group. ** p<0.01, *** p<0.001 when compared with the control group.

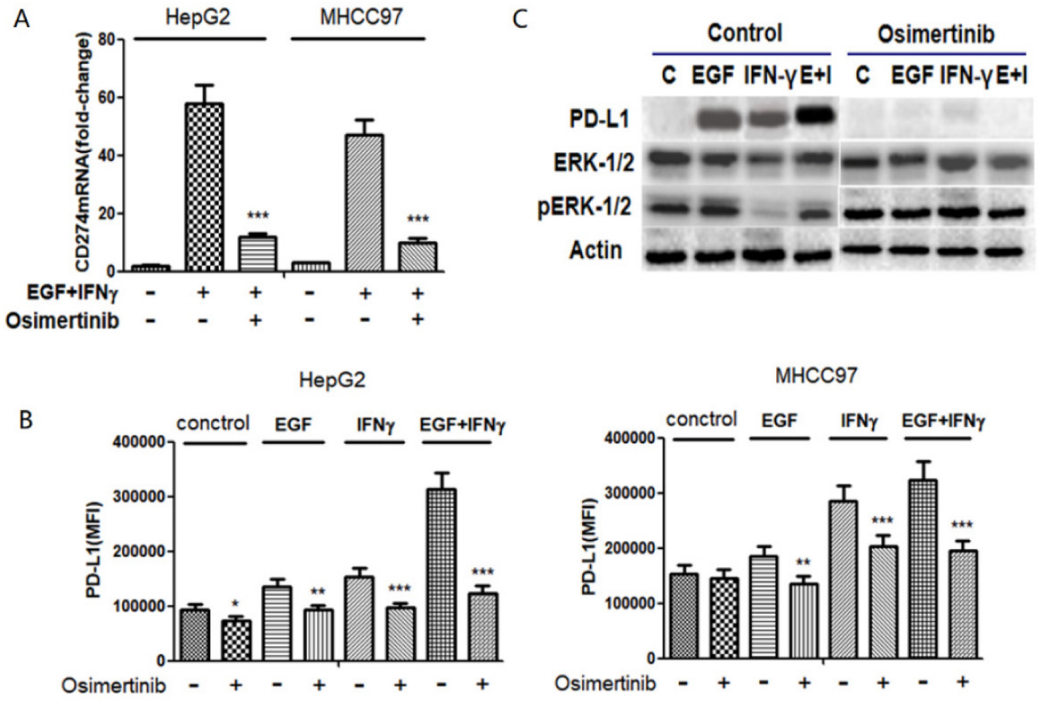

Figure 3. EGFR inhibitors reduced EGF and IFN-γ induced PD-L1 expression. **A:** HepG2 and MHCC97 cells were treated with 20 μg/ml of Ostinib [??] with or without 20 ng/ml of EGF and 20 ng/mL of IFN-γ for 24 h. **B:** PD-L1 mRNA levels in HepG2 and MHCC97 cells were measured using qRT-PCR, while the PD-L1 expression in membrane was detected by flow cytometry. **C:** The total protein of HepG2 cells was extracted by lysis, and cellular protein levels were measured using Western blotting with Actin as the loading control. Two-way ANOVA using Dunnett multiple comparison test: ** p<0.01, *** p<0.001 when compared with the untreated controls. ** p<0.01, *** p<0.001 when compared with the control group.

Statistics

Differences between cell line experiments and unpaired two-tailed Student's t-test or two-way ANOVA and Bonferroni *post-hoc* or Dunnett's test were assessed. The detection results were expressed as mean ± SD. P<0.05 was considered to be statistically significant. GraphPad Prism software (version 6.0 GraphPad software, San Diego, CA, USA) was utilised for statistical analyses.

Results

Expression of PD-L1 induced by IFN-γ

First, two HCC cell lines, HepG2 and MHCC97, were selected to investigate the relationship between EGFR and IFN-γ pathways in PD-L1 expression. Prior to this, the induction of PD-L1 expression by IFN-γ was verified by us. After overnight culture in cell resuscitation, IFN-γ was added to the incubation. Two days later, the total mRNA was extracted and tested by qRT-PCR. As shown in Figure 1, the gene level of PD-L1 was significantly increased in the group treated with IFN-γ. After that, we carried out flow experiment and inverted fluorescence photography of HepG2 to further validate the induced expression of PD-L1 by IFN-γ. The flow and inverted fluorescence results were consistent with previous qRT-PCR results.

EGF increased IFN-γ-induced PD-L1 expression

Here, concerning the PD-L1 expression, we explored the correlation between EGFR and IFN-γ pathway. We divided the cells into three groups, that is, the blank group, the IFN-γ treated group and the IFN-γ+EGF treated group. QRT-PCR and inverted fluorescence experiments were carried out after treatment. Analysis of the results as shown in Figure 2 demonstrated that EGF enhanced the expression of PD-L1 induced by IFN-γ. Subsequently, we performed the same treatment as cell grouping on HepG2 tumor-bearing nude mice, and injected the relevant drugs into the tail vein. Two days later, the tumor was dissected for immunohistochemical experiments. As shown in Figure 2, the protein expression was significantly enhanced in the EGF-treated group.

EGFR inhibitors reduced EGF and IFN-γ induced PD-L1 expression

To analyze the role of EGFR signaling in EGF and IFN-γ induced PD-L1 expression, we used Osimertinib (Ostinib), a known effective small-molecule inhibitor of EGFR, to intervene in EGF+ IFN-γ treated HepG2 and MHCC97 cells. QRT-PCR results revealed that Ostinib prevented the upregulation

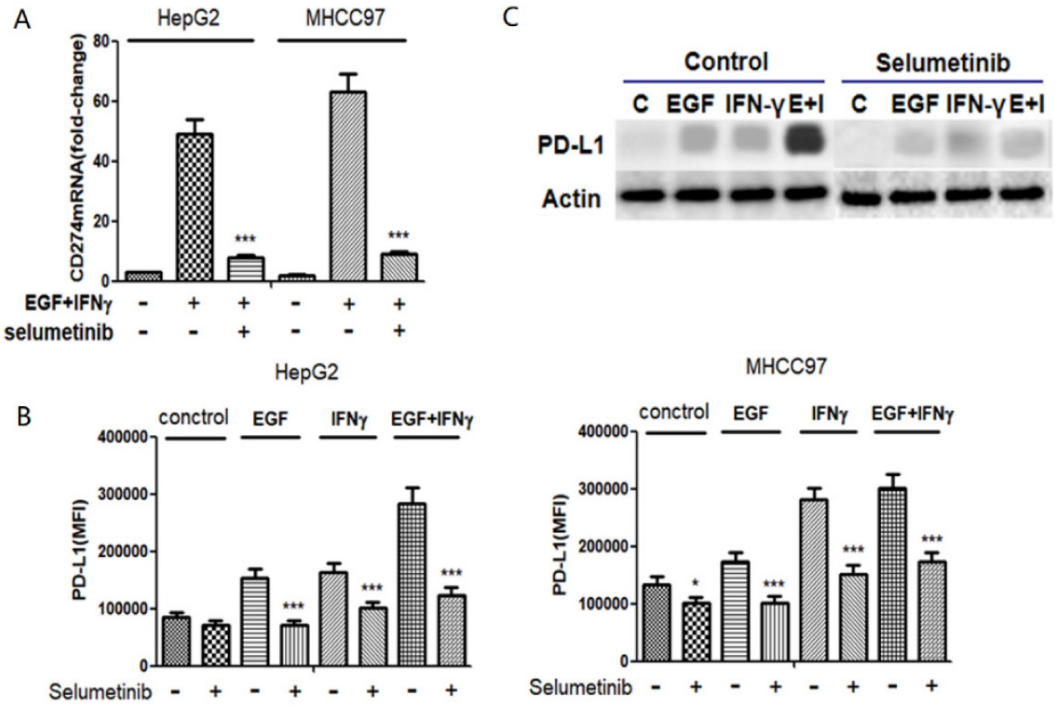

Figure 4. Smetinib effectively decreased EGF and IFN-γ induced PD-L1 expression. **A:** HepG2 and MHCC97 cells were treated with 10 μl/ml of mitterminib with or without 20 ng/ml of EGF and 20 ng/mL of IFN-γ for 24 hours. **B:** PD-L1 mRNA levels in HepG2 and MHCC97 cells were measured by qRT-PCR. **C:** The total protein of HepG2 cells was extracted by lysis, and cellular protein levels were measured using Western blotting with Actin as the loading control. Two-way ANOVA using Dunnett multiple comparison test: * p<0.05, *** p<0.001 when compared with the ligand-stimulated controls. C: untreated controls; E+I: EGF +IFN-γ.

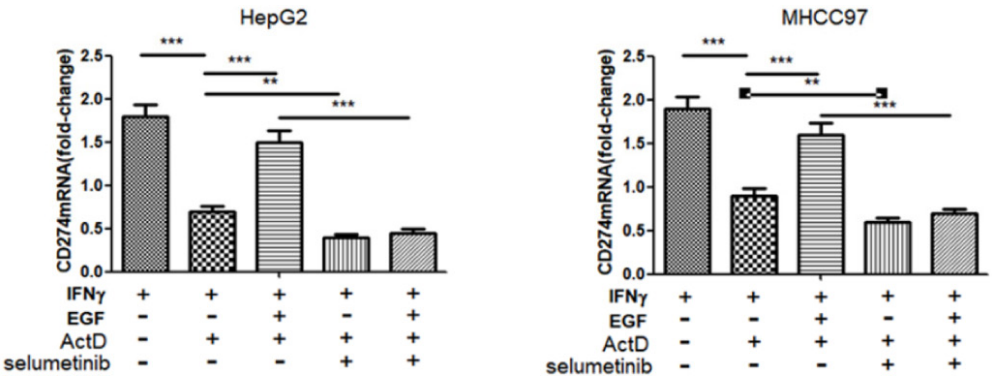

Figure 5. MAPK signaling enhanced CD274 mRNA stability. The cells were first treated with IFN-γ for 24 h, then treated with 5 μg/ml actinomycin D for 10 min, followed by 20ng/ml EGF or 10 μg/ml of selumatinib for 80 min. After that, qRT-PCR was applied to detect CD274 mRNA levels (PD-L1) in HepG2 and MHCC97 cells. Two-way ANOVA and Tukey test. ** p<0.01, *** p<0.001. ActD: actinomycin D.

of CD274 mRNA levels induced by EGF and IFN-γ alone and in combination, while flow cytometry and Western blotting showed that it also decreased PD-L1 expression on membrane and total PD-L1 protein levels. Notably, Ostinib also reduced basal PD-L1 membrane levels in HepG2 cells. The results of the above experimental data displayed that Ostinib had a similar effect on the increased expression of EGF and IFN-γ induced PD-L1 in the other two cell lines. As expected, EGFR inhibition effectively reduced EGF-dependent MAPK, which might upregulate the expression of PD-L1 by modulating MAPK signaling pathway by activating EGFR. All these suggested that EGFR-mediated signaling was responsible for the upregulation of total protein and membrane levels of CD274 mRNA and PD-L1 induced by EGF and IFN-γ. More details are shown in Figure 3.

Selumetinib effectively decreased EGF and IFN-γ induced PD-L1 expression

Next, we further evaluated the role of MAPK signaling in EGF and IFN-γ induced upregulation of PD-L1. The MEK1/2 inhibitor simotinib was applied, and the results demonstrated that it almost completely inhibited the induction of CD274 mRNA by EGF and IFN-γ in HepG2 and MHCC97 cells, and decreased the induction of total protein and membrane levels of PD-L1. Moreover, Selumetinib reduced basal PD-L1 membrane levels in MHCC97 cells, but had no significant effect on basal PD-L1 membrane levels in HepG2 cells. From the above data, it was apparent that the MAPK signaling pathway played a key role in the expression of PD-L1 induced by EGF and IFN-γ. Therefore, it could be concluded that IFN-γ activated the MAPK signaling pathway by activating EGFR and thus induced increased expression of PD-L1. More details are shown in Figure 4.

MAPK signaling pathway enhanced the stability of PD-L1 expression

Recent studies have exhibited that KRAS mutations were involved in post-transcriptional regulation of basal PD-L1 levels by regulating CD274 mRNA stability [13-16]. To explore whether MAPK signaling controlled the stability of IFN-γ-induced CD274 mRNA, KRAS wild-type and mutant cells were pretreated with IFN-γ, followed by the transcriptional blocker actinomycin D. CD274 levels were halved at 90 min after blocking. Interestingly, it was found that EGF-induced MAPK signaling activation counteracted the CD274 degradation. Therefore, inhibition of MAPK signaling by Selumetinib accelerated CD274 degradation. These results indicated that MAPK signaling affected the stability of CD274 mRNA and was conducive to the regulation of PD-L1 protein and membrane expression. More details were shown in Figure 5.

Discussion

In the present study, we revealed the correlation between MAPK signaling pathway in CD274 expression in HCC. Subsequently, we demonstrated the importance of MAPK signaling in upregulating PD-L1 by EGF and IFN-γ in HCC cell lines, which play an important role in regulating CD274 mRNA stability. What's more, we proved that the inhibition of the MAPK pathway prevented the upregulation of PD-L1 induced by EGF and IFN-γ. In a word, these results suggest that MAPK pathway can improve the tumor cell immunogenicity of HCC [17].

Targeting MAPK pathway is of special significance in HCC in that these tumors have more active MAPK pathway and certain KRAS mutation compared to other tumors [18-22]. More importantly, the expression of PD-L1 in tumor cells plays a decisive role in immunotherapy, and the latter is an

effective treatment method for cancer patients with lower therapeutic effect in advanced tumors [23-27]. Our study revealed that the MAPK pathway inhibited the induction of CD274 mRNA by EGF and IFN-γ in the following two aspects: Firstly, it inhibited EGFR or MEK1/2 reduced CD274 transcription, which was probably due to inhibition of the downstream effector eukaryotic initiation factor 4F (eIF4F) translation initiation complex of the MAPK pathway; Secondly, we observed a decrease in the stability of CD274 mRNA after inhibition of MEK1/2 or EGFR. These findings extended the early data of basal PD-L1 expression in KRAS mutant cell lines, in which MEK1/2 inhibited Tristetraprolin (TTP) activation, leading to CD274 mRNA degradation [28-30]. At the protein level, PD-L1 can be affected by several mechanisms, such as glycosylation and protein stability on the cell membrane [31-33]. However, we found that EGF or IFN-γ had no direct effect on protein levels of CKLF-like MARVEL transmembrane domain-containing protein 6 (CMTM6) in HepG2 and MHCC97 cells (data not shown).

Our experiments verified that both EGFR and MEK1/2 inhibitors could reduce the expression of EGF and IFN-γ induced PD-L1, which in turn might enhance the immunogenicity of HCC cells, whereas, since the MAPK pathway is the downstream of many growth factor receptors, downstream inhibition of MEK1/2 inhibitors may be more effective in regulating PD-L1 than inhibition of specific growth factor receptors. Studies have found that MEK1/2 suppresses the expression of HGF-induced PD-L1 in early renal cell carcinoma [34-37], but does not inhibit EGFR, while we have similarly proved that it blocks the expression of EGF and IFN-γ induced PD-L1 in HCC cells, indicating that MAPK signaling pathway plays an important role in the immunogenic regulation in multiple cancers. According to *in vitro* experiments in some studies, the downregulation of PD-L1 at some certain concentration of Selumetinib has little effect on cell growth. Although these *in vitro* experiments did not completely mimic the tumor microenvironment [38-40], the results revealed that the dose of Selumetinib might already be immunomodulatory compared to the dose used in cancer patients [41-43]. Today, the immunomodulatory effects of MAPK signaling has enjoyed increasing attention [44,45]. Studies using *in vivo* colon cancer models have shown that MEK inhibition enhances the antitumor immune response by preventing T cell apoptosis and reducing the levels of myeloid suppressor cells and regulatory T cells. When combined with PD-L1, PD-1 or CTLA-4 blocking therapy [45,46], it leads to persistent tumor suppression. Although in the Phase II and Phase III studies of Selumetinib in patients with NSCLC, the observed efficacy was not ideal [47-49], we observed its significant immunomodulatory effects, suggesting that the inhibition of the MAPK pathway can increase the efficacy of immunotherapy, while the particular importance of the modulation of PD-L1 expression in tumors lies in that they have more inflammatory tumor microenvironments and are better responsive to immunological checkpoint inhibitors than tumors with targeted genetic alterations (eg, EGFR mutations), and these combination strategies are also currently being tested in cancer patients [50-52].

To sum up, this study verifies the importance of EGF induced MAPK pathway signaling in PD-L1 expression in HCC, and provides fundamental guidance for exploring the combination therapy of MAPK pathway inhibitors with immunotherapy in this HCC subtype.

Authors' contributions

SX conceived the study and drafted the manuscript. SC acquired the data; XY and WH analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Affiliated Xiaolan Hospital, Southern Medical University, China. Patients and/or guardians who participated in this research signed the informed consent and had completed the clinical data.

Conflict of interests

The authors declare no conflict of interests.

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