High lymphocyte-to-monocyte ratio is associated with low α-fetoprotein expression in patients with hepatitis B virus-associated hepatocellular carcinoma

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Abstract. The association of the peripheral lymphocyte-to-monocyte ratio (LMR) with α-fetoprotein (AFP) status in patients with AFP-positive and AFP-negative hepatocellular carcinoma (HCC) has not been investigated in detail. The aim of the present study was to examine the association between the LMR and AFP status in these patients. The samples were obtained from patients with a hepatitis B virus (HBV) infection, who were negative for non-HBV hepatitis viruses and who did not suffer from autoimmune hepatitis. These patients were retrospectively reviewed and the differences of test indicators in the AFP-negative and AFP-positive groups were assessed. Flow cytometry was used to detect the expression levels of CD4, CD8 and programmed cell death protein 1 (PD-1), and ELISAs were used to analyze the expression levels of interleukin (IL)-10 and transforming growth factor (TGF)-β1. In addition, luciferase reporter assays were used to assess binding of the IL-10 promoter to the glucocorticoid receptor (GR) gene. Receiver operating characteristic curve and Spearman correlation analyses demonstrated that the AFP-negative HCC group exhibited a higher LMR, lower D-dimer and lower fibrin degradation products compared with the AFP-positive HCC group. The cut-off value of the LMR was 2.01 for AFP detection, with a sensitivity of 68.6% and a specificity of 75%. The high LMR noted in the AFP-negative HCC group was accompanied by a lower proportion of $CD4^+$ T lymphocytes and CD8-PD-1 expression compared with the corresponding levels of these parameters in the AFP-positive HCC group. Furthermore, the high levels of IL-10 and low levels of TGF-β1 were expressed in the AFP-positive HCC group. The data indicated that the IL-10-592 promoter exhibited a potent induction of luciferase activity in 293T cells cotransfected with a GR-overexpressing vector compared with the control cells. However, the relative luciferase activity was not altered following a mutation or polymorphism in the IL-10 gene. These results suggested that a high LMR was indicative of low AFP expression in HBV-associated HCC patients.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. These tumours primarily occur following a hepatitis B virus (HBV) infection or a medical history of chronic hepatitis viral infection and cirrhosis (1,2). Accumulating evidence has suggested that cytokine levels may predict the progression of HBV infections (3,4). Interleukin (IL)-10 is an important anti-inflammatory cytokine secreted by specific cells, including T regulatory lymphocytes (Treg cells), activated macrophages and T helper 2 cells. The expression levels of IL-10 regulate the immune function and determine the balance between cellular and humoral responses (5). In addition, the levels of fibrinogen (FIB) and D-dimer have been found to be effective predictors of adverse tumor profiles and outcomes for HCCs (6). Therefore, liver function and coagulation data analyses are useful in understanding the progression of HBV-associated HCC.

α-fetoprotein (AFP) is one of the most common diagnostic indicators of liver cancer and previous studies have shown that patients with AFP-positive liver cancer exhibit lower survival rates than those with AFP-negative liver cancer (7-9). Overall, an inflammatory microenvironment plays an important role in the progression of HCC. The lymphocyte-to-monocyte ratio (LMR) is a novel inflammatory biomarker that combines estimates of host immune homoeostasis and of the tumour microenvironment, and has been identified as a predictor of clinical outcomes for a variety of malignancies, including breast, renal, lung and colorectal cancers (10-14). Previously, it has been shown that the peripheral LMR possesses important prognostic value for patients with HCC (15-17).

$CD4^+$ T lymphocytes are associated with the development of liver cancer, while $CD8^+$ T lymphocytes are associated with the clearance of liver cancer cells (18). AFP can lead to tumor immune escape by affecting the ratio of $CD4^+/CD8^+$ T lymphocytes (19). However, it remains to be investigated

whether the numbers of $CD4^+$ and $CD8^+$ T lymphocytes are associated with AFP levels in HCC.

$CD4^+CD25^+$ Treg cells are a subgroup of $CD4^+$ T lymphocytes with negative immunoregulatory functions. They can exert inhibitory effects by secreting regulatory cytokines, such as IL-10 and transforming growth factor (TGF)-β. The secretion of these cytokines occurs via direct cell contact or via the pathway of programmed cell death protein 1 (PD-1)/PD-1 ligand (PD-L). The latter pathway contributes to the malignant transformation of the cells that escape immune surveillance and immune defense, which in turn results in the occurrence and development of tumors (20-22).

At present, the IL-10-592 polymorphism within the IL-10 gene promoter region has been shown to be associated with the susceptibility to occult HBV infection (OBI), a virological condition characterized by a low release of HBV from liver cells and low HBV-DNA levels in serum and/or liver tissue of HBV surface antigen (HBsAg)-negative subjects, which is the natural course for patients with a chronic HBV infection and patients who develop an HBV infection following immunosuppressive therapy (23-26). According to European guidelines on management of chronic HBV (CHB) infection, the natural history of CHB can be schematically divided into five, not necessarily sequential and stable, phases, the 5th of which is OBI that is defined as the presence of viral DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing negative for the HBsAg (27-29). The mechanism and clinical implications of OBI are not fully understood. Previous studies have reported that OBI can potentially contribute to acute exacerbation, cirrhosis and HCC (30-32). However, OBI prevalence and its natural course in the general population have been insufficiently investigated. HBV DNA without HBs antigenemia has been detected in the following clinical situations: i) Chronic, presumably viral, hepatitis unrelated to HCV, atypical alcoholic hepatitis and HCC; ii) viral reactivation following immunosuppression; and iii) Transmission via transplantation, transfusion or experimental transmission to chimpanzees (29,33-34). Among the immunosuppressive agents, glucocorticoids (GCs) are one of the earliest medications that can effectively inhibit the infection of HBV (35). During this type of infection, IL-10 expression is affected by the GC via ERK-mediated phosphorylation of the GC receptor (GR) in dendritic cells (36).

Conversely, the role of the LMR in patients with AFP-positive and AFP-negative HCC has not been previously explored. In the present study, the association between the LMR and AFP was investigated. In addition, peripheral blood $CD4^+$ and $CD8^+$ T lymphocyte numbers were analyzed in combination with PD-1, IL-10 and TGF-β levels. Concomitantly, the association between the IL-10 and GR expression was explored.

Materials and methods

Ethics and patients. The present study was reviewed and approved by the institutional review board of the Chongqing Medical University at The First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Written informed consent was obtained from all patients. The medical records of 266 in-patients with HBV-infections, who were negative for non-HBV hepatitis viruses and did not suffer from autoimmune hepatitis, were retrospectively reviewed. All patients were 35-55 years old and included 47% women and 53% men. The patients who were selected were treated between January 2010 and December 2016 at The First Affiliated Hospital of Chongqing Medical University. HBsAg, HBV e antigen (HBeAg), HBV-DNA and alanine aminotransferase (ALT) were the markers used to classify the patients into the various groups, along with the findings from histological examinations of their liver tissues. The patients were divided into 4 groups according to the guidelines for the prevention and treatment of chronic HBV in China (2015 edition) (37) and the diagnostic criteria for HCC, as evaluated by histologic examination of the liver. The groups consisted of 103 HBV carriers with normal liver function (ALT normal and HBsAg positive), 68 patients with chronic HBV infection (ALT normal, HBsAg positive and HBV-DNA positive), 51 patients with AFP-positive HCC (abnormal ALT levels, HBV-DNA positive, presence of a mass by histological analysis, AFP >20 ng/ml) and 44 patients with AFP-negative HCC (abnormal ALT levels, HBV-DNA positive, presence of a mass by histological analysis, AFP ≤20 ng/ml). This information is shown in Table I.

Case data analysis. All case data for the markers LMR, AFP, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), FIB, D-dimer, fibrin degradation products (FDP), serum ALT and aspartate aminotransferase (AST) were obtained from the medical laboratory of The First Affiliated Hospital of Chongqing Medical University. These data were statistically analyzed using SPSS software (version 17.0). The parameter LMR for the 4 groups were compared using one-way ANOVA with Scheffe's multiple comparison test ($P<0.05$). Spearman correlation analysis was performed. The parameters PT, APTT, TT, FIB, D-dimer, FDP, and ALT/AST for AFP-positive and AFP-negative HCC were compared using a student's t-test ($P<0.05$). The optimal cut-off value of the association of LMR with AFP was assessed using receiver operating characteristic (ROC) curves. The threshold for significance was set at 5% for Spearman correlation analysis.

Cell preparation. A total of 30 patients (age, 35-45 years; 13 women and 17 men) with HCC were diagnosed on the basis of HBV infection and selected from The First Affiliated Hospital of Chongqing Medical University between October 2017 and June 2018. Among them, 15 patients who were AFP-positive and had an LMR <2.01 were selected as the AFP-positive liver cancer group, and 15 patients who were AFP-negative and had an LMR >2.01 were selected as the AFP-negative liver cancer group. A total of 15 patients with an HBV infection without liver cancer were selected as the control group. All patients in the liver cancer group met the diagnostic criteria for primary liver cancer with HBV infection (38), and were excluded from other types of tumors and immune diseases, such as diabetes, arthritis and HIV as well as blood diseases, such as leukemia, lymphoma and other fungal and bacterial infections. All patients were treated with agents that did not affect the results of the study and any interventions affecting peripheral blood T lymphocyte subsets, such as surgery, minimally invasive operations, blood transfusion, radiotherapy or chemotherapy

Table I. Patient information among the four groups.

Group	No. patients	ALT	HBsAg	HBV-DNA	Hepatic histologic examination	AFP, ng/ml
Normal liver functional HBV carriers	103	Normal	+	-	-	NA
Chronic HBV infection	68	Normal	+	+	-	NA
AFP-positive HCC	51	Abnormal	+	+	+	≤20
AFP-negative HCC	44	Abnormal	+	+	+	>20

NA, not applicable; HBV, hepatitis B virus; AFP, α-fetoprotein; HCC, hepatocellular carcinoma; ALT; alanine aminotransferase; HBsAg, hepatitis B surface antigen.

Table II. Patient characteristics.

Factor	HBV+ NHCC (n=15)	AFP+ HCC (n=15)	AFP- HCC (n=15)
Age, years	49.4±7.68	55.3±15.59	55.1±12.46
ALT/AST	1.16±0.24	10.99±0.97	0.83±0.37
LMR	5.28±1.5	1.55±0.37	4.55±2.42
PLT	146±63	205±107	128±77
FDP	3.64±3.31	11.62±13.14	3.54±4.67
D-Dimer	1.58±0.86	6.10±5.63	0.72±0.81

Data are presented as the mean ± SD. AST/ALT, aspartate aminotransferase/aspartate aminotransferase ratio; LMR, lymphatic mononuclear ratio; PLT, platelet; FDP, fibrinogen degradation products; HBV, hepatitis B virus; AFP, α-fetoprotein; HCC, hepatocellular carcinoma; NHCC, non-hepatocellular carcinoma.

were conducted two months prior to enrollment. The clinical characteristics of the patients are summarized in Table II.

Flow cytometry for CD4, CD8 and PD-1 expression analysis. A total of 100 μl whole blood was collected from the patients and transferred to test tubes. Subsequently, 20 μl CD3-FITC, 5 μl CD4-BV510, 20 μl PD-1-PE (CD279-PE) and 5 μl CD8-BV421 antibodies were added. An equal volume of these antibodies were added to the non-HCC (NHCC) tubes. Mouse anti-human monoclonal antibodies CD3-FITC (cat. no. 561806), CD4-BV510 (cat. no. 562970), CD8-BV421 (cat. no. 562428) and PD-1-PE (CD279-PE; cat. no. 557946) were purchased from BD Biosciences, used according to the manufacturer's instruction ($1x10^6$ cells were used). The samples were mixed with the antibodies under oscillating conditions for 3-5 sec and incubated at room temperature in the dark for 30 min.

A total of 1,000 μl red blood cell lysis buffer (Beyotime Institute of Biotechnology) was added to the tubes and the samples were mixed gently with the antibodies. Following incubation at room temperature for 10 min, the samples were centrifuged for 4 min at 500 x g at room temperature and the supernatant was discarded. A total of 1,000 μl PBS were added and the samples were centrifuged for 4 min at 500 x g at room temperature, after which the milky white precipitate was collected. Following a final resuspension in 400 μl PBS, the samples were examined using a FACSCalibur™ flow cytometer (BD Biosciences). The percentage of the samples expressing a particular cell surface marker was analyzed on a FACSCalibur using the Cell Quest Pro™ software (v 5.2; BD Sciences).

ELISA analysis of the expression of IL-10 and TGF-β1. Human IL-10 ELISA kit (cat. no. BH-DB047) and Human TGF-β1 ELISA kit (cat. no. BH-ELISA 1805) were purchased from Shanghai Bohu Biotechnology Co. (https://bhbta.biomart.cn/) Serum samples were stored at -20˚C, thawed and then centrifuged (400 x g; 10 min; room temperature). Serum samples for TGF-β1 detection were acidified and neutralized according to the manufacturer's instructions to activate TGF-β1. A total of 100 μl sample or a standard of a known concentration was added to each well and the plate was incubated for 2 h at 37˚C. Subsequently, 100 μl Biotin-antibody (1X) was added to each well and incubated for 1 h at 37˚C. The samples were then aspirated and washed three times. A total of 100 μl horseradish peroxidase-avidin (1X) was added to each well and the samples were incubated for 1 h at 37˚C. The samples were aspirated and washed 5 times, then incubated with an additional 90 μl 3,3',5,5'-tetramethylbenzidine substrate in the dark for 15-30 min at 37˚C. Finally, 50 μl stop solution was added to each well and the absorbance was monitored at 450 nm within 5 min. A standard curve was produced according to the absorbance readings of the known concentration standards, which was then used to determine the concentration of the samples.

Preparation of human peripheral blood mononuclear cells (PBMCs). Human PBMCs were isolated from blood samples

Table III. Statistical analysis of the LMR among the four groups.

Comparison group	Comparison LMR (mean ± SD)
Normal liver functional HBV carriers vs. chronic HBV infection	4.52±2.6 vs. 4.17±1.99
Normal liver functional HBV carriers vs. AFP negative HCC	4.52±2.6 vs. 2.93±1.87[a]
Normal liver functional HBV carriers vs. AFP positive HCC	4.52±2.6 vs. 1.76±1.17[a]
Chronic HBV infection vs. AFP negative HCC	4.17±1.99 vs. 3.72±2.65[a]
Chronic HBV infection vs. AFP positive HCC	4.17±1.99 vs. 3.72±2.65[a]
AFP negative HCC vs. AFP positive HCC	2.93±1.87 vs. 1.76±1.17[a]

[a]P<0.05. HBV, hepatitis B virus; LMR, lymphatic mononuclear ratio; HCC, hepatocellular carcinoma; AFP, α-fetoprotein.

collected from AFP-positive and AFP-negative patients using the lymphocyte separation medium (TBD Science) according to the manufacturer's instructions. Subsequently, the cells were cultured in serum-free haematopoietic cell medium (Takara Bio, Inc.) at 37˚C in an incubator with 5% CO_2.

ELISA for determining the effect of dexamethasone or RU486 on IL-10 expression. Dexamethasone (Sigma-Aldrich; Merck KGaA) or RU486 (Sigma-Aldrich; Merck KGaA) at three different concentrations (3, 7 and 10 μmol/l), were added to 4-6x10^5 PBMCs (200 μl) in a 96-well plate and incubated for 48 h at 37˚C with 5% CO_2. The dexamethasone and RU486 concentrations were selected according to Chasserot-Golaz *et al* (39). Subsequently, the supernatant was collected and the expression levels of IL-10 were assessed using an ELISA kit (Human IL-10 ELISA kit; cat. no. CSB-E04593h; Cusabio Biotech Co., Ltd.) and MB-530 microplate reader to determine the cut-off value. Each group analysis was replicated 3 times.

IL-10-592 promoter binding site gene assay. The binding site of the IL-10-592 promoter to the GR required IL-10-592 wild-type and mutant promoters that were inserted into the pMCS-green-Renilla-luciferase plasmid. The GR gene was inserted into the pGL3-basic plasmid which was transferred into 293T cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) over the course of 48 h, according to the manufacturer's instructions.

Total RNA was extracted using Total RNA Isolation reagent (SuPerfecTRI™; cat. no. 3101-100; www.pufei.com) at 43.5˚C for 1 h and then 73.5˚C for 3 min. Each RNA sample was reverse transcribed according to the Promega M-MLV kit (cat. no. M1705) content. Then, 2 ul dNTPs were the mixture of 10 mM dATP, dCTP, dGTP and dTTP and 6 ul Nuclease-Free Water. The 5 μl buffer consisted of 250 mM Tris HCl, 375 mM HCl, 15 mM $MgCl_2$ and 50 mM DTT. The primers of GR used were as follows: 5'-GCTGTCGCGCTCACTGGCTGTC-3' and reverse, 5'-GCATCGTTCCGATCACTTCGCA-3'. Reverse transcription-quantitative PCR was performed on an ABI 7000 RT PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), using SYBR green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec, then dissociating at 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec. The reference gene was GAPDH. The following specific primers were used: GAPDH forward, 5'-CAATGACCCCTTCATTGACC-3' and reverse, 5'-GAT CTCGCTCCTGGAAGATG-3'; pGL3 forward, 5'-TGACTT CAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTT GCTGTAGCCAAA-3'; and pGL3-GR forward, 5'-GTCTTC ACCCTCACTGGCTGTC-3' and reverse, 5'-GGTCATTTC CCATCACTTTTGT-3'. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method of quantification (40).

The detection of luciferase activity was performed with the Dual-Glo Luciferase Assay System (Promega Corporation) after 48 h transfection according to the manufacturer's instructions. The compound-induced increases in luciferase activity were estimated by dividing the value of the firefly luminescence to the value of the *Renilla* luminescence (transfection efficiency control). The data were normalized to the luciferase activity of the non-treated controls, which were set to 1 (25%). Each concentration was tested in triplicate and the assay was conducted three times.

Statistical tests for flow cytometry, ELISA and the value of the luminescence assay. These data were statistically analyzed using the SPSS software (version 17.0; SPSS, Inc.). All experimental results are presented as the mean ± SD. They were compared using one-way ANOVAs with Scheffe's multiple comparison tests. P<0.05 was considered statistically significant.

Results

Comparison of the LMR for the four groups. The LMR was decreased in the treated groups compared with the levels noted in the normal liver function HBV carrier group (Table III). However, the results of the chronic HBV group did not differ significantly from those of the normal liver function HBV carrier group. A marked decline was noted in the LMR of the AFP-positive and AFP-negative HCC groups. These results indicated that the LMR was higher in the non-HCC groups than that of the HCC groups. Therefore, the normal liver function of the HBV carrier group (non-HCC group) was compared with the chronic HBV group. Both the AFP-positive and AFP-negative HCC groups exhibited significant differences with regards to the LMR compared with the non-HCC group (Table IV).

Statistical analysis of liver function and coagulation data for AFP-negative and AFP-positive HCC groups. The liver

Table IV. Statistical analysis of the LMR among three groups.

Comparison group	Comparison LMR (mean ± SD)
Non-HCC vs. AFP positive	4.37±2.36 vs. 1.76±1.17[a]
Non-HCC vs. AFP negative	4.37±2.36 vs. 2.93±1.87[a]
AFP positive vs. negative	1.76±1.17 vs. 2.93±1.87[a]

[a]P<0.05. LMR, lymphatic mononuclear ratio; HCC, hepatocellular carcinoma; AFP, α-fetoprotein.

Table V. Statistical analysis of data among two groups.

Factor	AFP positive HCC (n=44)	AFP negative HCC (n=51)
APTT	37.25±6.59	37.23±8.76
PT	14.83±1.55	14.38±1.80
FIB	3.40±1.35	3.70±1.43
D-Dimer	3.89±6.83	1.51±1.38[a]
FDP	9.32±11.67	4.79±4.15[a]
AST/ALT	1.91±1.83	1.30±0.79[a]

Data are presented as the mean ± SD. [a]P<0.05 vs. AFP-positive HCC group. HCC, hepatocellular carcinoma; AFP, α-fetoprotein; APTT activated partial fibrinogen; PT, prothrombin time; FDP, fibrinogen degradation products; AST/ALT, aspartate aminotransferase/aspartate aminotransferase ratio; HCC, hepatocellular carcinoma; FIB, Fibrinogen.

Table VI. Spearman analysis of the LMR between D-Dimer, FDP and AST/ALT.

Value	D-Dimer	FDP	AST/ALT
P-value	<0.001	<0.001	0.059
R-value	-0.42	-0.432	-0.107

LMR, lymphatic mononuclear ratio; FDP, fibrinogen degradation products; AST/ALT, aspartate aminotransferase/aspartate aminotransferase ratio.

function and coagulation were assessed in the AFP-negative and -positive HCC groups. Notably, the following parameters, AST/ALT, PT, APTT, FIB, D-dimer and FDP were assessed. The levels of PT, APTT or FIB between the two groups were not significantly altered, whereas significant between-group differences were noted with regards to the AST/ALT, D-dimer and FDP (Table V). Spearman correlation analysis (Table VI) indicated that D-dimer and FDP were associated with the LMR in the AFP-negative group. This association was not noted for the AST and ALT markers.

Optimal cut-off values of the LMR for AFP-negative and -positive groups. Based on the aforementioned results, the associations of the LMR with the AFP-negative and AFP-positive patient groups were examined. The optimal

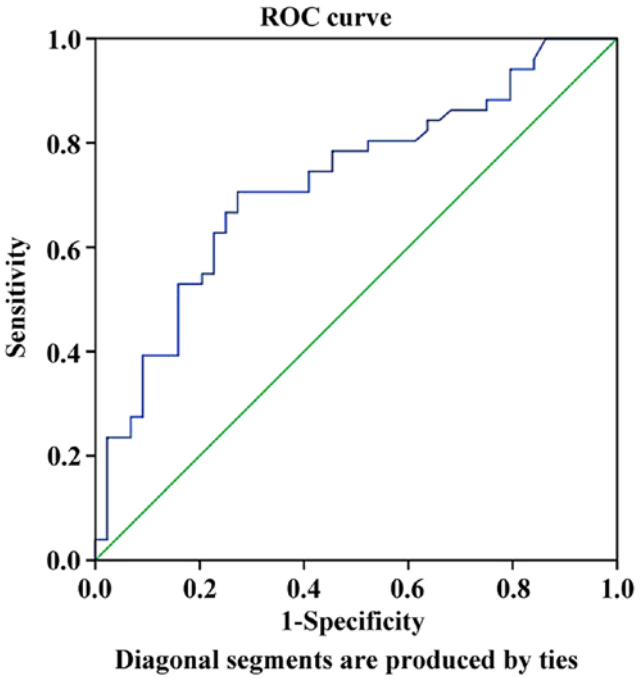

Figure 1. ROC curve analysis of the association between the lymphocyte to monocyte ratio and AFP status for AFP-positive and -negative hepatocellular carcinoma groups. AFP, α-fetoprotein; ROC, receiver operating characteristic.

cut-off value of LMR for AFP was 2.01 with an area under the curve of 0.724, a sensitivity of 68.6% and a specificity of 75.0% (Fig. 1). The mean LMR was associated with the AFP status in the AFP-negative and -positive groups. Therefore, Spearman correlation analysis was used to assess the LMR values in these two groups and the results indicated significant correlations (R=0.387, P<0.001).

Statistical analysis of $CD4^+$ and $CD8^+$ T lymphocytes among the three groups. Based on the cutoff value, the proportion of $CD4^+$ T and $CD8^+$ T lymphocytes in the peripheral blood of patients in the HBV+ non-liver cancer, AFP-positive and AFP-negative groups was assessed by flow cytometry. The percentages of $CD4^+$ T lymphocytes for the aforementioned groups were 55±10, 65±8 and 61±13, respectively (Fig. 2A and D). A significant difference was noted among the three groups (P<0.05) and the highest increase was evident in the AFP-positive group indicating that $CD4^+$ T lymphocyte number correlated with AFP levels. The percentages of $CD8^+$ T lymphocytes in the three groups were 30±9, 27±8 and 27±11, respectively (Fig. 2A and E). The number of $CD8^+$ T lymphocytes was markedly reduced in the liver cancer group, but no significant differences were noted in the AFP-positive and negative groups (P>0.05), indicating that the number of $CD8^+$ T lymphocytes did not exhibit a correlation with the AFP concentration.

Statistical analysis of CD4-PD-1 and CD8-PD-1 in each group. The expression levels of PD-1 in $CD4^+$ T and $CD8^+$ T lymphocytes from the peripheral blood of patients in the non-liver cancer, AFP-positive and AFP-negative groups were assessed by flow cytometry. The expression levels of CD4-PD-1 were 18±8, 20±10 and 22±9, respectively (Fig. 2B and F). The

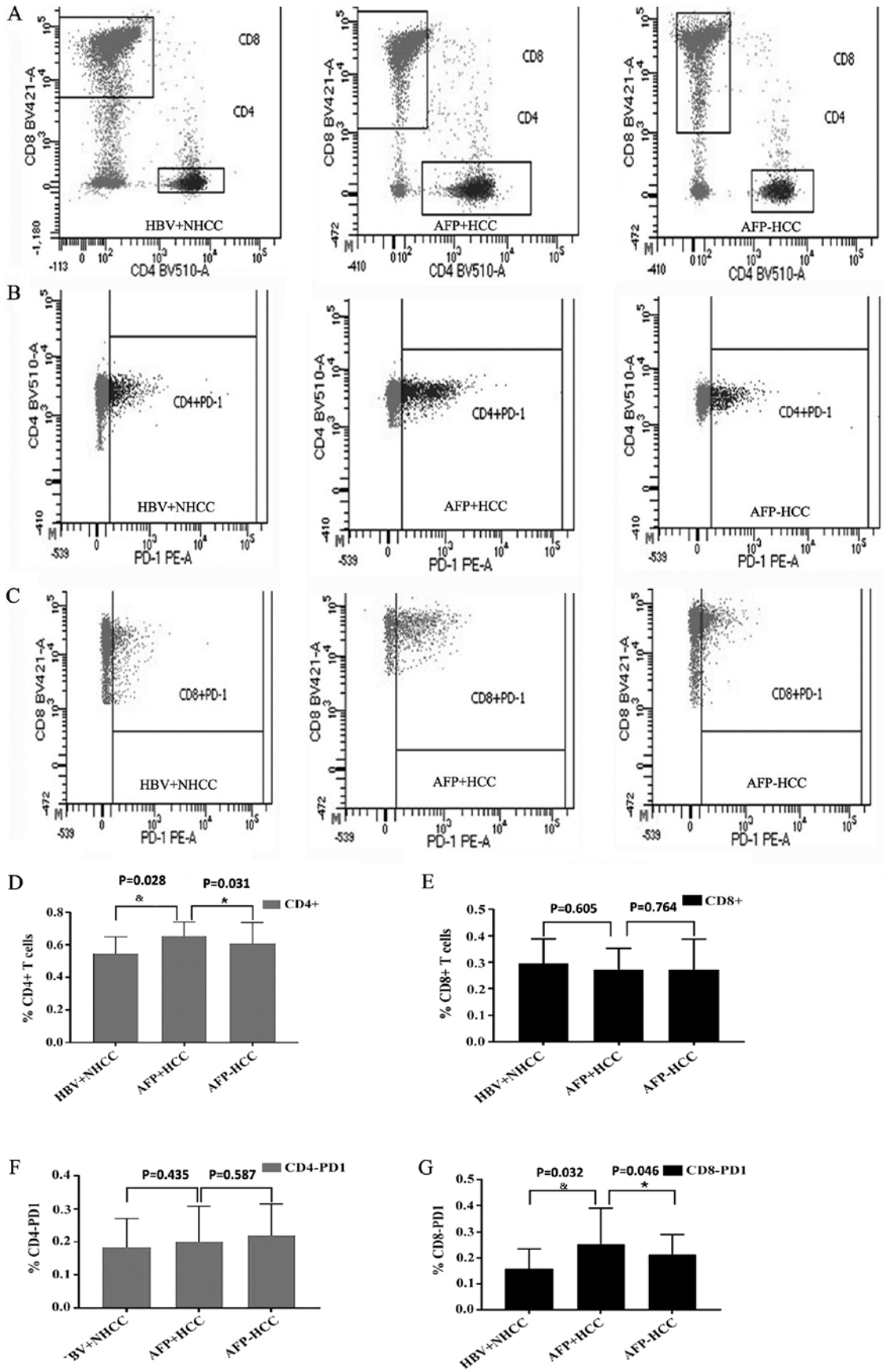

Figure 2. Flow cytometry analysis of the percentage of $CD4^+$, $CD8^+$, CD4-PD-1 and CD8-PD-1 lymphocytes in the AFP-positive and -negative groups. AFP, α-fetoprotein; (A) Percentage of $CD4^+$ and $CD8^+$ lymphocytes in these three groups. (B) Percentage of CD4-PD-1 lymphocytes in these three groups. (C) Percentage of CD8-PD-1 lymphocytes in these three groups. (D and E) Statistical analysis of the expression of $CD4^+$ and $CD8^+$ lymphocytes in these three groups. (F) Statistical analysis of the expression of CD4-PD-1 lymphocytes in the three groups. (G) Statistical analysis of the expression of CD8-PD-1 lymphocytes in the three groups. $^*P<0.05$ vs. AFP-negative groups. $^\&P<0.05$ vs. HBV+NHCC. HCC, hepatocellular carcinoma. PD-1, programmed cell death protein 1; NHCC, non-hepatocellular carcinoma; HBV, hepatitis B virus.

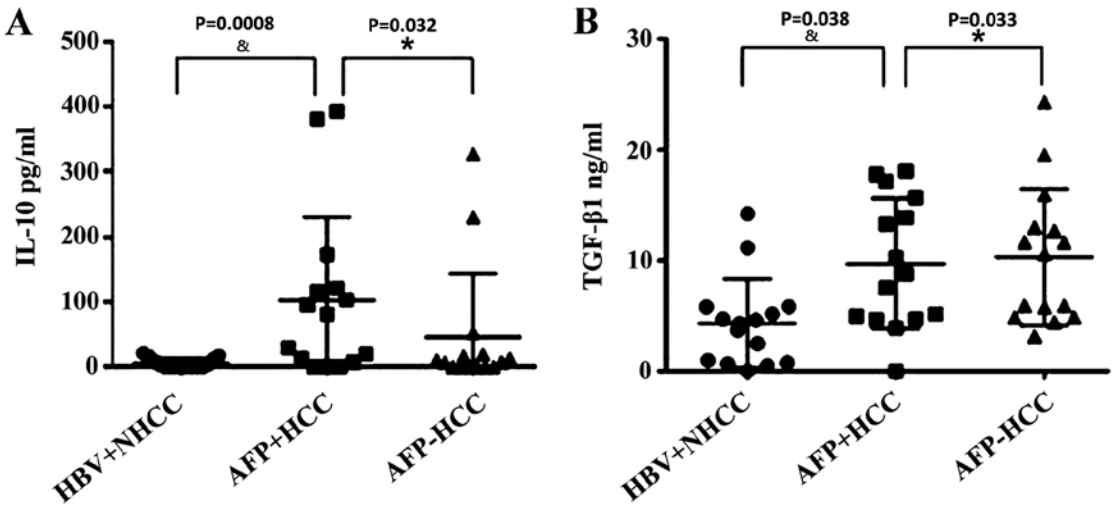

Figure 3. ELISA analysis of the levels of (A) IL-10 and (B) TGF-β1 in the AFP-positive and -negative groups. IL, interleukin; TGF, transforming growth factor; AFP, α-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NHCC non-hepatocellular carcinoma. &P<0.05 vs. HBV+NHCC. *P<0.05 vs. AFP-positive groups.

expression levels of CD4-PD-1 appeared to be increased in the liver cancer group, but no significant differences were noted in the three groups (P>0.05), suggesting that CD4-PD-1 was independent of AFP expression. The expression levels of CD8-PD-1 in the three groups were 16±7, 25±13 and 21±7, respectively (Fig. 2C and G). The expression levels of CD8-PD-1 in the liver cancer group were significantly increased compared with the HBV+NHCC group (P<0.05), whereas the expression levels of the AFP-positive group were higher than those of the AFP-negative group, indicating that CD8-PD-1 was associated with AFP levels.

Statistical analysis of serum IL-10 and TGF-β in each group. The serum levels of IL-10 and groups were measured using ELISAs. The IL-10 concentration levels were 5.58±7.30, 102.11±123.56 and 45.52±94.23, respectively (Fig. 3A). The TGF-β1 levels in the aforementioned groups were 4.38±3.89, 9.74±5.69 and 10.33±5.98, respectively (Fig. 3B). The one-way ANOVA analysis indicated that IL-10 and TGF-β1 levels exhibited significant differences between the three groups (P<0.05). IL-10 and TGF-β1 exhibited the lowest levels in the HBV+ non-liver cancer group, and the highest of IL-10 in the AFP-positive group, indicating that IL-10 expression levels were associated with AFP levels. However, TGF-β1 levels in AFP-positive group were lower compared with the AFP-negative group (P<0.05).

Dexamethasone promotes the expression of IL-10 in AFP-positive patients. ELISAs indicated that following incubation of the samples with 3, 7 and 10 μmol/l dexamethasone for 48 h, the optical density values of IL-10 were higher in the AFP-positive group than in the AFP-negative group (Fig. S1). These results indicated that the expression levels of IL-10 were higher in the AFP-positive group than in the AFP-negative group.

RU486 inhibits the expression of IL-10 in HBV-infected patients. The samples of the AFP-negative and AFP-positive group were incubated with RU486 for 24 h, an inhibitor of dexamethasone (41). The expression levels of IL-10 were gradually decreased in aforementioned two groups with increasing concentrations of RU486, but the expression of IL-10 was not markedly decreased at 7 μmol (Fig. S2).

IL-10 promoter is the binding site for GR regardless of the presence of the polymorphic sites. To determine whether the IL-10-592 promoter is the binding site for GR, luciferase reporter activity was assessed following the overexpression of GR (Fig. 4A). The IL-10 promoter produced a strong induction of relative luciferase activity that peaked at 48 h in the 293T-cotransfected luciferase cells that overexpressed GR (Fig. 4B). The relative luciferase activity caused by the IL-10 promoter was higher than that of the control cells (P<0.05). However, following cotransfection of the cells with the IL-10-592 polymorphic promoter and the GR gene, a similar induction in the relative luciferase activity was noted compared with that noted for the wild type variant (P=0.15). These results indicated that the IL-10 promoter was the binding site for GR, regardless of the presence of the IL-10-592 polymorphism.

Discussion

In the present study, a cohort of 266 in-patients with HBV infections were examined, who were negative for non-HBV hepatitis viruses and did not suffer from autoimmune hepatitis. The data indicated that a high LMR correlated with AFP negativity and lower levels of D-dimer and FDP in the HCC group.

Previous studies have demonstrated that AFP contributes to the development, growth, invasion and metastasis of HCC (42-45). For example, Bihari *et al* (42) reported that increased levels of AFP following HCC therapy were indicative of incomplete response or recurrence, which indicated that the release of AFP from HCC cells to the circulation was a major source of HCC metastasis. In addition, Yang *et al* (44) reported that HCC patients with no contraindications for surgery and serum AFP levels ≤20 ng/ml could mainly benefit from hepatectomy as a primary treatment as opposed to HCC patients with serum AFP levels >20 ng/ml who required comprehensive therapy other

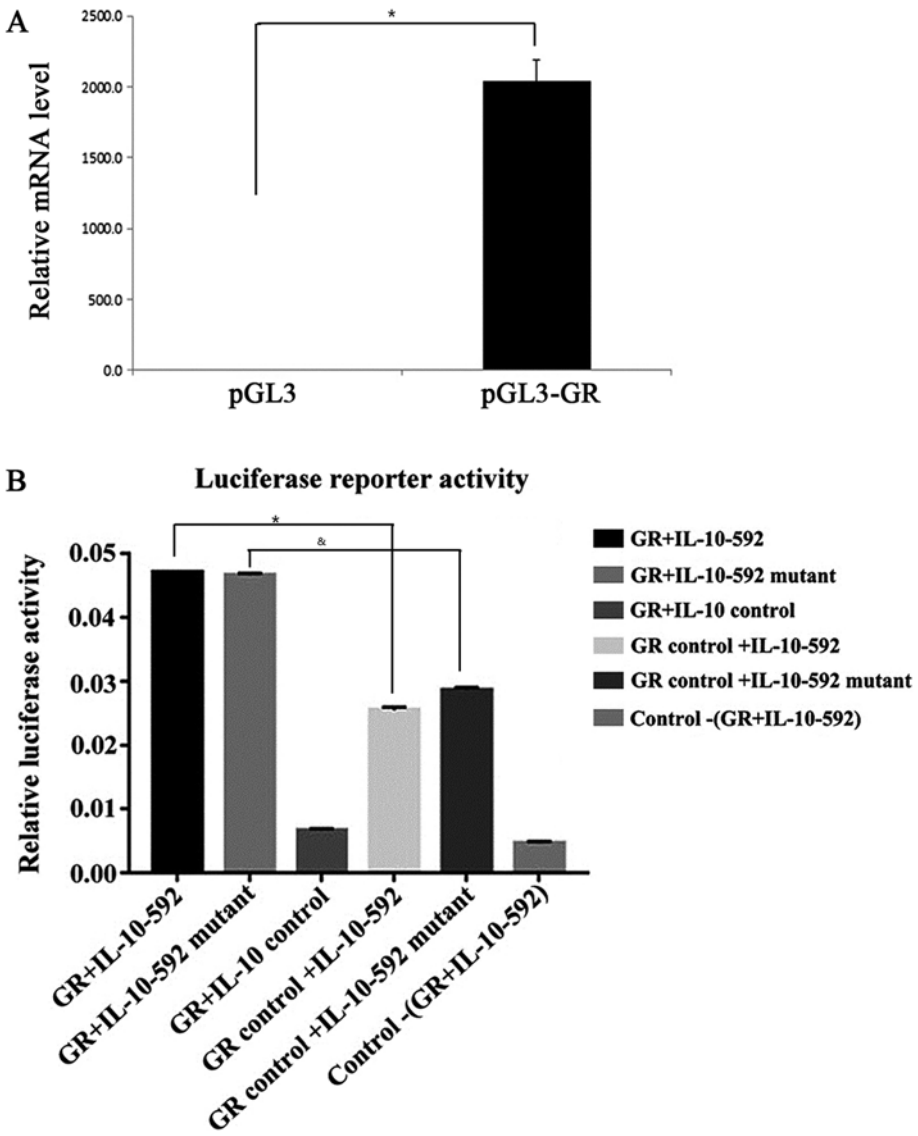

Figure 4. Relative mRNA expression levels of GR is overexpressed in 293T cells and luciferase reporter cells respond to the IL-10-592 promoter binding to GR with increased luciferase activity. (A) The relative mRNA expression levels were analysed by reverse transcription-quantitative PCR. pGL3-GR was overexpressed (2035.66±151.02) relative to the negative control vector pGL3 (1.00±0.01). *P<0.05 vs. negative control vector. (B) Wild-type and mutant IL-10-592 promoters were transfected into 293T cells that overexpressed GR and were incubated for 48 h. The relative activity was measured using luciferase assays. The data are presented as the mean ± SD of three individual experiments. *P<0.05 vs. GR control + IL-10-592 control cells; &P<0.05 vs. GR control + IL-10-592 mutant. IL, interleukin; GR, glucocorticoid receptor.

than surgical resection and close follow-up. Moreover, several studies have suggested that the D-dimer can predict survival of several types of malignancies (42,46,47). To the best of the authors' knowledge, the present study is the largest scale study to date that has explored the association between the LMR and the clinicopathological parameters for AFP. The results suggested that patients with AFP-negative HCC exhibited higher LMR and lower levels of D-dimer and FDP. Additional studies have reported that AFP-negative HCC patients with high LMR exhibit optimal disease-free survival and lower metastasis rate than AFP-positive patients with low LMR (48,49).

Multiple mechanisms may be associated with HCC invasion and metastasis, including recombinant human AFP (rhAFP)-induced expression of matrix metallopeptidase 9; C-C chemokine receptor type 5 and AFP mRNA levels; and absent in melanoma 2 protein levels (43,50,51). The measurement of the levels of these markers in various neoplasms, limits their wider application for tracking the disease status during the clinical course of treatment (41,49). In contrast to these markers, LMR is a readily measured biomarker that can be used to predict the HCC status (49,50,52). The present study indicated that the LMR correlated significantly with the D-dimer and FDP. Patients with HCC who were AFP-negative and had low levels of D-dimer and FDP, exhibited a high LMR compared with patients with HCC who were AFP-positive. Liu *et al* (53) further reported that FIB and D-dimer levels were elevated following carcinogenesis. However, in the present study, the FIB levels of patients with HCC who were AFP-positive or -negative, were not significantly different. In addition, the cut-off value used in the present study for the LMR, which was selected by ROC curve analysis, was not the same as that reported by Hong *et al* (54). Nonetheless, the present study contains specific limitations. Firstly, selection bias may exist due to the retrospective nature of the study.

Secondly, diabetes mellitus, ischemia, renal disease and systemic inflammation, which could potentially affect LMR, were not evaluated. Thirdly, the correlations of FIB with HCC and the cut-off value of LMR were different than those reported in other studies due to different endpoints assessed and different populations enrolled (47,48). Therefore, the conclusions of the present study may need to be validated by a prospective investigation.

Various *in vitro* studies have shown that AFP can play an immunosuppressive role in the tumor microenvironment. Previously, AFP has been shown to induce apoptosis in immune cells, such as lymphocytes and dendritic cells, by upregulating the expression of the pro-apoptotic genes, including Bax, BH3 interacting-domain death agonist, Bad and apoptotic protease activating factor 1. Concomitantly, AFP has been shown to enhance the activity of pro-apoptotic factors [Fas, Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand] in immune cells. These pro-apoptotic markers are usually induced to increase forkhead box 3 expression in $CD4^+$ T lymphocytes and promote the transformation of $CD4^+$ $CD25^-$ T lymphocytes into $CD4^+$ $CD25^+$ Treg cells (55).

Previous clinical studies have shown that serum AFP levels are closely associated with the development, recurrence rate and survival rate of primary liver cancer (44,55). The malignant degree and long-term recurrence rate of AFP negative liver cancer are low, which results in high survival rate and optimal prognosis (44). In the present study, it was concluded that $CD4^+$ T lymphocytes were significantly different from each other among the three groups. The high level of $CD4^+$ T lymphocytes was associated with poor prognosis of liver cancer in the AFP positive group. The majority of previous studies have reported that $CD4^+$ $CD25^+$ T lymphocytes are involved in immunosuppression (44,55,56). $CD4^+$ $CD25^+$ T lymphocytes are a subgroup of T lymphocytes with negative immunoregulatory functions. They exert negative effects on tumor immunity by inhibiting T lymphocytes and may also act as a prognostic factor for patients with liver cancer (44). It has been shown that higher Treg cell numbers are associated with various clinical pathological parameters, such as increased AFP levels, presence of multiple tumors, poor differentiation, advanced tumor stage and poor prognosis of vascular infiltration (56). In the present study, the cell surface marker molecule CD25 was not detected, whereas the $CD25^+$ Treg usually accounted for 5-10% of $CD4^+$ T lymphocytes (57). Therefore, the number of $CD4^+$ T lymphocytes detected in the present study was of particular importance.

It also has been reported that PD-1/PD-L can upregulate the expression of Fas and FasL in Treg cells, inhibit the proliferation of $CD8^+$ T lymphocytes and promote the apoptosis of $CD8^+$ T cells (56-58). In the present study, flow cytometry and ELISA assays indicated that the expression levels of CD8-PD-1 and IL-10 were different in the three groups ($P<0.05$). The expression levels of the aforementioned markers were significantly increased in the liver cancer group, whereas the expression levels in the AFP-positive group were higher than those of the AFP-negative group. PD-1 was expressed in a variety of activated immune cells and played a key role in immune regulation. The combination of PD-1 and PD-L inhibited the activation and proliferation of T lymphocytes, induced apoptosis of T cells and caused tumor cells to evade immune attack, which showed that the PD-1/PD-L ratio played a negative role in the anti-tumor immune response. In addition, PD-1 is significantly unregulated in tumor-infiltrated lymphocytes, notably in effector $CD8^+$ T cells (58,59). Due to the inhibition of antiviral and anticancer immune function, the increase in the expression levels of PD-1/PD-L may lead to the poorer prognosis of HCC patients and may promote tumor invasion and postoperative recurrence in liver cancer patients (59). Previously, the expression levels of PD-1 in Treg cells were investigated in combination with the expression levels of Fas and FasL. These biomarkers were shown to promote the secretion of IL-10 via the PD-1/PD-L pathway in order to exert negative immune regulation. IL-10 inhibits the stimulation of immune cells. In addition, IL-10 can assist the differentiation of infantile T cells into Treg cells, while Treg cells can secrete IL-10. Positive feedback regulation between Treg cells and IL-10 may promote immune tolerance (60-62). Therefore, AFP can cause upregulation of Fas and FasL expression in Treg cells and promote the secretion of IL-10 by PD-1/PD-L signaling, leading to the inhibition of the proliferation of $CD8^+$ T lymphocytes. TGF-β1 is an important cytokine, which induces the production of Treg cells. It has been shown that Treg cell infiltration is higher in cells with high expression of TGF-β1 than that in low TGF-β1 expression cells, suggesting that the expression of TGF-β1 in tumor tissues may increase the local Treg cell infiltration in the tumor, while the infiltration of Treg cells in the liver tissue is often associated with poor prognosis. In addition to the direct effect on tumor progression and infiltration, TGF-β exhibits a potent immunosuppressive effect. TGF-β1 can inhibit the proliferation of effector T cells, control the differentiation of various $CD4^+$ subtypes and reduce the generation of effector T cells. In a previous study, following the stimulation of TGF-β1, $CD4^+$ immature T cells were differentiated into Treg cells and the number of Treg cells in liver cancer tissues was positively associated with the expression of TGF-β1 (20,63). TGF-β1 promotes tumor immune escape by maintaining a normal Treg cell number and enhancing the induction of Treg cell differentiation (20). In the present study, ELISA indicated that TGF-β1 levels in the liver cancer group were significantly higher than those in the non-liver cancer group, while the TGF-β1 levels in the AFP-negative group were slightly higher than those in the AFP-positive group. Therefore, the immunosuppressive effect of TGF-β1 and its association with AFP expression requires further investigation. Taken collectively, the data suggested that AFP may upregulate the expression of Fas and FasL on Treg cells and promote the secretion of IL-10 via the PD-1/PD-L signaling pathway, thereby inhibiting the proliferation of $CD8^+$ T lymphocytes. Therefore, the decrease in the number of $CD8^+$ T lymphocytes should be consistent with the increase in the AFP levels. However, the results of the present study indicated that the number of $CD8^+$ T lymphocytes was significantly different in the liver cancer and non-liver cancer groups, whereas no significant difference was noted in the AFP-negative and AFP positive groups. Unfortunately, it has been reported that only 20% of patients with liver cancer are sensitive to PD-1/PD-L treatment (20). Moreover, certain patients that respond to the treatment still relapse (64). This suggests that other factors play a role in the suppression of $CD8^+$ T lymphocytes. Inhibition of PD-1/PD-L

signaling may not solely account for the number of $CD8^+$ T lymphocytes. Therefore, it was concluded that the number of $CD8^+$ T lymphocytes exhibited no significant differences between the AFP-positive and AFP-negative groups.

It has been proposed that upregulation of IL-10 expression may be a potential mechanism by which GR exerts its beneficial effects (62). Therefore, the association of the -592 polymorphism, within the promoter of the IL-10 gene, with the GR gene was examined. Increased luciferase activity was noted due to the presence of the IL-10-592 promoter. The results indicated that there were no significant differences in luciferase activity between the cells with the mutant IL-10-592 promoter and the cells with the wild-type IL-10-592 promoter. Moreover, it has been previously shown that GR recognizes a specific nucleotide sequence in the HBV genome and that ERK-mediated phosphorylation of the GR modulates the expression of IL-10 following infection of dendritic cells with HBV (38,65). In addition, the binding site of the GR with IL-10 is a factor that has to be considered. The results suggested that the expression levels of IL-10 were increased by the association of the GR with the IL-10 promoter, regardless of the presence of the polymorphism. LMR could be affected by other diseases, including diabetes mellitus (63). Therefore, the association of the GR with the IL-10 promoter in HCC requires further investigation to exclude the influence of diabetes mellitus and other factors.

In conclusion, AFP-negative HCC patients exhibited higher LMR and lower levels of D-dimer and FPD compared with those noted in AFP-positive HCC patients. The number of $CD4^+$ and CD8-PD-1 T lymphocytes and the expression levels of IL-10 in the serum of HCC patients were consistent with the expression levels of AFP. The latter may be involved with the secretion of IL-10 via PD-1/PD-L signaling, thereby inhibiting the proliferation of $CD8^+$ T lymphocytes. Furthermore, IL-10 expression was associated with GR regardless of the presence of the polymorphism. In conclusion, the number of peripheral blood T lymphocytes, the expression of PD-1 and the content of AFP could be used as prognostic indicators for patients with liver cancer, which is essential for understanding the immunopathological mechanisms of liver cancer and for the development of effective therapeutic strategies.

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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

HW designed the study. HW, SL and XL wrote the manuscript. HW and YX performed the reverse transcription-quantitative PCR experiments and luciferase activity assay. LL and SL performed the case data analysis. XL and HW performed the ELISA and flow cytometry experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of the Institutional Review Board of Chongqing Medical University First Affiliated Hospital. (Clinical trial registration no.: 2014-71). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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