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ORIGINAL ARTICLE

Programmed cell death-1 in patients with primary liver cancer and its effect on prognosis

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Summary

Purpose: *To detect the expression of programmed cell death-1 (PD-1) in peripheral blood T lymphocytes of patients with primary liver cancer and its effect on their prognosis.*

Methods: *The medical records of 42 patients with primary liver cancer, 36 patients with chronic hepatitis B (CHB) and 38 healthy volunteers composed the liver cancer group, benign lesion group and control group, respectively. Fluorescence quantitative RT-PCR (qRT-PCR) was used for detecting the expression level of PD-1 mRNA in peripheral blood T lymphocytes of subjects in the three groups, and flow cytometry was used for detecting the positive expression of PD-l protein on the surface of T lymphocytes of subjects.*

Results: *Patients in the control group and the benign lesion group had lower expression level of PD-1 mRNA than those in the liver cancer group (p<0.05). Patients in the control group had lower expression level of PD-1 mRNA than those in the benign lesion group (p<0.05). Patients in the control group and the benign lesion group had lower positive expression rate of PD-1 protein on the surface of T lymphocytes than those in the liver cancer group (p<0.05).*

Conclusion: *The expression of PD-1 in the peripheral blood was higher in patients with primary liver cancer. Patients in the PD-1 low expression group had significantly better prognosis than those in the PD-1 high expression group. PD-1 may be related to the occurrence and development of primary liver cancer and is worthy of further study.*

Key words: *expression, primary liver cancer, prognosis, programmed cell death-1, T lymphocyte*

Introduction

Primary liver cancer is a common digestive tract tumor, mainly composed of hepatocellular carcinoma, intrahepatic cholangiocarcinoma, hepatocytes and cholangiocarcinoma. Hepatocellular carcinoma accounts for more than 90% of primary liver cancers [1,2]. Primary liver cancer ranks fourth as cause of death worldwide, only behind lung cancer, colorectal cancer and gastric cancer [3]. Its onset is concealed, with inconspicuous early symptoms. Clinically over 70% of liver cancer patients, who are already in advanced stage at the time of diagnosis, miss the golden chance of operation or other treatments [4,5]. More notably, the incidence and mortality of most cancers have declined in recent years with the advancement of medical technology and health concepts, but those of primary liver cancer have been rising [6]. Primary liver cancer has now become an important factor threatening human life and health.

PD-1, a member of B7-CD28 co-stimulatory receptor family, is expressed mainly on activated T lymphocytes, B lymphocytes and monocytes. Its ligand, PD-L1, is mainly expressed on the surface of cancer cells. After binding to PD-L1, PD-1 causes

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T cell inactivation or death, which inhibits T cell-induced cellular immune responses and promotes tumor cell growth [7-9]. At present, clinical studies find that PD-1 is also highly expressed in gastric cancer and upper urothelial carcinoma, and its expression may be one of the indicators that prognose survival of patients [10,11]. However, few reports are currently available about the expression of PD-1 in liver cancer. It is suspected that PD-1 expression is also related to patients' prognosis. Therefore, in this study, the expression of PD-1 in peripheral blood T lymphocytes of liver cancer patients has been focally investigated, and the relationship between its expression and prognosis has been analyzed.

Methods

General information

The medical records of 42 patients with primary liver cancer (the liver cancer group) first diagnosed in this hospital from April 2014 to June 2015 and 36 CHB patients (the benign lesion group) at the same period were collected. Another 38 healthy volunteers formed the control group. There were 37 males and 5 females in the liver cancer group, aged 25-72 years (mean 45.72±7.84); 28 males and 8 females in the benign lesion group, aged 23-75 years (mean 43.68±8.13); and 26 males and 12 females in the control group, aged 22-68 years (mean 42.74±8.75).

Inclusion criteria: (1) Patients pathologically diagnosed with liver cancer were included in the liver cancer group. (2) Patients diagnosed with hepatitis B infection by clinical and pathological characteristics examination were included in the benign lesion group (excluded were those with hepatitis A, C and D virus complicated with infection). (3) Healthy volunteers with no hepatitis virus infection and normal liver function were included in the control group.

Exclusion criteria: (1) Patients complicated with severe infection or immune system diseases in other systemic systems; (2) Patients with cardiopulmonary, brain and kidney dysfunction; (3) Patients treated with radiotherapy, chemotherapy and other related treatments 6 months prior to enrollment.

This study was approved by the Medical Ethics Committee of the affiliated Hospital of Weifang Medical University. All subjects were informed of the experimental contents. Subjects or their family members signed a complete informed consent form.

Experimental reagents and instruments

TRIzol (Chongqing Pulike Biotechnology Co., Ltd., Chongqing, China, 15596026); reverse transcription kit (Beijing Huada Protein Research and Development Center Co., Ltd., BPI01030); ABI-7900 quantitative PCR instrument (Tianjin Jinside Biotechnology Co., Ltd., China, ABI7900); TransScript Green Two -Step qRT-PCR SuperMix (Beijing Quanshijin Biotechnology Co., Ltd., AQ132); Nuclease-Free Water (Shanghai Haoyang Biotechnology Co., Ltd., 129114); EDTA (Beijing Baiao Laibo Technology Co., Ltd., QN0717-DZH); fetal bovine serum (FBS) (Weisente Biotechnology (China) Co., Ltd., 085-110); dimethyl sulfoxide (DMSO) (Shanghai Shifeng Biotechnology Co., Ltd., A5852); UV spectrophotometer (Tuomogen Biotechnology Co., Ltd., Beijing, China, MD2000); human lymphocyte separation solution (Shanghai Langdun Biotechnology Co., Ltd., LTS1077); 4% paraformaldehyde (Shanghai Zeye Biotechnology Co., Ltd., ZY131248-250); BD flow cytometry (FACSCantoTMII, Aikesen (Beijing) Technology Co., Ltd.; PD1 antibody (PE) (Xiamen Research Biotechnology Co., Ltd., Fujian, China, ORBYK124519).

Experimental primers

Experimental primers were designed by Primer Premier 5.0 (Premier, Palo Alto CA, USA). Primer design software, generated by Shanghai Boya Co., Ltd. GAPDH was used as an experimental reference gene. Primer sequences are shown in Table 1.

Table 1. Primer sequences

Primer	*Sequence (5'→3')*
GAPDH	
Upstream	GGTCATCCATGACAACTTTGGTATC
Downstream	ATATTTGGCAGGTTTTTCTAGACGG
PD-1	
Upstream	AGCTCAGGGTGACAGAGAGAA
Downstream	AACCACCAGGGTTTGGAACTG

Cell culture and lymphocyte separation

Three ml of venous blood were taken from all subjects after 8h of fasting with a vacuum blood lancet, and anti-coagulated with EDTA. Density gradient centrifugation was used for isolating peripheral blood T lymphocytes, lymphocyte separation solution for purifying cell suspension, 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) for preparing cell stock solution. The separated cells were added to the culture solution and stored in liquid nitrogen.

qRT-PCR detection of expression level of PD-1 mRNA in peripheral blood T lymphocytes

The Trizol extraction reagent was used for extracting total RNA from the sample in strict accordance with the operating instructions. UV spectrophotometer and agarose gel electrophoresis was used for detecting the purity, concentration and integrity of the extracted total RNA. The extracted total RNA was reversely transcribed into cDNA according to the instructions of the reverse transcription kit, and a part was taken for subsequent experiments. The ABI7900 PCR instrument was used for amplification. The PCR reaction system was as follows: total 10μl of 2×TransScript® Top Green qRT-PCR SuperMix + DyeI,

0.4μl of each of upstream and downstream primers, and Nuclease-Free Water added to 20μL. PCR reaction conditions were as follows: pre-denaturation at 94°C for 30s, denaturation at 94°C for 5s, annealing at 50-60°C for 15s and extension at 72°C for 10s, for a total of 40 cycles in this experiment. GAPDH was used as an internal reference, and the independent experiment was performed for 3 times. The Ct value was recorded and RQ=$2^{-\Delta Ct}$ was used for statistical analysis.

Flow cytometry detection of positive expression of PD-1 protein on the surface of T lymphocytes

The fluorescein antibody PE-PD-1 was mixed with 100μl of peripheral blood T lymphocytes at a concentration of 1×109/L, stained at 4°C for 30min, washed twice with PBS solution, and then fixed with 4% paraformaldehyde solution. Flow cytometry was used for analysis and CellQuest software for analyzing the expression of PD-1 protein. The number of T cells positively expressed by PD-1 protein was calculated.

Statistics

SPSS19.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for statistically analysed of the experimental data and GraphPad Prism7 (Beijing Huanzhong Ruichi Technology Co., Ltd., Beijing, China) for plotting the experimental data. Count data were expressed as percents, and chi-square test was used for comparison between groups. Measurement data were expressed as mean±SD, and independent sample *t*-test was used for comparison between groups. One-way ANOVA was used for comparison among multiple groups. The reactive oxygen species (ROC) curve was plotted, and Kaplan-Meier method was used for survival analysis. Log-rank test was used to compare differences between groups. $P<0.05$ denoted statistically significant difference.

Results

Comparison of general information

As shown in Table 2, based on the Child-Pugh classification [12], 17 patients had grade A Child-Pugh, 14 patients had grade B and 11 patients had grade C in the liver cancer group. Based on the pathological classification, 13 tumors were highly differentiated, 17 moderately differentiated and 12 poorly differentiated. Based on the Barcelona staging [13], 25 patients had in 0-B stage and 17 C-D stage. There were no differences in the age, body mass index and gender of patients among the liver cancer group, the benign lesion group and the con-

Table 2. Comparison of general clinical data

	Liver cancer group (n=42) *n(%)*	*Benign lesion group (n=36)* *n (%)*	*Control group (n=38)* *n (%)*	*F/t/x²*	*p*
Age (years) mean±SD	45.72±7.84	43.68±8.13	42.74±8.75	1.377	0.257
Body mass index (kg/m²) mean±SD	23.59±3.58	24.16±3.10	22.96±4.28	0.979	0.379
Gender				4.581	0.101
Male	37 (88.10)	28 (77.78)	26 (68.42)		
Female	5 (11.90)	8 (22.22)	12 (31.58)		
AFP (μg/L)				56.54	<0.001
≤400	15 (35.71)	35 (97.22)	38 (100.00)		
>400	21 (50.00)	7 (19.44)	0 (0.00)		
HBsAg				10.98	0.001
Positive	31 (73.81)	36 (100.00)	-		
Negative	11 (26.19)	0 (0.00)	-		
Child-Pugh classification					
A	17 (40.38)	-	-		
B	14 (33.33)	-	-		
C	11 (26.19)	-	-		
Pathological grade					
Highly differentiated	13 (30.95)	-	-		
Moderately differentiated	17 (40.480	-	-		
Poorly differentiated	12 (28.57)	-	-		
Barcelona stage					
0-B	25 (59.52)	-	-		
C-D	17 (40.48)	-	-		

trol group (p>0.05). The number of patients with serum alpha fetoprotein (AFP) level >400μg/L was significantly lower in the control group than that in the liver cancer group and the benign lesion group (p<0.05). The number of serum AFP level >400μg/L was significantly lower in the benign lesion group than that in the liver cancer group (p<0.05). The number of patients with positive HBsAg was significantly lower in the liver cancer group compared with the benign lesion group (p<0.05).

Comparison of expression level of PD-1 mRNA in peripheral blood T lymphocytes of patients in three groups

The results of qRT-PCR showed that the mean expression level of PD-1 mRNA of patients was 8.58±4.28 in the liver cancer group, 6.54±3.73 in the benign lesion group and 4.38±1.95 in the control group with significant difference in the expression level of PD-1 mRNA of patients among the three groups (p<0.05). Patients in the control group and the benign lesion group had significantly lower expression level of PD-1 mRNA than those in the liver cancer group (p<0.05). Patients in the control group had lower expression level of PD-1 mRNA than those in the benign lesion group (p<0.05; Figure 1).

Comparison of positive expression rate of PD-1 protein on the surface of T lymphocytes of patients in three groups

The positive expression rate of PD-1 protein on the surface of T lymphocytes of patients was detected by flow cytometry. The positive mean expression rate of PD-1 protein on the surface of T lymphocytes of patients was 21.47±8.84 in the liver cancer group, 15.36±7.23 in the benign lesion group and 3.68±0.42 in the control group, with significant difference in the positive expression rate of PD-1 protein on the surface of T lymphocytes of patients among the three groups (p<0.05). Patients in the control group and the benign lesion group had significantly lower positive expression rate of PD-1 protein on the surface of T lymphocytes than those in the liver cancer group (p<0.05). Patients in the control group had significantly lower positive expression rate of PD-1 protein on the surface of T lymphocytes than those in the benign lesion group (p<0.05; Figure 2).

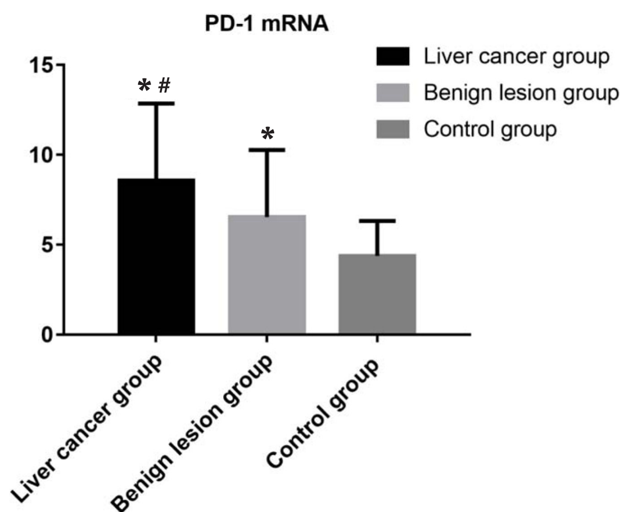

Figure 1. Comparison of expression level of PD-1 mRNA in peripheral blood T lymphocytes of patients among liver cancer group, benign lesion group and control group. The results of qRT-PCR showed that there was a significant difference in the expression level of PD-1 mRNA in peripheral blood T lymphocytes of patients among the liver cancer group, the benign lesion group and the control group (p<0.05). Patients in the control group and the benign lesion group had significantly lower expression level of PD-1 mRNA than those in the liver cancer group (p<0.05). Patients in the control group had significantly lower expression level of PD-1 mRNA than those in the benign lesion group (p<0.05). *indicates that compared to the control group, p<0.05. # indicates that compared to the benign lesion group, p<0.05.

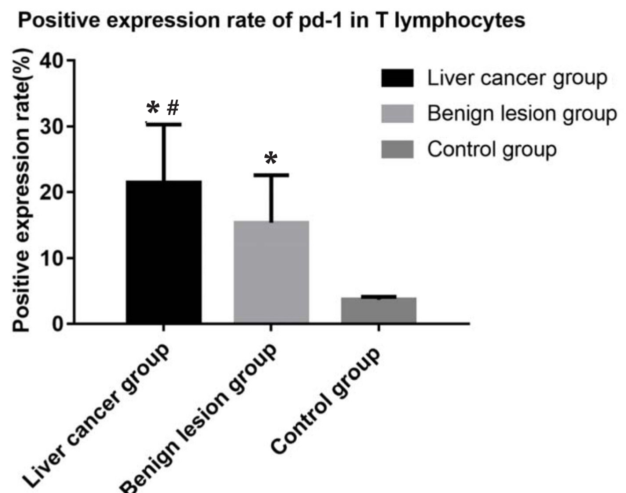

Figure 2. Comparison of positive expression rate of PD-1 protein on the surface of peripheral blood T lymphocytes of patients among liver cancer group, benign lesion group and control group. The positive expression rate of PD-1 protein on the surface of T lymphocytes of patients was detected by flow cytometry. There was a significant difference in the positive expression rate of PD-1 protein on the surface of T lymphocytes of patients among the liver cancer group, the benign lesion group and the control group (p<0.05). Patients in the control group and the benign lesion group had significantly lower positive expression rate of PD-1 protein on the surface of T lymphocytes than those in the liver cancer group (p<0.05). Patients in the control group had significantly lower positive expression rate of PD-1 protein on the surface of T lymphocytes than those in the benign lesion group (p<0.05). *indicates that compared to the control group, p<0.05. #indicates that compared to the benign lesion group, p<0.05.

ROC curve

The ROC curve was plotted according to the expression level of PD-1 mRNA in peripheral blood T lymphocytes of patients in the liver cancer group and the control group. The AUC was 0.883 (range 0.808-0.958), the diagnostic sensitivity was 97.37%, the specificity was 69.05%, the Youden index was 0.664, and the corresponding diagnostic threshold was 7.636 (p<0.001; Figure 3).

Survival analysis

According to ROC curve, the diagnostic threshold of PD-1 mRNA was 7.657. On this basis, the liver cancer group was divided into the high expression group (29 patients) and the low expression group (13 patients). Patients in the two groups were followed up for a median of 18 months. The median survival time of patients was 16 months in the PD-1 mRNA high expression group, and 27 months in the PD-1 mRNA low expression group, with the overall survival rates of 10.34 and 38.46%, respectively. The survival of patients in the low expression group was significantly better than that in the high expression group (p<0.05; Figure 4).

Discussion

Primary liver cancer is a relatively common digestive tract malignant tumor. Its incidence and mortality are relatively high worldwide, which still have an upward trend in many industrialized countries [14]. Studies found that most primary liver cancers are mainly correlated with chronic hepatitis B virus (HBV) or chronic hepatitis C virus (HCV) infection, and alcoholic cirrhosis, smoking, obesity and alcoholism are also common risk factors for primary liver cancer [15]. In addition, patients with primary liver cancer have generally poor prognosis. Studies reported that the 5-year relative survival rate of liver cancer patients in Europe is only 12%, and primary liver cancer has seriously affected human life and health [16]. Primary liver cancer is difficult to treat. On the one hand, the existing treatments are costly with poor compliance, so few liver cancer patients in clinical practice can be treated by completely curing hepatitis infection. On the other hand, the treatment options for patients with advanced liver cancer are often limited, and common chemotherapeutics are accompanied by toxic side effects [17]. Therefore, seeking a new treatment is critical for the treatment and prognosis of patients with primary liver cancer.

At present, immunotherapy has become the fourth common cancer treatment after operation, radiotherapy and chemotherapy. Blocking immunotherapy for PD-1 is one of the hotspots of current tumor immunotherapy. Antibodies against PD-1 have been approved for the clinical treatment of melanoma and non-small cell lung cancer in the United States, Europe and Japan [18]. Studies found

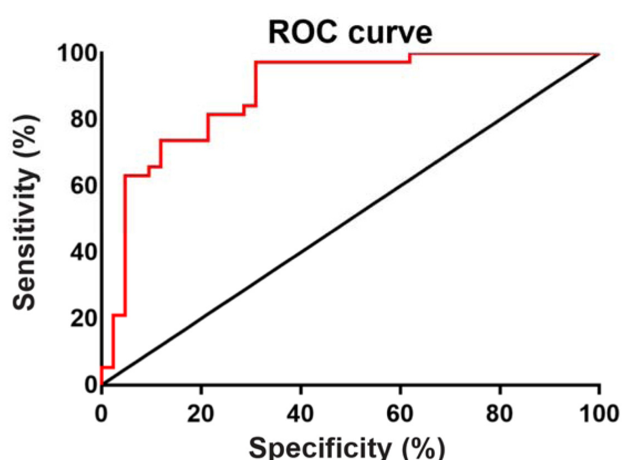

Figure 3. Analysis of the diagnostic value of PD-1 for liver cancer. The ROC curve was plotted according to the expression level of PD-1 mRNA in the peripheral blood T lymphocytes of patients in the liver cancer group and the control group. The AUC was 0.883 (0.808-0.958), the diagnostic sensitivity was 97.37%, the specificity was 69.05%, the Youden index was 0.664, and the corresponding diagnostic threshold was 7.636 (p<0.001).

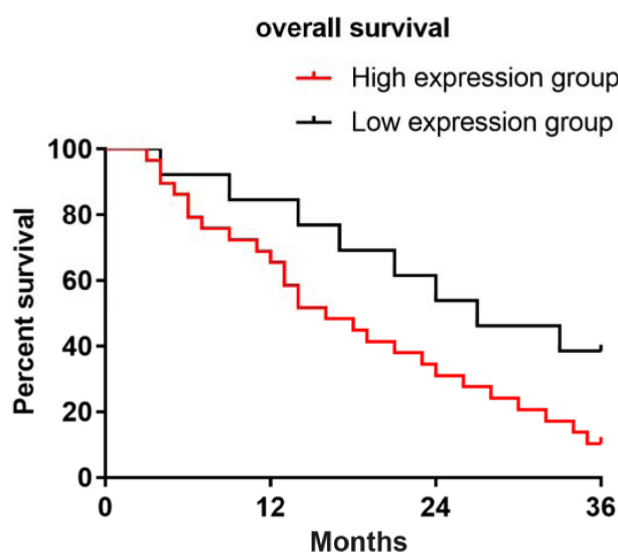

Figure 4. Survival comparison of patients between PD-1 high expression group and PD-1 low expression group. The diagnostic threshold of PD-1 mRNA was 7.657. On this basis, the liver cancer group was divided into the high expression group and the low expression group. The median survival time of patients was 16 months in the PD-1 high expression group, and 27 months in the PD-1 low expression group, with the overall survival rates of 10.34 and 38.46%, respectively. The survival of patients in the low expression group was significantly better than that in the high expression group (p<0.05).

that tumor cells activate PD-1 on the surface of lymphocytes by expressing PD-L1, thereby evading immune surveillance and conducting PD-1/PD-L1 signal. The expression level of PD-1 has different degrees of effect on the function of T cells [19]. Blocking the PD-1/PD-L signaling pathway with PD-1 antibodies shows anti-tumor activity in a variety of malignant tumors, with fewer adverse events during use [20]. In addition, PD-1 is abnormally expressed in various cancers, the expression of which can be used as a marker for the poor prognosis of breast cancer and thymic carcinoma [21,22]. However, there are currently few studies on the expression of PD-1 in liver cancer. It is suspected that PD-1 has similar performance in primary liver cancer. Therefore, in this study, the expression level of PD-1 mRNA and the positive expression rate of PD-1 protein in peripheral blood T lymphocytes of liver cancer patients were detected and evaluated, and their relationship with prognosis of patients with primary liver cancer was analyzed.

The results of this study showed that patients in the control group and the benign lesion group had lower expression level of PD-1 mRNA in peripheral blood T lymphocytes than those in the liver cancer group. Patients in the control group and the 11 benign lesion group had lower positive expression rate of PD-1 protein on the surface of T lymphocytes than those in the liver cancer group. Patients in the control group had lower expression level of PD-1 mRNA and positive expression rate of PD-1 protein in T lymphocytes than those in the benign lesion group. The ROC curve was plotted according to the expression level of PD-1 mRNA in peripheral blood T lymphocytes of patients in the liver cancer group and the control group. The AUC was 0.883 (0.808-0.958), the diagnostic sensitivity was 97.37%, the specificity 69.05%, the Youden index 0.664, and the corresponding diagnostic threshold 7.636. The diagnostic threshold was used to distinguish the high and low expressions of PD-1 mRNA. The median survival time of patients was 16 months in the high expression group, and 27 months in the low expression group, with the overall survival rates of 10.34% and 38.46%, respectively. The survival of patients in the low expression group was better than that in the high expression group. This indicates that the expressions of PD-1 mRNA and PD-1 protein in peripheral blood T lymphocytes are higher in liver cancer patients than those in healthy volunteers and CHB patients. Patients with high expression of PD-1 have poorer prognosis than those with low expression of PD-1. Firstly, as described above, PD-1 is mainly expressed on activated T lymphocytes in humans. The deletion of PD-1 can induce proliferation of adenovirus-infected liver effector T cells, thereby rapidly clearing viruses. However, after binding to PD-L1, PD-1 can trigger inhibition of signals at the downstream of T cell receptor and reduce the killing function of T cells, so that cancer cells can protect themselves from immune cell-mediated killing, thereby promoting tumor progression [23,24]. This also indicates from both sides that PD-1 positive patients have faster tumor progression and poorer survival than PD-1 negative patients, which complements our findings, i.e. non-cancer patients have lower PD-1 expression than cancer patients. Secondly, Shen et al. [25] and other authors have detected the expression of PD-1 protein in peripheral blood and T cells of pancreatic ductal adenocarcinoma. The results showed that the expression of PD-1 protein in peripheral blood T lymphocytes and tumor tissue infiltrating T lymphocytes of patients was higher than that in healthy volunteers and adjacent normal tissues. The authors speculate that the persistent overexpression of PD-1 on T lymphocytes may be related to the high postoperative recurrence rate. In addition, researchers have conducted a meta-analysis on about 12 epithelial-derived malignant tumors [26]. The results showed that patients with PD-1 or PD-L1 positive expression have significantly shorter overall survival time, and PD-1 or PD-L1 expression is an indicator for judging the prognosis of patients. Due to limited experimental conditions, the recurrence of patients with primary liver cancer was not evaluated in this study, but these two studies support our views on the other hand, i.e. patients in the PD-1 high expression group have poorer prognosis. Finally, the literature suggests that the expression of PD-1 varies with the course of HBV infection, is related to the number of HBV viral vectors and liver function, and can be used as a potential indicator for viral replication and liver injury [27]. This also indicates that PD-1 may be involved in the occurrence and development of primary liver cancer, the expression of which may be used as an indicator to distinguish between healthy people and benign lesion and between benign lesion patients and liver cancer patients. This explains from another perspective that PD-1 is highly expressed in patients with primary liver cancer, and the prognosis of patients in the high expression group is poorer.

There are still many shortcomings in this study. One is that the sample size is not large enough, which makes the results of survival analysis not so objective, so other authors need to supplement or expand the study scope for multi-center sample collection. The other is that this study does not

explain the specific mechanism on how PD-1 mediates primary liver cancer, which needs further investigation.

In summary, the expression of PD-1 in the peripheral blood is higher in patients with primary liver cancer than that in healthy volunteers and benign lesion patients. Patients in the PD-1 low expression group have significantly better prognosis than those in the PD-1 high expression group. The study indicates that PD-1 may be related to the occurrence and development of primary liver cancer and is worth of further investigation.

Conflict of interests

The authors declare no conflict of interests.

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