TNF-α-mediated epithelial-to-mesenchymal transition regulates expression of immune checkpoint molecules in hepatocellular carcinoma

RITU SHRESTHA[1,2], KIM R. BRIDLE[1,2], DARRELL H.G. CRAWFORD[1,2] and APARNA JAYACHANDRAN[1,2]

[1]University of Queensland, Faculty of Medicine and [2]Liver Cancer Unit, Gallipoli Medical Research Institute, Greenslopes Private Hospital, Brisbane, QLD 4120, Australia

Received June 12, 2019; Accepted January 31, 2020

DOI: 10.3892/mmr.2020.10991

Abstract. Hepatocellular carcinoma (HCC) is the fastest growing cause of cancer-related deaths globally. Epithelial-to-mesenchymal transition (EMT) is a cellular process that confers HCC tumor cells with the ability to evade the immune system. Immune escape in most tumors, including HCC, is controlled by immune checkpoint molecules. The aim of the present study was to investigate the association between EMT and immune checkpoint in HCC, and identify novel therapeutic targets for HCC. An *in vitro* model of reversible EMT was utilized based on cytokine tumor necrosis factor (TNF)-α treatment of HCC cell lines Hep3B and PLC/PRF/5. Hep3B and PLC/PRF/5 cells were treated with TNF-α, and the EMT status and the expression of immune checkpoint molecules was assessed by reverse transcription-quantitative PCR, western blotting and immunofluorescence. To confirm an association between EMT and immune modulators, cells were exposed to culture medium with TNF-α for 3 days to induce EMT, following which a reversal assay was performed. The expression of immune modulators and mesenchymal-to-epithelial transition (MET) status was investigated upon reversal of EMT. Furthermore, SurvExpress, a web-based platform was utilized to analyze survival and recurrence in a dataset of patients with HCC. TNF-α treatment for 3 days induced EMT in Hep3B and PLC/PRF/5 cells, as demonstrated by the downregulation of epithelial markers along with upregulation in mesenchymal markers. An EMT reversal assay was able to induce MET by increasing epithelial markers and decreasing mesenchymal markers. TNF-α-induced EMT led to the upregulation of immune modulators, including programmed death receptor ligand (*PD-L)1*, *PD-L2*, *CD73* and *B7-H3*. In contrast, reversal of EMT suppressed the expression of *PD-L1*, *PD-L2*, *CD73* and *B7-H3*. In addition, high expression of TNF-α and PD-L1 in 422 patients with HCC was associated with poor overall survival. The coordinate expression of TNF-α with PD-L2 in this patient cohort was associated with increased HCC recurrence. In conclusion, the present study demonstrated a close association between immune modulator expression and EMT induction/reversal driven by TNF-α.

Correspondence to: Dr Aparna Jayachandran, Liver Cancer Unit, Gallipoli Medical Research Institute, Greenslopes Private Hospital, 100 Newdegate Street, Greenslopes, Brisbane, QLD 4120, Australia
E-mail: a.jayachandran@uq.edu.au

Key words: epithelial-to-mesenchymal transition, hepatocellular carcinoma, immune checkpoint molecules, tumor necrosis factor-α, programmed death ligand-1, mesenchymal-to-epithelial transition

Introduction

Hepatocellular carcinoma (HCC) is a primary liver malignancy accounting for 80-90% of all liver cancer (1). According to the latest global cancer data, the incidence of HCC has rapidly risen with ~782,000 deaths in 2018 (2). The majority of HCC is resistant to treatment as it is often diagnosed at advanced stages of disease, so the overall median survival is <1 year (3). To the best of the authors' knowledge, sorafenib and levatinib are the only first-line systemic therapeutic agents available for the treatment of advanced HCC. Several second-line drugs, including regorafenib, ramucirumab and cabozantinib, have been approved for patients with HCC who have previously been treated with sorafenib, but the overall survival (OS) rate remains poor (4,5).

Immune checkpoint blockade therapies have emerged as potential treatments in cancer immunotherapy (6). The cytotoxic T-cell mediated immune response is accompanied by co-stimulatory signals and co-inhibitory signals or immune checkpoints (7,8). The immune checkpoint molecules are often overexpressed or activated in the tumor microenvironment, thus resulting in immune evasion by tumor cells (7-9). Immune checkpoint inhibitors (ICIs) that block programmed cell death protein-1 (PD-1) or its ligand, programmed death receptor ligand (PD-L)1, have shown therapeutic potential in several cancer types, including HCC (10,11). Anti-PD-1 ICIs, nivolumab and pembrolizumab, have been approved by The Food and Drug Administration for the treatment of HCC in patients where treatment using sorafenib has failed (12). There are a number of ongoing clinical trials on ICIs targeting PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab) in HCC (7,13). Due to the success of anti-PD-1/PD-L1

immunotherapy clinical studies, understanding the underlying mechanisms regulating PD-L1 expression has attracted increasing attention. Previous research by the authors and other researchers demonstrated an increased expression of other immune checkpoint molecules in HCC (8,14). However, the mechanisms regulating the expression of these and other immune checkpoint molecules in HCC remains unclear (8).

A key process in HCC progression is epithelial-to-mesenchymal transition (EMT). EMT is a complex molecular and cellular process that allows epithelial cells to gain mesenchymal features, including migration, invasiveness and increased resistance to immune evasion (15,16). Mesenchymal-to-epithelial transition (MET) is the reverse process of EMT that offers phenotypic plasticity for conversion of mesenchymal cells to epithelial derivatives (15). EMT status is closely associated with cancer metastasis, stemness, immune escape and drug resistance in HCC (17,18). It was previously identified that EMT is involved in the regulation of PD-L1 in several cancer types, including breast cancer (19,20), lung cancer (17,21), pancreatic cancer (22), esophageal cancer (23) and salivary adenoid cystic carcinoma (24). The authors previously demonstrated that EMT is associated with PD-L1 expression in patients with HCC (8).

In the present study, it was hypothesized that EMT is associated with the ability of HCC tumor cells to escape detection and destruction by the immune response, by regulating the expression of immune checkpoint molecules, such as PD-L1. In the present study, the changes in immune checkpoint expression during the EMT process were examined by incorporating a reversible model of EMT based on tumor necrosis factor (TNF)-α treatment of the HCC cell lines Hep3B and PLC/PRF/5. TNF-α was selected as an inducer of EMT, as it has been previously demonstrated to play an important role in promoting EMT in HCC (25). This *in vitro* system may provide an improved understanding of the modulation of immune checkpoints during both EMT and MET.

Materials and methods

Cell culture and reagents. The human HCC cell line Hep3B was provided by Professor V. Nathan Subramaniam, The Queensland University of Technology. The human HCC cell line PLC/PRF/5 was purchased from CellBank Australia (cat. no. 85061113). Both cell lines were mycoplasma-tested using the MycoAlert™ mycoplasma detection kit (Lonza Group, Ltd.) and cultured in DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.), and incubated at 37˚C under a humidified atmosphere with 5% CO_2 in air (26). The cytokine TNF-α was purchased from PeproTech, Inc.

EMT reversal assay. An EMT reversal assay was performed to determine the association between EMT and immune checkpoint expression. Firstly, EMT was induced by using culture medium with 20 ng/ml TNF-α for 3 days at 37˚C and then reversal of EMT was induced by changing the culture medium without TNF-α for the next 3 days.

RNA extraction and cDNA synthesis. RNA was isolated from Hep3B and PLC/PRF/5 cells, as previously described, using Isolate II Bioline RNA synthesis kit (Bioline), according to the manufacturer's protocol (26). The quantity and purity of RNA was confirmed using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized by reverse transcribing 1 μg RNA into cDNA using the Bioline SensiFAST cDNA synthesis kit (Bioline) with the following thermocycling conditions: Primer annealing at 25˚C for 10 min, reverse transcription at 42˚C for 15 min, inactivation at 85˚C for 5 min and a final extension 4˚C for 30 min.

Table I. List of primers for reverse transcription-quantitative PCR.

Gene	Primer sequence, 5'-3'	
β-actin	F:	CCAACCGCGAGAAGATGA
	R:	CCAGAGGCGTACAGGGATAG
E-cadherin	F:	AGGCCAAGCAGCAGTACATT
	R:	ATTCACATCCAGCACATCCA
N-cadherin	F:	TCCTTGCTTCTGACAATGGA
	R:	TTCGCAAGTCTCTGCCTCTT
Occludin	F:	TAGTCAGATGGGGGTGAAGG
	R:	CATTTATGATGAGCAGCCCC
Vimentin	F:	CTTCAGAGAGAGGAAGCCGA
	R:	ATTCCACTTTGCGTTCAAGG
Snai2	F:	TGGTTGCTTCAAGGACACAT
	R:	GTTGCAGTGAGGGCAAGAA
Fibronectin	F:	CAGTGGGAGACCTCGAGAAG
	R:	TCCCTCGGAACATCAGAAAC
PD-L1	F:	TGCCGACTACAAGCGAATTACTG
	R:	CTGCTTGTCCAGATGACTTCGG
PD-L2	F:	ACCGTGAAAGAGCCACTTTG
	R:	GCGACCCCATAGATGATTATGC
CD73	F:	TTGGAAATTTGGCCTCTTTG
	R:	ACTTCATGAACGCCCTGC
B7-H3	F:	CTGGCTTTCGTGTGCTGGAGAA
	R:	GCTGTCAGAGTGTTTCAGAGGC
VISTA	F:	AGATGCACCATCCAACTGTGTGG
	R:	AGGCAGAGGATTCCTACGATGC
VTCN1	F:	CTCACAGATGCTGGCACCTACA
	R:	GCAAGGTCTCTGAGCTGGCATT

PD-L, programmed death receptor ligand; *Snai2*, snail family transcriptional repressor 2; F, forward; R, reverse; *VTCN1*, V-set domain-containing T-cell activation inhibitor 1; *VISTA*, V-domain immunoglobulin suppressor of T-cell activation.

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed using SensiFast™ SYBR® Lo-ROX kit (Bioline) on a ViiA7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), as previously described (26). A three-step cycle procedure was applied with 40 cycles of the following conditions: 95˚C for 5 sec, 63˚C for 20 sec and 75˚C for 20 sec. β-actin was used as an internal control. The primer sequences used are listed in Table I. Data were analyzed using

Table II. List of antibodies.

A, Primary antibodies

Name	Cat. no.	Manufacturer	Dilution	
			Western blotting	Immuno-fluorescence
E-cadherin	ab76055	Abcam	1:1,000	1:100
N-cadherin	SC-53488	Santa Cruz Biotechnology, Inc.	1:200	-
Occludin	SC-133255	Santa Cruz Biotechnology, Inc.	1:200	1:50
Vimentin	ab92547	Abcam	1:1,000	1:600
CD73	ab133582	Abcam	1:250	
CD73	ab175396	Abcam	-	1:100
PD-L1	ab238697	Abcam	2 μg/ml	2 μg/ml
B7-H3	14058S	Cell Signaling Technology, Inc.	1:1,000	-
PD-L2	82723S	Cell Signaling Technology, Inc.	1:1,000	-
GAPDH	MAB376	EMD Millipore	1:100,000	-

B, Secondary antibody

Name	Cat. no.	Manufacturer	Dilution	
			Western blotting	Immuno-fluorescence
Goat anti-mouse HRP	6421040	Invitrogen; Thermo Fisher Scientific, Inc.	1:50,000	-
Goat anti-rabbit HRP	656120	Invitrogen; Thermo Fisher Scientific, Inc.	1:50,000	-
Alexafluor 488 anti-mouse	A21202	Thermo Fisher Scientific, Inc.	-	1:200
Alexafluor 594 anti-rabbit	A21207	Thermo Fisher Scientific, Inc.	-	1:200

PD-L, programmed death receptor ligand; HRP, horseradish peroxidase; -, not applicable.

the $2^{-\Delta\Delta Cq}$ method where β-actin was assigned as the housekeeping gene. The results are expressed as relative mRNA expression to the control (27).

Western blot analysis. Cells were seeded ($2x10^5$ cells/well) in 6-well plates and subsequently lysed using RIPA buffer (Thermo Fisher Scientific, Inc.) with complete protease inhibitors and PhosSTOP™ phosphatase inhibitors (both purchased from Roche Diagnostics) on ice to extract the total protein. The total protein was measured with a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 10 μg protein was used for 4% SDS-PAGE, following which proteins were transferred to a PVDF membrane. After blocking with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 h, the membranes were incubated with primary antibodies at 4˚C overnight. An enhanced chemiluminescence reagent, SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.), was used to detect the protein adhering to the membranes following incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. All antibodies used are listed in Table II, including the dilutions, cat. nos. and suppliers. GAPDH was selected as the housekeeping control in each group. The images were captured with ImageQuant™ LAS 500 and quantified with Image Studio™ Lite v5.2 software (LI-COR Biosciences).

Immunofluorescence. Cells ($5x10^3$ cells/well) were cultured in 8-well tissue culture treated chamber slides. Cells were washed with 1X PBS and then fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Inc.) at room temperature for 20 min followed by treatment with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA). The cells were then blocked with 5% FBS at room temperature for 1 h, followed by overnight incubation with primary antibodies at 4˚C. The cells were incubated with secondary antibodies at room temperature for 1 h, followed by 10 min incubation with DAPI (Thermo Fisher Scientific, Inc.) at room temperature. All antibodies used are listed in Table II, including the dilutions, cat. nos. and suppliers. The slides were then mounted in ProLong® Diamond (Thermo Fisher Scientific, Inc.) for observation with a Nikon C2 confocal microscope (magnification, x40), and captured and analyzed with NIS-Elements-AR software (version 5.01.00; Nikon Corporation).

SurvExpress bioinformatics tool. An online tool, SurvExpress, was used to generate survival analysis using datasets of patients with HCC, as previously described (8,28) A total of six publically available datasets of patients with HCC were used: Hoshida Golub Liver GSE10143 (162 patients), Tsuchiya Rusyn Liver GSE17856 (95 patients), Hoshida Golub Liver GSE10186 (118 patients), Liver Hepatocellular Carcinoma The Cancer Genome Atlas (TCGA; 12 patients), TCGA-Liver-Cancer

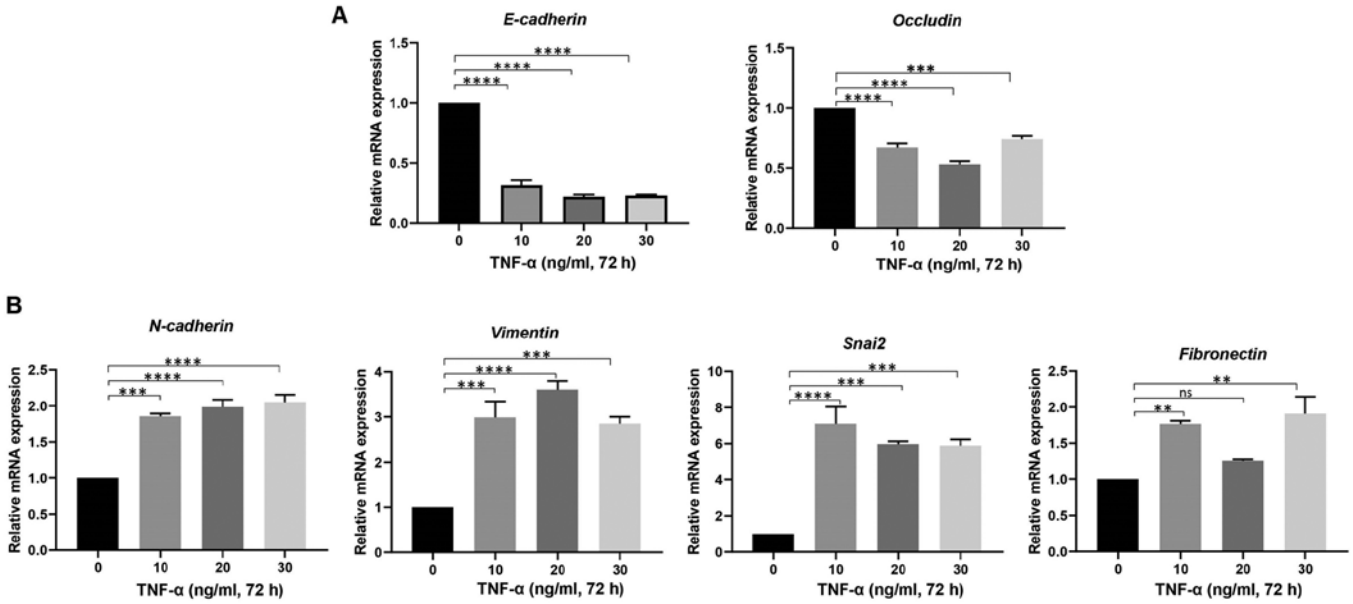

Figure 1. TNF-α induces epithelial-to-mesenchymal transition in Hep3B cells at various doses. Reverse transcription-quantitative PCR analysis demonstrated (A) lower expression of *E-cadherin* and *Occludin*, and (B) higher expression of *N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin* upon treatment with TNF-α at concentrations of 0, 10, 20 and 30 ng/ml for 72 h. n=3. **P<0.01, ***P<0.005, ****P<0.001. TNF-α, tumor necrosis factor-α; *Snai2*, snail family transcriptional repressor 2; ns, not significant.

(422 patients) and LIHC-TCGA-Liver hepatocellular carcinoma June 2016 (361 patients) (8,28-31). The gene expression of immune checkpoint molecules in combination with TNF-α were examined and their association with the survival of patients was analyzed (Cox regression analyses) using the patient database with patient survival information (8). The survival times were estimated using Kaplan-Meier curves (8).

Statistical analysis. All experiments were repeated at least three times and representative results are presented. Statistical analyses were performed using Prism software version 8.00 (GraphPad Software, Inc.). Data for dose concentration and time course experiments were analyzed using one-way ANOVA followed by Dunnet's multiple comparisons test. Comparisons of TNF-α-induced EMT and MET following reversal assay were performed using ANOVA followed by Sidak's multiple comparisons test. Gene expression differences between control and TNF-α treated cells were analyzed using Student's t-test. The results are presented as the mean ± SEM. Error bars indicate SEM. P<0.05 was considered to indicate a statistically significant difference.

For survival analyses using SurvExpress, a log-rank test was used for testing the P-value of survival curves, and deviance residuals were applied for the correlation coefficient (8,32). Moreover, the hazard ratio (HR) between the groups were estimated using another Cox model (8).

Results

TNF-α induces EMT in human HCC cells. Several cytokines are known to induce EMT in HCC cells, including TNF-α. In order to induce EMT, Hep3B and PLC/PRF/5 cells were treated with various concentrations of TNF-α for 72 h to assess EMT induction. EMT induced by TNF-α in Hep3B cells was determined by downregulation of epithelial markers (*E-cadherin* and *Occludin*) and upregulation of mesenchymal markers [*N-cadherin*, *Vimentin*, snail family transcriptional repressor 2 (*Snai2*) and *Fibronectin*]. The optimal EMT effects of TNF-α were observed at 20 ng/ml (Fig. 1). Thus, the concentration of 20 ng/ml was selected for all further studies. Furthermore, Hep3B cells were treated with 20 ng/ml TNF-α at various time-points to observe optimal EMT induction. The qPCR results showed that TNF-α treatment at various time-points was able to induce EMT in Hep3B cells (Fig. 2). At 72 h, robust EMT marker changes were observed; therefore, this time-point was selected for further study. Similarly, robust EMT in PLC/PRF/5 cells treated with 20 ng/ml of TNF-α at 72 h was observed. EMT induction was demonstrated by the downregulation of epithelial markers (*E-cadherin* and *Occludin*) and an upregulation of mesenchymal markers (*N-cadherin* and *Vimentin*; Fig. S1). No expression of *Snai2* and *Fibronectin* was detected in this cell line. The ability of TNF-α to induce EMT in HCC was further validated at the protein level by western blot analysis in Hep3B (Fig. 3A) and PLC/PRF/5 (Fig. 3B) cells.

TNF-α-mediated EMT upregulates immune checkpoint expression. To examine whether TNF-α-mediated EMT influenced the expression of immune modulators, Hep3B and PLC/PRF/5 cells were treated with 20 ng/ml TNF-α for 72 h. The cells were then evaluated for expression of immune modulators *PD-L1*, *PD-L2*, *CD73*, *B7-H3*, V-set domain-containing T-cell activation inhibitor 1 (*VTCN1*) and V-domain immunoglobulin suppressor of T-cell activation (*VISTA*). These immune regulators were assessed as our group previously demonstrated that these immune checkpoint molecules are associated with a poor prognosis in patients with HCC (8). The qPCR results revealed upregulation of four immune modulators (*PD-L1*, *PD-L2*, *CD73* and *B7-H3*) and downregulation of two immune modulators (*VTCN1* and *VISTA*) upon treatment

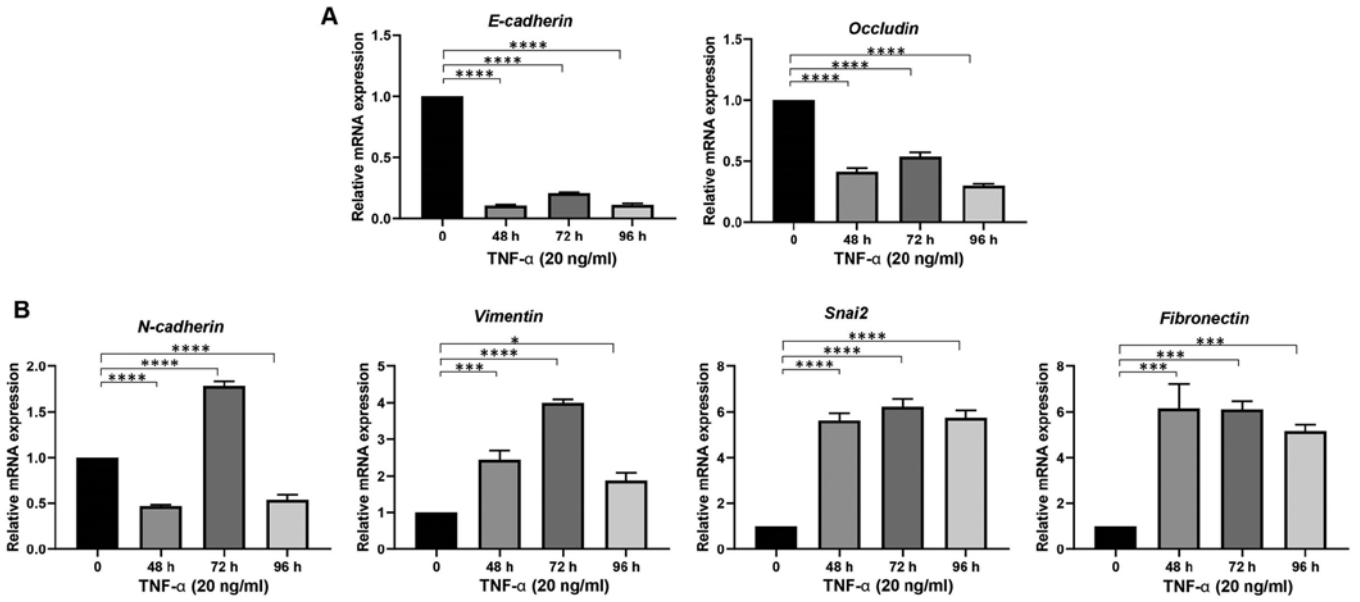

Figure 2. TNF-α induces epithelial-to-mesenchymal transition in Hep3B cells at various time-points. Reverse transcription-quantitative PCR analysis demonstrated (A) lower expression of *E-cadherin* and *Occludin*, and (B) higher expression of *N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin* upon treatment with 20 ng/ml TNF-α at time-points of 48, 72 and 96 h. n=3. *P<0.05, ***P<0.005, ****P<0.001. TNF-α, tumor necrosis factor-α; *Snai2*, snail family transcriptional repressor 2.

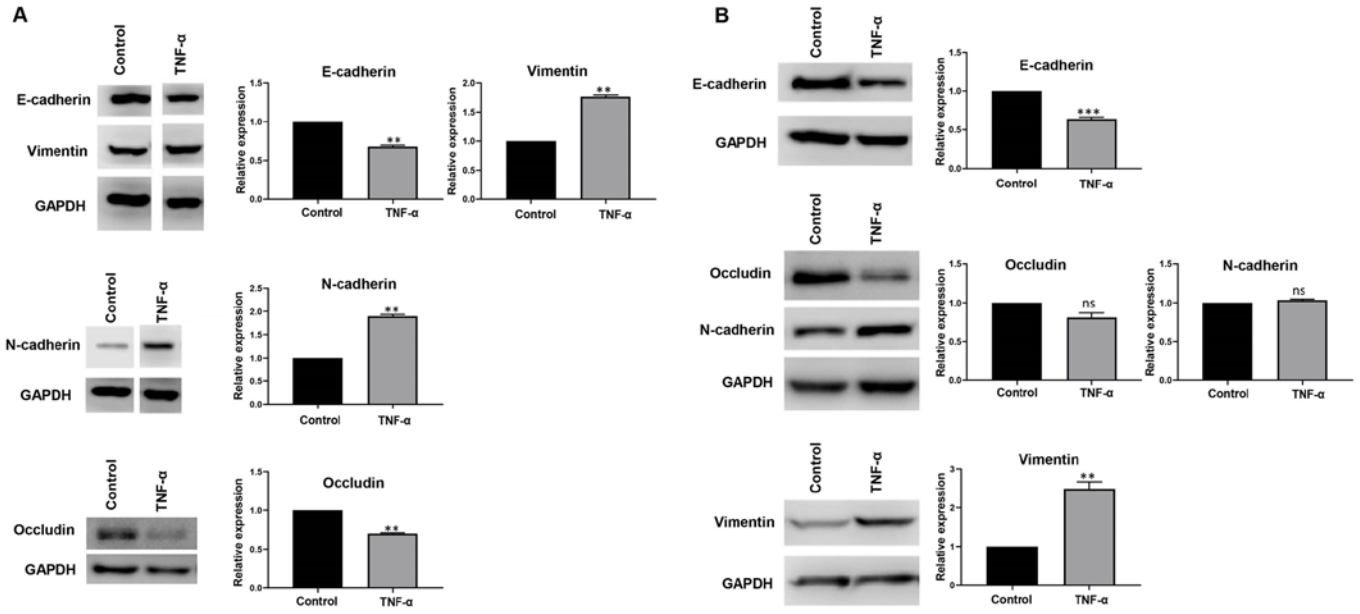

Figure 3. TNF-α induces epithelial-to-mesenchymal transition in hepatocellular carcinoma cells. Western blot analysis demonstrated lower expression of E-cadherin and Occludin, and higher expression of N-cadherin and Vimentin in (A) Hep3B and (B) PLC/PRF/5 cells upon treatment with 20 ng/ml TNF-α for 72 h. n=3. **P<0.01, ***P<0.005 vs. control. TNF-α, tumor necrosis factor-α; ns, not significant.

with TNF-α in Hep3B cells (Fig. 4A and B). The upregulation of immune checkpoints by TNF-α-mediated EMT in Hep3B cells was validated at the protein level by western blot analysis (Fig. 4C).

Similarly, upregulation of immune modulators *PD-L1*, *CD73* and *B7-H3* was observed upon treatment with TNF-α in PLC/PRF/5 cells (Fig. 5A). *PD-L2*, *VTCN1* and *VISTA* were not detected in PLC/PRF/5 cells. This upregulation of immune modulators by TNF-α in PLC/PRF/5 cells was further confirmed by western blot analysis (Fig. 5B). This demonstrated that TNF-α has a potentially important role in modulating immune checkpoints and EMT in HCC.

Reversal of TNF-α-mediated EMT reverses immune checkpoint expression. In order to assess the association between TNF-α-mediated EMT and immune modulator expression, an EMT reversal assay was performed. The induction of MET was evidenced by the increase in epithelial markers (*E-cadherin* and *Occludin*) and a decrease in mesenchymal markers (*N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin*) in

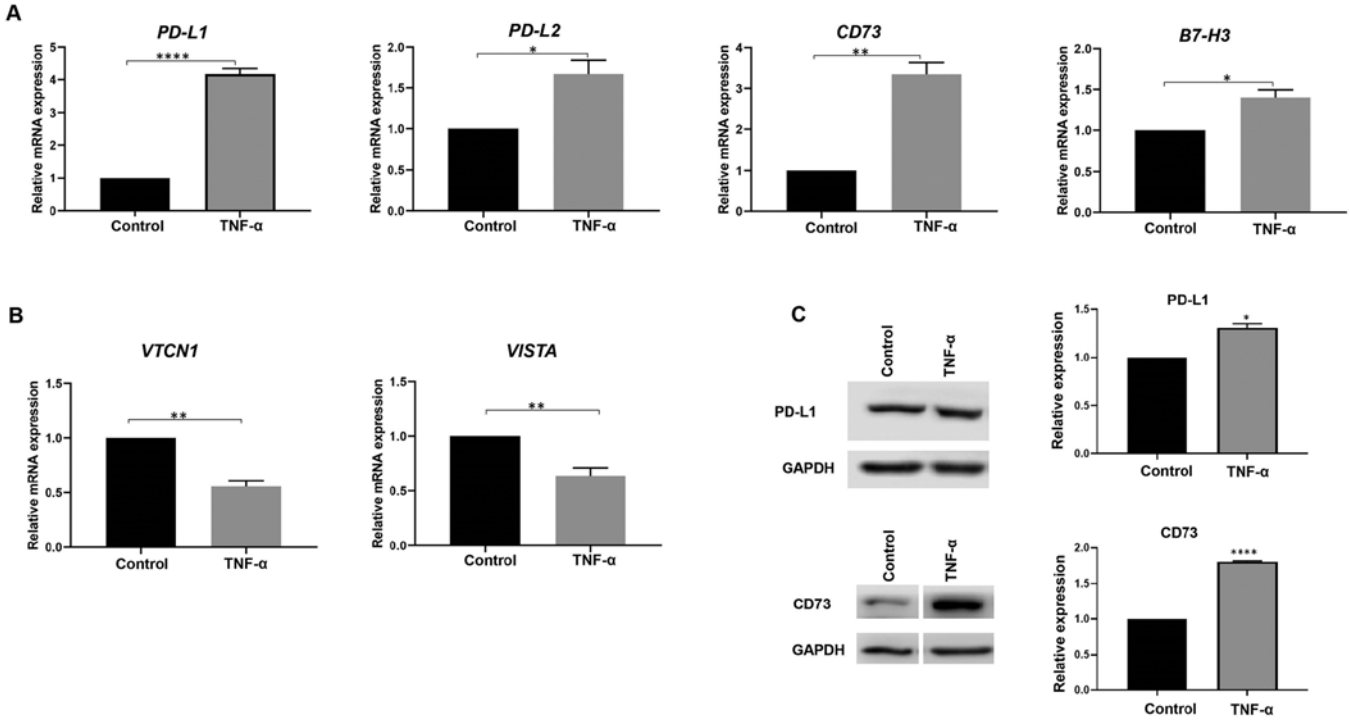

Figure 4. TNF-α-induced epithelial-to-mesenchymal transition regulates expression of immune checkpoint molecules in Hep3B cells. Reverse transcription-quantitative PCR analysis demonstrated (A) higher expression of *PD-L1*, *PD-L2*, *CD73* and *B7-H3*, and (B) lower expression of *VTCN1* and *VISTA* upon treatment with 20 ng/ml TNF-α for 72 h. n=3. *P<0.05, **P<0.01, ****P<0.001. (C) Western blot analysis demonstrated upregulation of PD-L1 and CD73 in Hep3B cells upon treatment with 20 ng/ml TNF-α for 72 h. n=3. *P<0.05, ****P<0.001 vs. control. GAPDH was used as the loading control. TNF-α, tumor necrosis factor-α; *VTCN1*, V-set domain-containing T-cell activation inhibitor 1; *VISTA*, V-domain immunoglobulin suppressor of T-cell activation; PD-L, programmed death receptor ligand.

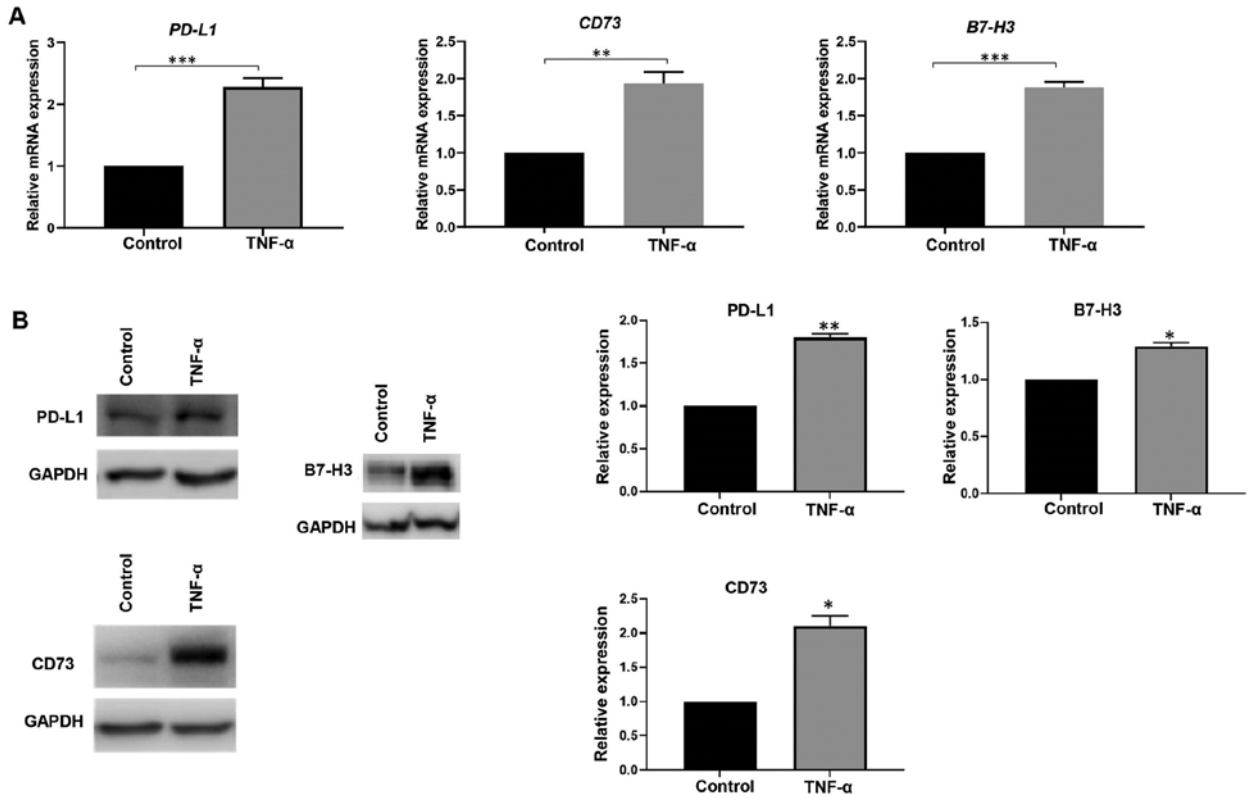

Figure 5. TNF-α-induced epithelial-to-mesenchymal transition regulates expression of immune checkpoint molecules in PLC/PRF/5 cells. (A) Reverse transcription-quantitative PCR analysis demonstrated higher expression of *PD-L1*, *CD73* and *B7-H3* in PLC/PRF/5 cells upon treatment with 20 ng/ml TNF-α for 72 h. n=3. **P<0.01, ***P<0.005. (B) Western blot analysis demonstrated upregulation of PD-L1, CD73 and B7-H3 in PLC/PRF/5 cells upon treatment with 20 ng/ml TNF-α for 72 h. n=3. *P<0.05, **P<0.01 vs. control. GAPDH was used as the loading control. TNF-α, tumor necrosis factor-α; PD-L, programmed death receptor ligand.

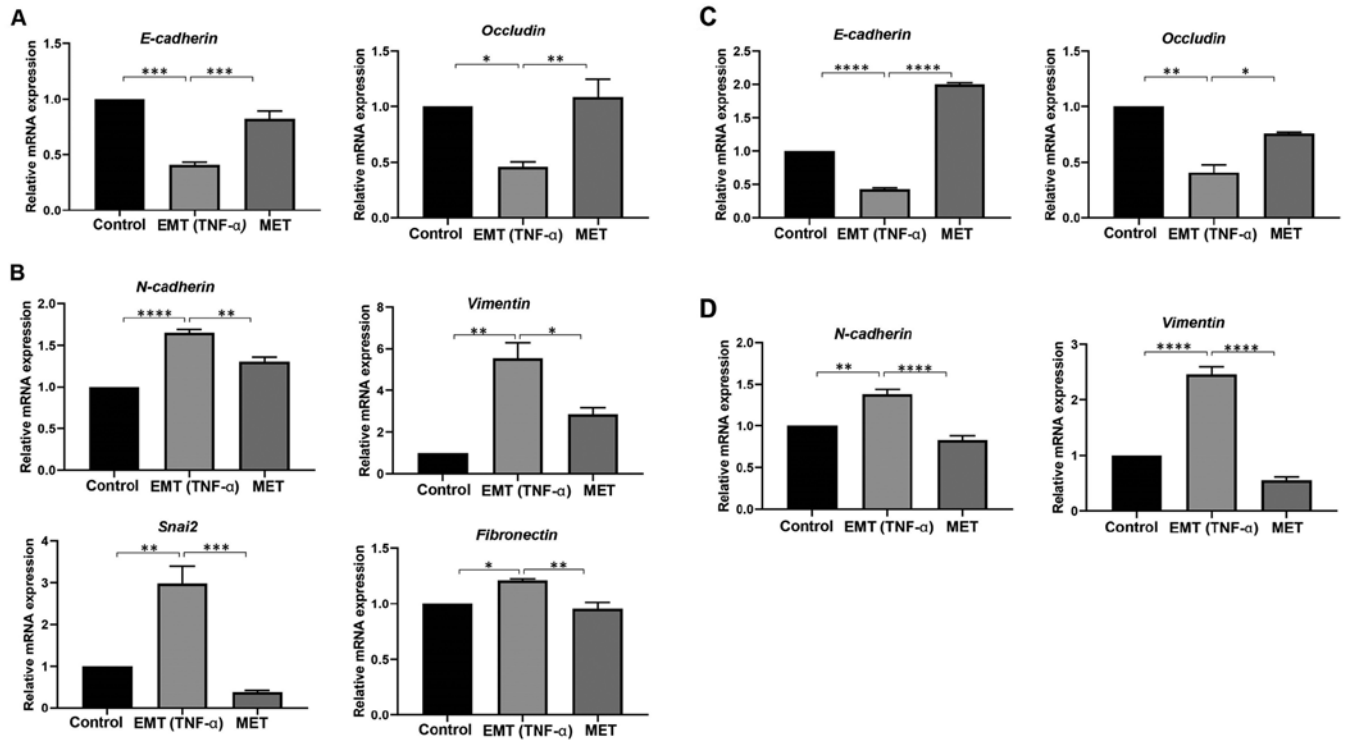

Figure 6. TNF-α-induced EMT in hepatocellular carcinoma cells is reversible. RT-qPCR analysis demonstrated (A) higher expression of *E-cadherin* and *Occludin*, and (B) lower expression of *N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin* in TNF-α treated Hep3B cells following reversal assay. n=3. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001. RT-qPCR analysis presented (C) higher expression of *E-cadherin* and *Occludin*, and (D) lower expression of *N-cadherin* and *Vimentin* in TNF-α treated PLC/PRF/5 cells following reversal assay. n=3. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001. EMT, epithelial-to-mesenchymal transition; TNF-α, tumor necrosis factor-α; RT-qPCR, reverse transcription-quantitative PCR; *Snai2*, snail family transcriptional repressor 2; MET, mesenchymal-to-epithelial transition.

Hep3B cells (Fig. 6A and B). Similar MET was observed in PLC/PRF/5 cells, with an increase in epithelial markers (*E-cadherin* and *Occludin*) and a decrease in mesenchymal markers (*N-cadherin* and *Vimentin*; Fig. 6C and D). The changes in the EMT status observed by the reversal assay in Hep3B cells was further confirmed by immunofluorescence and western blot analysis (Fig. 7). The reversal of EMT status in the reversal assay in PLC/PRF/5 cells was also demonstrated by immunofluorescence and western blot analysis (Fig. S2).

The expression of immune checkpoints was also detected following the reversal assay. Downregulation of *PD-L1*, *PD-L2*, *CD73* and *B7-H3* was observed in Hep3B cells upon removal of TNF-α (Fig. 8A). Downregulation of PD-L1, CD73 and B7-H3 was additionally observed in PLC/PRF/5 cells upon removal of TNF-α (Fig. 8B). The reversal in immune checkpoint expression upon reversal of TNF-α-mediated EMT suggested that TNF-α is involved in regulation of immune checkpoint expression.

To further investigate the association between TNF-α-mediated EMT and PD-L1 expression, immunofluorescence and western blot analysis were performed upon TNF-α treatment followed by the reversal assay. The immunofluorescence staining demonstrated upregulated expression of PD-L1 and CD73 during TNF-α-induced EMT and decreased expression upon reversal of EMT in Hep3B cells (Fig. 9A and B). In addition, western blot analysis showed increased expression of PD-L1, CD73, B7-H3 and PD-L2 during TNF-α-induced EMT and decreased expression upon reversal of EMT in Hep3B cells (Fig. 9C).

Furthermore, the immunofluorescence staining showed elevated expression of PD-L1 and CD73 during TNF-α-induced EMT and decreased expression upon reversal of EMT in PLC/PRF/5 cells (Fig. 10A and B). Moreover, western blot analysis showed increased expression of PD-L1, CD73 and B7-H3 during TNF-α-induced EMT and decreased expression upon reversal of EMT in PLC/PRF/5 cells (Fig. 10C). Overall, the present data demonstrated that TNF-α simultaneously induced EMT and immune checkpoint expression in HCC cells.

Coordinate expression of TNF-α and immune modulators in HCC. A total of six different HCC datasets within SurvExpress were used to analyze the recurrence free survival and OS of immune checkpoint molecules in combination with TNF-α in HCC patients. It was observed that coordinate expression of PD-L1 in combination with TNF-α resulted in a significantly worse OS in 422 patients [HR: 1.45; confidence interval (CI): 1.03-2.02; Log-Rank Equal Curves P=0.02948; Fig. 11A]. Similarly, PD-L2 when combined with TNF-α showed a significantly worse recurrence free survival (HR: 1.44; CI: 1.00-2.06; Log-Rank Equal Curves P=0.04606; Fig. 11B). There was no significant difference in recurrence free survival and OS in patients with HCC who showed coordinate expression of TNF-α with CD73 or B7-H3 (data not shown). The present data suggested a significant association between TNF-α and immune checkpoint expression in patients with HCC, as they showed an adverse prognosis with this profile.

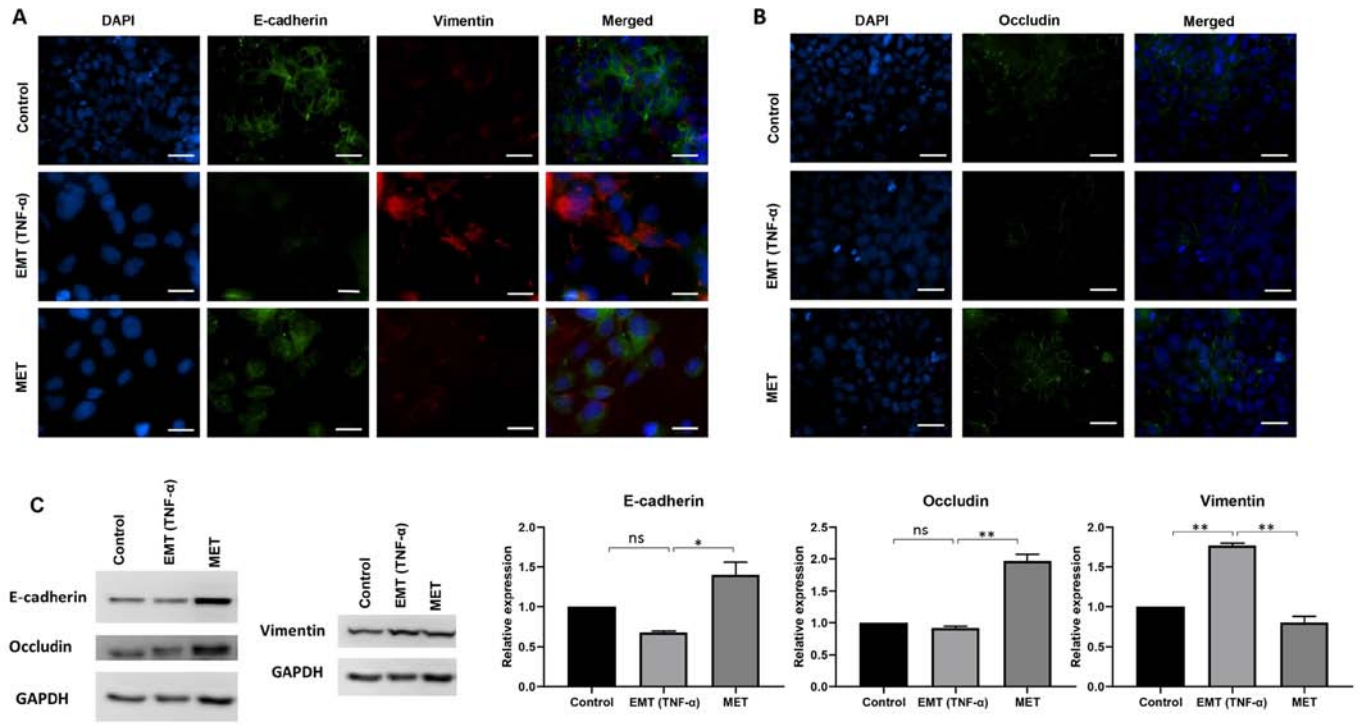

Figure 7. TNF-α-induced EMT in Hep3B cells is reversible. Fluorescence microscopy identified (A) suppression of E-cadherin and upregulation of Vimentin during EMT, and upregulation of E-cadherin and decreased expression of Vimentin during MET. (B) During EMT, repression of Occludin was observed and during MET, higher expression of Occludin was observed. Scale bar, 100 μm. Magnification, x40. (C) Western blot analysis demonstrated upregulation of E-cadherin and Occludin, and downregulation of Vimentin during MET. n=3. *P<0.05, **P<0.01. GAPDH was used as loading control. EMT, epithelial-to-mesenchymal transition; TNF-α, tumor necrosis factor-α; MET, mesenchymal-to-epithelial transition; ns, not significant.

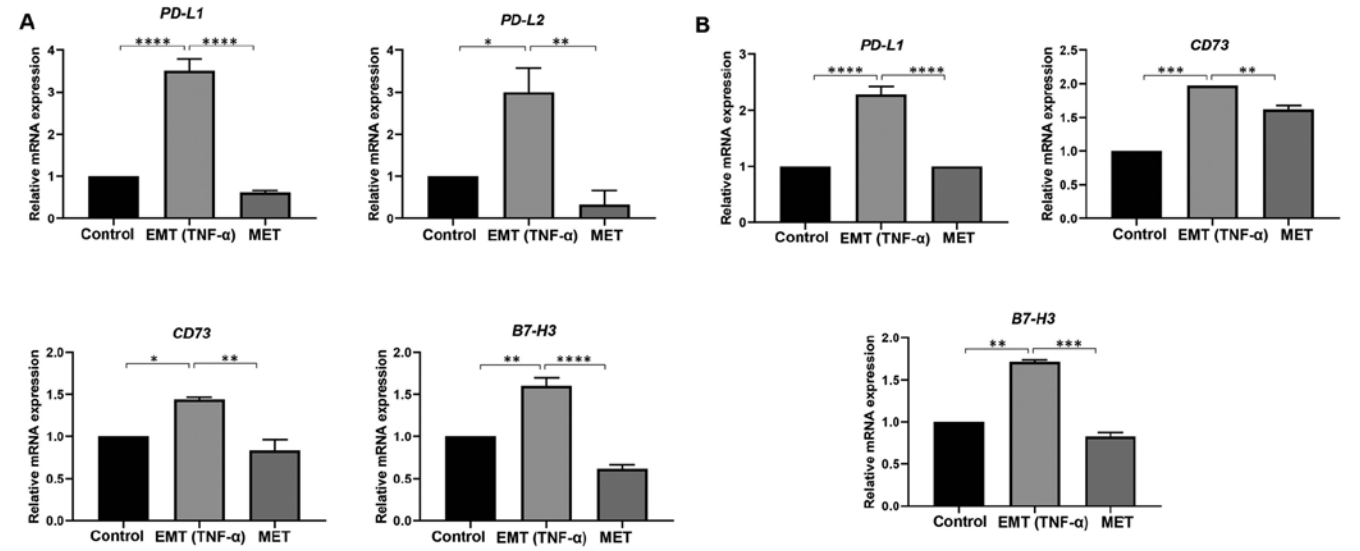

Figure 8. Reversal of EMT returns immune checkpoint expression to control levels in hepatocellular carcinoma cells. Reverse transcription-quantitative PCR analysis demonstrated (A) lower expression of immune checkpoint molecules *PD-L1*, *PD-L2*, *CD73* and *B7-H3* in EMT induced Hep3B cells and (B) lower expression of immune checkpoint molecules *PD-L1*, *CD73* and *B7-H3* in EMT induced PLC/PRF/5 cells following reversal assay. n=3. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001. EMT, epithelial-to-mesenchymal transition; TNF-α, tumor necrosis factor-α; MET, mesenchymal-to-epithelial transition; *PD-L*, programmed death receptor ligand.

Discussion

In the present study, an association between EMT and immune checkpoint molecules in HCC was identified. It was demonstrated that TNF-α simultaneously induced EMT and the expression of immune checkpoint molecules PD-L1, PD-L2, CD73 and B7-H3 in Hep3B cells, along with increased expression of PD-L1, CD73 and B7-H3 in PLC/PRF/5 cells. Moreover, it was demonstrated that EMT status is closely associated with immune checkpoint upregulation as MET induced by the reversal of TNF-α-mediated EMT attenuated the expression of immune checkpoints. Notably, a significant association between

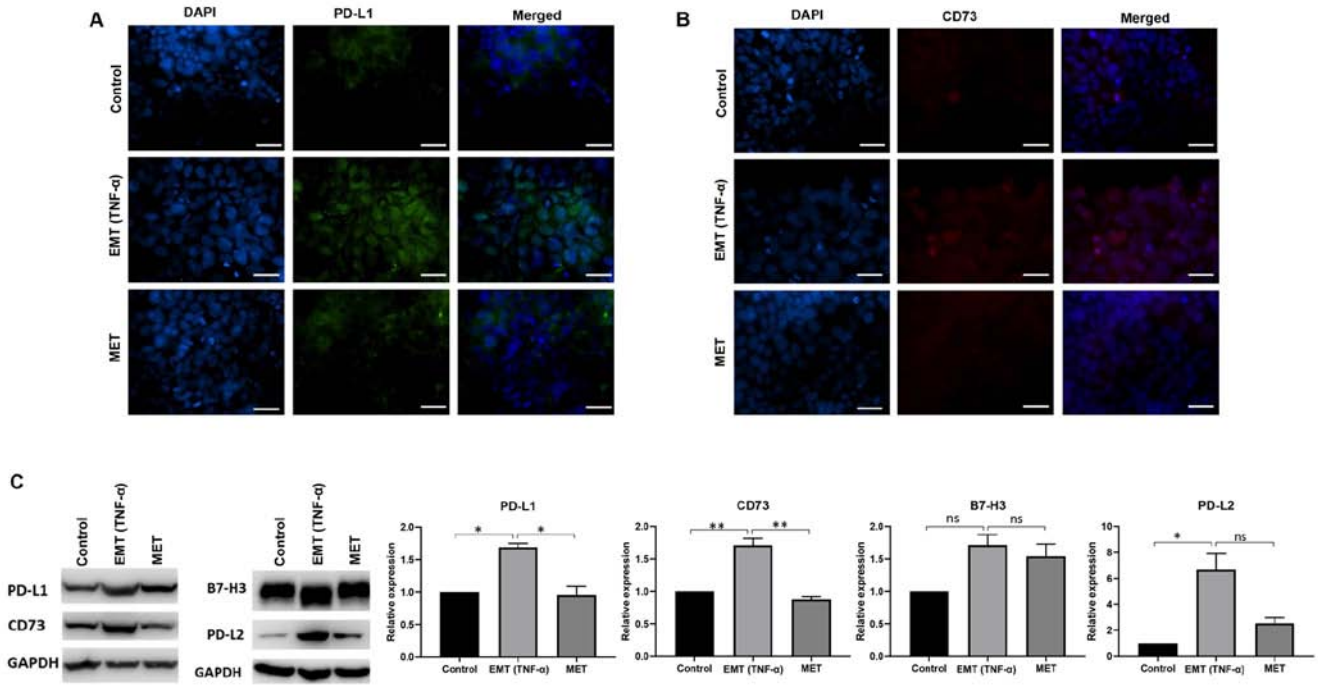

Figure 9. Reversal of EMT returns immune checkpoint expression to control levels in Hep3B cells. Fluorescence microscopy showed (A) higher expression of PD-L1 upon EMT induction and lower expression of PD-L1 following reversal assay and (B) higher expression of CD73 upon EMT induction and lower expression of CD73 following reversal assay in Hep3B cells. Scale bar, 100 μm. Magnification, x40. (C) Western blot analysis demonstrated higher expression of PD-L1, CD73, B7-H3 and PD-L2 upon EMT induction and lower expression following reversal assay in Hep3B cells. n=3. *P<0.05, **P<0.01. GAPDH was used as the loading control. EMT, epithelial-to-mesenchymal transition; TNF-α, tumor necrosis factor-α; MET, mesenchymal-to-epithelial transition; PD-L, programmed death receptor ligand.

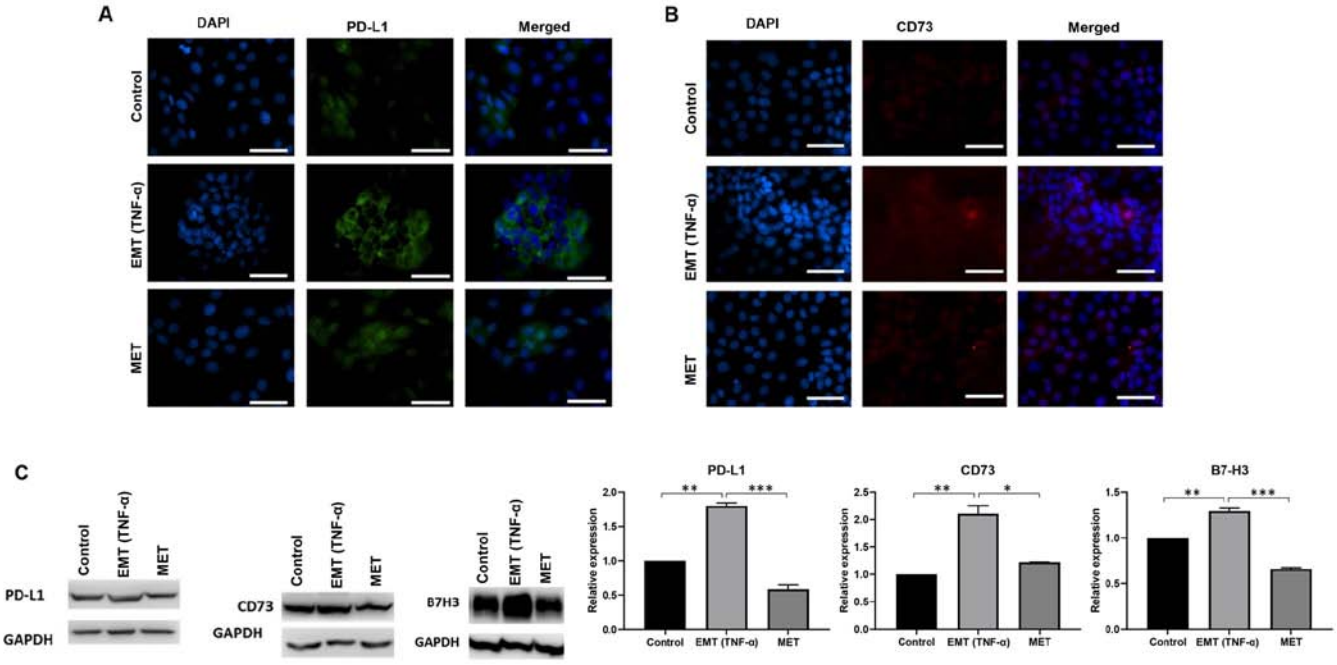

Figure 10. Reversal of EMT reverses immune checkpoint expression in PLC/PRF/5 cells. Fluorescence microscopy revealed (A) higher expression of PD-L1 upon EMT induction and lower expression of PD-L1 following reversal assay, and (B) higher expression of CD73 upon EMT induction and lower expression of CD73 following reversal assay in PLC/PRF/5 cells. Scale bar, 100 μm. Magnification, x40. (C) Western blot analysis demonstrated higher expression of PD-L1, CD73 and B7-H3 upon EMT induction and lower expression following reversal assay in PLC/PRF/5 cells. n=3. *P<0.05, **P<0.01, ***P<0.005. GAPDH was used as the loading control. EMT, epithelial-to-mesenchymal transition; TNF-α, tumor necrosis factor-α; MET, mesenchymal-to-epithelial transition; PD-L, programmed death receptor ligand.

TNF-α and immune checkpoint levels was identified in patients with HCC. Patients with increased expression of both TNF-α and PD-L1 showed poor OS, whereas patients with high expression of TNF-α and PD-L2 showed high rates of recurrence.

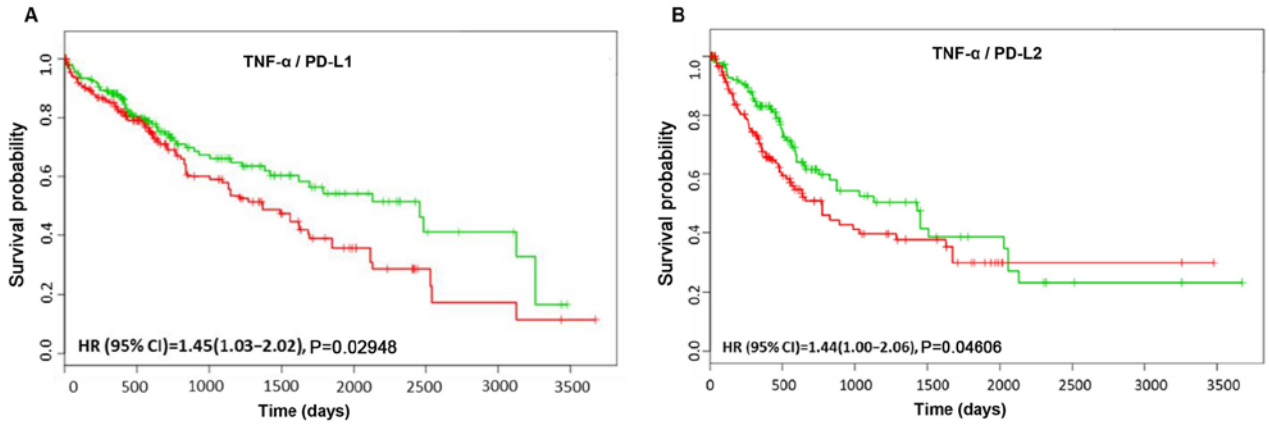

Figure 11. Association of TNF-α in combination with immune checkpoints and survival in patients with HCC. Kaplan-Meier survival curves for the analysis of (A) gene expression of PD-L1/TNF-α and overall survival, and (B) gene expression of PD-L2/TNF-α and recurrence free survival in samples from patients with HCC. Red curve represents the high-risk group, while green curve represents the low-risk group (8). Markers (+) represent censoring samples. TNF-α, tumor necrosis factor-α; PD-L, programmed death receptor ligand; HR, hazard ratio; HCC, hepatocellular carcinoma.

Various systems have been utilized to induce EMT in human HCC cell lines (33,34). Stimulation of HCC cells with TNF-α leads to the induction of EMT (35). TNF-α is known to induce EMT alone or in combination with other cytokines such as transforming growth factor (TGF)-β in several cancer types, such as breast cancer, lung cancer and HCC (19,21,25). In the present study, a TNF-α based *in vitro* model was used to induce EMT in a reversible manner. To the best of the authors' knowledge, the present study is the first study to evaluate the role of EMT in the regulation of immune checkpoint expression in HCC.

Despite the promising results of ICIs from clinical trials, these therapies have failed in several instances due to mutations that alter immunogenicity, expression of alternative immune checkpoint molecules and dysregulated T-cell infiltration (36). Understanding the underlying molecular biological mechanisms regulating immune checkpoints may result in developing new and effective treatment strategies. In the present study it was noted that the expression of immune checkpoint molecules PD-L1, PD-L2, CD73 and B7-H3 was upregulated following TNF-α-induced EMT. PD-L1 is an essential and widely studied immunomodulatory ligand that is aberrantly upregulated in several cancer types, has roles in promoting tumor escape and is associated with poor prognosis (37-41). Our group and other researchers have reported the association between upregulation of PD-L1 and poor survival in patients with HCC (8,42). Several previous studies have reported different mechanisms involved in the regulation of PD-L1 expression in cancer cells (19,43-45). However, the mechanism of PD-L1 expression in HCC still remains unclear (46).

In previous years, previous studies have demonstrated cytokine-induced EMT, in particular TNF-α, TGF-β and interferon-γ are responsible for elevating the expression of PD-L1 in cancer, such as non-small cell lung carcinoma and breast cancer (17,19,21). The present finding that TNF-α is involved in the upregulation of PD-L1 is consistent with these studies. Another previous study demonstrated that PD-L1 was upregulated in EMT positive human esophageal tumor samples compared with the EMT negative samples (23). The relationship between EMT and PD-L1 was further examined in human breast cancer cells by Noman *et al* (20). This previous study identified increased expression of PD-L1 in cells having undergone EMT by EMT-transcription factors (zinc finger E-box binding homeobox 1, microRNA 200 or Snai1) and PD-L1 rendered EMT-activated cells resistant to cytotoxic T-lymphocytes-mediated lysis (20). In patients with HCC, our group previously identified an association between PD-L1 expression and EMT status (8). High PD-L1 expression was closely associated with high expression of the mesenchymal maker Vimentin and low expression of epithelial marker E-cadherin (8). However, to the best of the authors' knowledge, no studies have reported the association between EMT and other immune checkpoint in HCC.

PD-L2 is a second ligand that binds to PD-1 to prevent cytokine production, cell adhesion and T-cell proliferation (47). Our group and other researchers have previously reported that PD-L2 expression is associated with poor survival and recurrence of HCC in patients (8,48). A previous meta-analysis study by Yang *et al* (49) identified that upregulation of PD-L2 predicted poor OS in HCC.

CD73 is also reported to be a novel prognostic biomarker for HCC (8,50,51). However, to the best of the authors' knowledge, the regulation of CD73 expression has not been studied in the context of EMT, and the present study is the first to report that TNF-α-induced EMT regulates CD73 expression. In a recent triple-negative breast cancer study by Qiao *et al* (52), an anti-CD73 antibody was demonstrated to inhibit lung metastasis *in vivo*.

B7-H3 is mostly upregulated in several cancer types, including liver, breast, bladder, colorectal, cervical, glioma, esophageal and gastric cancer (53). Previous studies have also demonstrated that dysregulation of B7-H3 in HCC results in impaired T-lymphocyte function, thus leading to poor prognosis and recurrence (54,55). In muscle-invasive bladder cancer, high expression of B7-H3 was associated with a poor clinicopathological status and poor prognosis (56). Most notably, B7-H3 is known to promote EMT by repressing E-cadherin expression, and upregulating Vimentin and N-cadherin expression in colorectal cancer (57). To the best of

the authors' knowledge, the present study is the first to report the regulation of B7-H3 expression by EMT in HCC.

Further mechanistic and functional studies are necessary to examine the molecular biology of immune checkpoints and its precise role in EMT in HCC. However, given the association between EMT, TNF-α and immune checkpoint in HCC, it is conceivable that combining EMT or TNF-α inhibitors with ICIs in patients with HCC may be a future therapeutic approach for the management of HCC.

Acknowledgements

The authors would like to thank Mrs Lesley-Anne Jaskowski, Gallipoli Medical Research Institute, Greenslopes Private Hospital, for her technical assistance during the revision of the manuscript.

Funding

The present study was funded by Gallipoli Medical Research Foundation (project no. 017401).

Availability of data and materials

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

Authors' contributions

RS, KRB, DHGC and AJ designed the study, performed data acquisition, analysis and interpretation, and wrote the manuscript. RS, AJ, KRB and DHGC critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chacko S and Samanta S: 'Hepatocellular carcinoma: A life-threatening disease'. Biomed Pharmacother 84: 1679-1688, 2016.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
3. Sayiner M, Golabi P and Younossi ZM: Disease burden of hepatocellular carcinoma: A global perspective. Dig Dis Sci 64: 910-917, 2019.
4. Kudo M: A new era of systemic therapy for hepatocellular carcinoma with regorafenib and lenvatinib. Liver Cancer 6: 177-184, 2017.
5. Mir N, Jayachandran A, Dhungel B, Shrestha R and Steel JC: Epithelial-to-mesenchymal transition: A Mediator of sorafenib resistance in advanced hepatocellular carcinoma. Curr Cancer Drug Targets 17: 698-706, 2017.
6. Sharma P and Allison JP: The future of immune checkpoint therapy. Science 348: 56-61, 2015.
7. Pardoll DM: The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12: 252-264, 2012.
8. Shrestha R, Prithviraj P, Anaka M, Bridle KR, Crawford DH, Dhungel B, Steel JC and Jayachandran A: Monitoring immune checkpoint regulators as predictive biomarkers in hepatocellular carcinoma. Front Oncol 8: 269, 2018.
9. Hato T, Goyal L, Greten TF, Duda DG and Zhu AX: Immune checkpoint blockade in hepatocellular carcinoma: Current progress and future directions. Hepatology 60: 1776-1782, 2014.
10. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WE, Poddubskaya E, Antonia S, Pluzanski A, Vokes EE, Holgado E, *et al*: Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med 373: 123-135, 2015.
11. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, Hoeller C, Khushalani NI, Miller WH Jr, Lao CD, *et al*: Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): A randomised, controlled, open-label, phase 3 trial. Lancet Oncol 16: 375-384, 2015.
12. Jindal A, Thadi A and Shailubhai K: Hepatocellular carcinoma: Etiology and current and future drugs. J Clin Exp Hepatol 9: 221-232, 2019.
13. Kudo M: Immune checkpoint inhibition in hepatocellular carcinoma: Basics and ongoing clinical trials. Oncology 92 (Suppl 1): 50-62, 2017.
14. Li Z, Li N, Li F, Zhou Z, Sang J, Chen Y, Han Q, Lv Y and Liu Z: Immune checkpoint proteins PD-1 and TIM-3 are both highly expressed in liver tissues and correlate with their gene polymorphisms in patients with HBV-related hepatocellular carcinoma. Medicine (Baltimore) 95: e5749, 2016.
15. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420-1428, 2009.
16. Jayachandran A, Dhungel B and Steel JC: Epithelial-to-mesenchymal plasticity of cancer stem cells: Therapeutic targets in hepatocellular carcinoma. J Hematol Oncol 9: 74, 2016.
17. David JM, Dominguez C, McCampbell KK, Gulley JL, Schlom J and Palena C: A novel bifunctional anti-PD-L1/TGF-β Trap fusion protein (M7824) efficiently reverts mesenchymalization of human lung cancer cells. OncoImmunology 6: e1349589, 2017.
18. David JM, Dominguez C and Palena C: Pharmacological and immunological targeting of tumor mesenchymalization. Pharmacol Ther 170: 212-225, 2017.
19. Alsuliman A, Colak D, Al-Harazi O, Fitwi H, Tulbah A, Al-Tweigeri T, Al-Alwan M and Ghebeh H: Bidirectional crosstalk between PD-L1 expression and epithelial to mesenchymal transition: Significance in claudin-low breast cancer cells. Mol Cancer 14: 149, 2015.
20. Noman MZ, Janji B, Abdou A, Hasmim M, Terry S, Tan TZ, Mami-Chouaib F, Thiery JP and Chouaib S: The immune checkpoint ligand PD-L1 is upregulated in EMT-activated human breast cancer cells by a mechanism involving ZEB-1 and miR-200. Oncoimmunology 6: e1263412, 2017.
21. Asgarova A, Asgarov K, Godet Y, Peixoto P, Nadaradjane A, Boyer-Guittaut M, Galaine J, Guenat D, Mougey V, Perrard J, *et al*: PD-L1 expression is regulated by both DNA methylation and NF-κB during EMT signaling in non-small cell lung carcinoma. Oncoimmunology 7: e1423170, 2018.
22. Imai D, Yoshizumi T, Okano S, Itoh S, Ikegami T, Harada N, Aishima S, Oda Y and Maehara Y: IFN-γ promotes epithelial-mesenchymal transition and the expression of PD-L1 in pancreatic cancer. J Surg Res 240: 115-123, 2019.
23. Chen L, Xiong Y, Li J, Zheng X, Zhou Q, Turner A, Wu C, Lu B and Jiang J: PD-L1 expression promotes epithelial to mesenchymal transition in human esophageal cancer. Cell Physiol Biochem 42: 2267-2280, 2017.
24. Wang Y, Hu J, Wang Y, Ye W, Zhang X, Ju H, Xu D, Liu L, Ye D, Zhang L, *et al*: EGFR activation induced Snail-dependent EMT and myc-dependent PD-L1 in human salivary adenoid cystic carcinoma cells. Cell Cycle 17: 1457-1470, 2018.
25. Chen Y, Wen H, Zhou C, Su Q, Lin Y, Xie Y, Huang Y, Qiu Q, Lin J, Huang X, *et al*: TNF-α derived from M2 tumor-associated macrophages promotes epithelial-mesenchymal transition and cancer stemness through the Wnt/β-catenin pathway in SMMC-7721 hepatocellular carcinoma cells. Exp Cell Res 378: 41-50, 2019.

26. Jayachandran A, Shrestha R, Dhungel B, Huang IT, Vasconcelos MY, Morrison BJ, Ramlogan-Steel CA and Steel JC: Murine hepatocellular carcinoma derived stem cells reveal epithelial-to-mesenchymal plasticity. World J Stem Cells 9: 159-168, 2017.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Aguirre-Gamboa R, Gomez-Rueda H, Martínez-Ledesma E, Martínez-Torteya A, Chacolla-Huaringa R, Rodriguez-Barrientos A, Tamez-Peña JG and Treviño V: SurvExpress: An online biomarker validation tool and database for cancer gene expression data using survival analysis. PLoS One 8: e74250, 2013.
29. Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, *et al*: Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res 69: 7385-7392, 2009.
30. Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, *et al*: Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 359: 1995-2004, 2008.
31. Tsuchiya M, Parker JS, Kono H, Matsuda M, Fujii H and Rusyn I: Gene expression in nontumoral liver tissue and recurrence-free survival in hepatitis C virus-positive hepatocellular carcinoma. Mol Cancer 9: 74, 2010.
32. Awan FM, Naz A, Obaid A, Ali A, Ahmad J, Anjum S and Janjua HA: Identification of Circulating Biomarker Candidates for Hepatocellular Carcinoma (HCC): An Integrated Prioritization Approach. PLoS One 10: e0138913, 2015.
33. Lee TK, Poon RT, Yuen AP, Ling MT, Kwok WK, Wang XH, Wong YC, Guan XY, Man K, Chau KL, *et al*: Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. Clin Cancer Res 12: 5369-5376, 2006.
34. Xu Z, Shen MX, Ma DZ, Wang LY and Zha XL: TGF-beta1-promoted epithelial-to-mesenchymal transformation and cell adhesion contribute to TGF-beta1-enhanced cell migration in SMMC-7721 cells. Cell Res 13: 343-350, 2003.
35. Zhu Y, Cheng Y, Guo Y, Chen J, Chen F, Luo R and Li A: Protein kinase D2 contributes to TNF-α-induced epithelial mesenchymal transition and invasion via the PI3K/GSK-3β/β-catenin pathway in hepatocellular carcinoma. Oncotarget 7: 5327-5341, 2016.
36. Xu F, Jin T, Zhu Y and Dai C: Immune checkpoint therapy in liver cancer. J Exp Clin Cancer Res 37: 110, 2018.
37. Afreen S and Dermime S: The immunoinhibitory B7-H1 molecule as a potential target in cancer: Killing many birds with one stone. Hematol Oncol Stem Cell Ther 7: 1-17, 2014.
38. Mu CY, Huang JA, Chen Y, Chen C and Zhang XG: High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. Med Oncol 28: 682-688, 2011.
39. Muenst S, Schaerli AR, Gao F, Däster S, Trella E, Droeser RA, Muraro MG, Zajac P, Zanetti R, Gillanders WE, *et al*: Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 146: 15-24, 2014.
40. Shi SJ, Wang LJ, Wang GD, Guo ZY, Wei M, Meng YL, Yang AG and Wen WH: B7-H1 expression is associated with poor prognosis in colorectal carcinoma and regulates the proliferation and invasion of HCT116 colorectal cancer cells. PLoS One 8: e76012, 2013.
41. Thompson RH, Gillett MD, Cheville JC, Lohse CM, Dong H, Webster WS, Krejci KG, Lobo JR, Sengupta S, Chen L, *et al*: Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. Proc Natl Acad Sci USA 101: 17174-17179, 2004.
42. Calderaro J, Rousseau B, Amaddeo G, Mercey M, Charpy C, Costentin C, Luciani A, Zafrani ES, Laurent A, Azoulay D, *et al*: Programmed death ligand 1 expression in hepatocellular carcinoma: Relationship with clinical and pathological features. Hepatology 64: 2038-2046, 2016.
43. Crane CA, Panner A, Murray JC, Wilson SP, Xu H, Chen L, Simko JP, Waldman FM, Pieper RO and Parsa AT: PI(3) kinase is associated with a mechanism of immunoresistance in breast and prostate cancer. Oncogene 28: 306-312, 2009.
44. Dey N, Crosswell HE, De P, Parsons R, Peng Q, Su JD and Durden DL: The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration. Cancer Res 68: 1862-1871, 2008.
45. Ghebeh H, Tulbah A, Mohammed S, Elkum N, Bin Amer SM, Al-Tweigeri T and Dermime S: Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells. Int J Cancer 121: 751-758, 2007.
46. Funaki S, Shintani Y, Kawamura T, Kanzaki R, Minami M and Okumura M: Chemotherapy enhances programmed cell death 1/ligand 1 expression via TGF-β induced epithelial mesenchymal transition in non-small cell lung cancer. Oncol Rep 38: 2277-2284, 2017.
47. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, *et al*: PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2: 261-268, 2001.
48. Jung HI, Jeong D, Ji S, Ahn TS, Bae SH, Chin S, Chung JC, Kim HC, Lee MS and Baek MJ: Overexpression of PD-L1 and PD-L2 is associated with poor prognosis in patients with hepatocellular carcinoma. Cancer Res Treat 49: 246-254, 2017.
49. Yang H, Zhou X, Sun L and Mao Y: Correlation Between PD-L2 expression and clinical outcome in solid cancer patients: A meta-analysis. Front Oncol 9: 47, 2019.
50. Ma XL, Shen MN, Hu B, Wang BL, Yang WJ, Lv LH, Wang H, Zhou Y, Jin AL, Sun YF, *et al*: CD73 promotes hepatocellular carcinoma progression and metastasis via activating PI3K/AKT signaling by inducing Rap1-mediated membrane localization of P110β and predicts poor prognosis. J Hematol Oncol 12: 37, 2019.
51. Sciarra A, Monteiro I, Ménétrier-Caux C, Caux C, Gilbert B, Halkic N, La Rosa S, Romero P, Sempoux C and de Leval L: CD73 expression in normal and pathological human hepatobiliopancreatic tissues. Cancer Immunol Immunother 68: 467-478, 2019.
52. Qiao Z, Li X, Kang N, Yang Y, Chen C, Wu T, Zhao M, Liu Y and Ji X: A Novel specific Anti-CD73 antibody inhibits triple-negative breast cancer cell motility by regulating autophagy. Int J Mol Sci 20: E1057, 2019.
53. Dong P, Xiong Y, Yue J, Hanley SJB and Watari H: B7H3 as a promoter of metastasis and promising therapeutic target. Front Oncol 8: 264, 2018.
54. Kang FB, Wang L, Jia HC, Li D, Li HJ, Zhang YG and Sun DX: B7-H3 promotes aggression and invasion of hepatocellular carcinoma by targeting epithelial-to-mesenchymal transition via JAK2/STAT3/Slug signaling pathway. Cancer Cell Int 15: 45, 2015.
55. Zheng Y, Liao N, Wu Y, Gao J, Li Z, Liu W, Wang Y, Li M, Li X, Chen L, *et al*: High expression of B7 H2 or B7 H3 is associated with poor prognosis in hepatocellular carcinoma. Mol Med Rep 19: 4315-4325, 2019.
56. Xu ZL, Zhang Y, Wang L, Li F, Man HW, Li PF and Shan BE: B7 H3 promotes malignant progression of muscle invasive bladder cancer. Oncol Rep 40: 2722-2733, 2018.
57. Jiang B, Zhang T, Liu F, Sun Z, Shi H, Hua D and Yang C: The co-stimulatory molecule B7-H3 promotes the epithelial-mesenchymal transition in colorectal cancer. Oncotarget 7: 31755-31771, 2016.