

The expression of PD-L1 APE1 and P53 in hepatocellular carcinoma and its relationship to clinical pathology

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Abstract. – OBJECTIVE: To study the expression of programmed death-ligand1 (PD-L1) in hepatocellular carcinoma and its relationship with clinicopathological characteristics and, prognosis of hepatocellular carcinoma and APE1, P53 protein expression levels.

PATIENTS AND METHODS: A total of 128 patients with hepatocellular carcinoma were enrolled in this study. The expression of PD-L1, APE1 and P53 were detected by immunohistochemistry. Use immunohistochemical ABC staining method to detect the expression levels of PD-L1, APE1 and P53 protein in the hepatocellular carcinoma of 128 cases.

RESULTS: Positive The positive expression rates levels of PD-L1, APE1, and P53 protein in hepatocellular carcinoma tissues are were 82.03%, 92.19%, and 60.94%. PD-L1 positive expression were significantly associated with clinical stage, The PD-L1 protein has a high expression in patients with I ~ II stage liver cancer. HBV infection positive and nonportal vein thrombosis ($p=0.041$; $p=0.030$; $p=0.014$). It is inversely correlated with P53 and PD-L1 expression (correlation coefficient -0.227 , $p=0.010$), and positively correlated with APE1 expression (correlation coefficient 0.189 , $p=0.032$). The expression of PD-L1 is associated with the survival time of patients with hepatocellular carcinoma, and the median survival time of patients with high expression of PD-L1 is ten months. The median survival time of patients with low expression is five months ($p=0.001$). The relationship between the expression of APE1 and P53 protein and overall survival time of patients with hepatocellular carcinoma has not been found.

CONCLUSIONS: The PD-L1 and APE1 expression in hepatocellular carcinoma are related to the level of the expression of P53 protein. The expression state of PD-L1 may be a prognostic factor in hepatocellular carcinoma.

Key Words:

Hepatocellular carcinoma, PD-L1, APE1, P53.

Introduction

Hepatocellular carcinoma is one of the most common malignant tumors in the world. Because of the difficulty of early diagnosis, many traditional treatment methods cannot obtain satisfactory results. This results in the patient's five-year survival rate of only 3%-5% and about 600,000 people die of this disease every year in China¹. With the rapid development of molecular biology, people are aware that the occurrence and development of a tumor is a complex mechanism of multi genes interaction like mesh. At present, molecular targeted therapy Nexavar has become the focus of current studies, and single gene and single point studies are transformed into a joint analysis of multiple genes and multiple points².

Research shows that programmed death-ligand1 (PD-L1) is an important negative immune regulatory molecule. As a member of the costimulatory molecule B7 family expressed by antigen presenting cells, its main role is to inhibit T cell activation and proliferation, promote apoptosis of T cells, and play an important role in tumor immune escape^{3,4}. The PD-L1 is found in a variety of tumor tissue, and the link between its expression and clinical pathological features of patients has been proven. In breast, gastric, esophageal, lung, and bladder cancer, and renal cell carcinoma, we find the expression of PD-L1⁵⁻¹³. Recent studies have shown that on tumor cells, the disorder expression of PD-L1 destructs antitumor immunity of the body, which causes the immune escape of tumor cells¹⁴. It uses soluble PD-1 (sPD-1) to block the PD-1/PD-L1 pathway and is expected to be an effective means for the immunotherapy of hepatocellular carcinoma^{15,16}. At present in China, PD-L1 immune targeted clinical trials for the treatment of

lung cancer is on the way. Results show that the treatment effect of PD-L1 antibody in lung cancer is related to histologic types, and further analysis finds that the expression of PD-L1 is related to genes¹⁷. The results provide the pathological stratified basis to PD-L1 antibody treatment for patients. In the future, combined targeted therapy may have an important significance in cancer therapy.

The apurinic/aprimidinic endonuclease 1 (APE1) has a dual function of the DNA damage repair and oxidative reduction, and is a multifunctional protein, which plays an important role in maintaining DNA stability and regulating the expression of cytokines¹⁸. In lung cancer, the abnormal expression, distribution and function change of the APE1 protein is closely related to anti-apoptosis and tumorigenesis¹⁹⁻²¹. The P53 gene is a cell growth "monitor", that plays a negative regulatory role in the cell cycle to regulate cell proliferation and differentiation. Studies have shown that, in HCC cells, the APE1 redox function can enhance the P53 DNA binding activity²². In gastric carcinoma tissues, there is a correlation between the expression of PD-L1 and APE1. Therefore, the research detects the PD-L1, APE1 and P53 protein expression in hepatocellular carcinoma by immunohistochemical method, and focuses on the expression of PD-L1 and its relationship with the clinical pathology and prognosis of patients with hepatocellular carcinoma. The correlation with PD-L1, APE1, and P53 expression provides a basis for futural clinical PD-L1 antibodies in the treatment of liver cancer.

Patients and Methods

One hundred and twenty-eight cases of primary hepatocellular carcinoma tissues were obtained from surgical specimens of hospitalized patients in the hospital from January 2001 to May 2011, and confirmed by pathological diagnosis as hepatocellular carcinoma. There were 108 males and 20 females, and the median age was 54 years (17-75 years of age). Pathological Grading was divided into four grades by the classical Edmondson-Steiner: Grade I, 24 cases (18.75%), grade II, 76 cases (59.38%), grade III, 12 cases (9.38%), and grade IV, 16 cases (12.50%). The maximum tumor ≤ 5 cm 38 cases, >5 cm 90 cases; solitary nodular hepatocellular

carcinoma 126 cases, multiple nodules two cases. According to TNM classification standard of International Union Against Cancer (UICC): stage I 8 cases (3.13%), stage II 24 cases (18.75%), stage III 68 cases (53.13%), stage IV 28 cases (21.88%). HBV positive 112 cases (87.5%), negative 16 cases (12.5%), portal vein embolization 44 cases (34.38%), non-cancer embolism 84 cases (65.63%). 66 cases for alpha fetoprotein (AFP) >400 $\mu\text{g/L}$ (51.56%), 62 cases for AFP ≤ 400 $\mu\text{g/L}$ (48.44%) (Table I).

No patients had received preoperative radiotherapy or chemotherapy. Since the patient received operation therapy and began follow-up, until death or last follow-up, obtain the overall survival (OS) of the patient. Follow-up every three months, follow-up deadline was June 2014; the median follow-up time was ten months.

Immunohistochemical Staining and Result Determination.

All tissues were fixed by 10% neutral formaldehyde solution, conventional dehydration, and paraffin embedding. The polyclonal anti-human PD-L1 antibody was purchased from USA GeneTex Company (Irvine, CA, USA). The rabbit anti-human APE1 monoclonal antibody was purchased from USA Santa Cruz Company, and the mouse anti-human P53 monoclonal antibody was purchased from Beijing Zhongshan Jinqiao Biological Technology Co., Ltd. Immunohistochemical staining was performed using the ElivisionTM two-step method (Zymed, CO, USA) according to the instructions. The serial sections of the paraffin-embedded tissue 3 μm pieces were oven baked at 60°C for more than two hours, then dehydrated and dewaxed. Then we used 3% H_2O_2 - (1:9) methanol for 10 min to block the endogenous peroxidase activity, and washed twice with phosphate buffered saline (PBS) buffer solution (pH7.4). Where APE1 and P53 dyeing needed heat repair, the steps were to soak the slide by sodium citrate (0.01 M, pH6.0), and place it in a pressure cooker gas for 1 min and, then, cool it to room temperature for 20 min. The PD-L1 was closed in a room by 10% sheep serum, APE1 and P53 were closed in room by 5% sheep serum for 10 min. Add first antibody (concentration of PD-L1 was 1:100; the concentration of APE1 was 1:3000; P53 working concentration was 1:50) and stay overnight in a wet box of 4°C refrigerator. Wash with PBS for 5 min*2, according to the source

of first antibody, add second antibody, diaminobenzidine (DAB) showed color, after hematoxylin counterstain, mount.

The PD-L1 is positive if there are brown granules in the membrane and cytoplasm, APE1 is positive if there are brown granules in the nucleus and cytoplasm, and P53 is positive if the nucleus is brown. P53 APE1, PD-L1 in view of high magnification randomly counted 1000 tumor cells, and was scored according to the percentage of positive cells: the positive cell rate <10% records 0 point, the positive cell rate 10%-25% records 1 point, the positive cell rate 25%-50% records 2 points, the positive cell rate ≥50% records 3 points. The PD-L1 and APE1 define 0-1 point that means negative (-), and 2-3 points mean high expression. P53 defines 0 point that means negative (-), and 1-3 points mean positive (+).

Statistical Analysis

The statistical analysis was performed using SPSS19.0 (IBM SPSS Statistics) to analyze the data. The relationship between PD-L1 expression

and the features of clinical pathology was exactly tested by with Pearson χ^2 and Fisher's, and the correlation analysis among PD-L1, APE1 and P53 is done by Pearson correlation coefficient analysis. Survival analysis was analyzed by the Kaplan-Meier curve method; the factor levels comparison was tested by Log-rank, various clinicopathological factors that affect prognosis were analyzed by Cox regression (forward). $p < 0.005$ was considered statistically significant.

Results

In HCC, the PD-L1 expression characteristics of 128 primary liver cancer patients, PD-L1 in cancer tissues is positive in 105 cases, negative in 23 cases, the positive rate is 82.03%. PD-L1 is located in the cell membrane and cytoplasm, and its color is between light yellow and brown with granular (Figure 1). For the vast majority of HCC cases, PD-L1 positive cells are evenly distributed throughout the specimen, similar to the expression way of glioma nine and ovarian cancer²³. In

Table I. Relationship between PD-L1 expression and clinical pathological features of hepatocellular carcinoma.

Item	Cases (%)	PD-L1 expression level (n)		P ¹
		Positive (%)	Negative (%)	
Sex				
Male	108 (84.38)	88 (81.48)	20 (18.52)	1.000 ²
Female	20 (15.62)	17 (85.00)	3 (15.00)	
Age	54 (17-75)			0.622
≤ 50	56 (43.75)	47 (83.93)	9 (16.07)	
> 50	72 (56.25)	58 (80.56)	14 (19.44)	
Differential degree				0.403 ²
High differentiation	28 (11.72)	25 (89.29)	3 (10.71)	
Medium, low differentiation	100 (88.28)	80 (80.00)	20 (20.00)	
Tumor size				0.209 ²
≤ 5 cm	38 (29.69)	34 (89.47)	4 (10.53)	
> 5 cm	90 (70.31)	71 (78.89)	19 (21.11)	
Clinical stages				0.188 ²
Stage I-II	32 (25.00)	29 (90.63)	3 (9.37)	
Stage III-IV	96 (75.00)	76 (79.17)	20 (20.83)	
HBV infection				0.004
Negative	16 (12.50)	9 (56.25)	7 (43.75)	
Positive	112 (87.50)	96 (85.71)	16 (14.29)	
Portal vein thrombosis				0.003
No	84 (65.63)	75 (89.29)	9 (10.71)	
Yes	44 (34.37)	30 (68.18)	14 (38.82)	
The level of serum AFP				0.948
≤ 400 μg/L	62 (48.44)	51 (82.26)	11 (17.74)	
> 400 μg/L	66 (51.56)	54 (81.82)	12 (18.18)	

¹Person χ^2 test; ²Fisher's exact test.

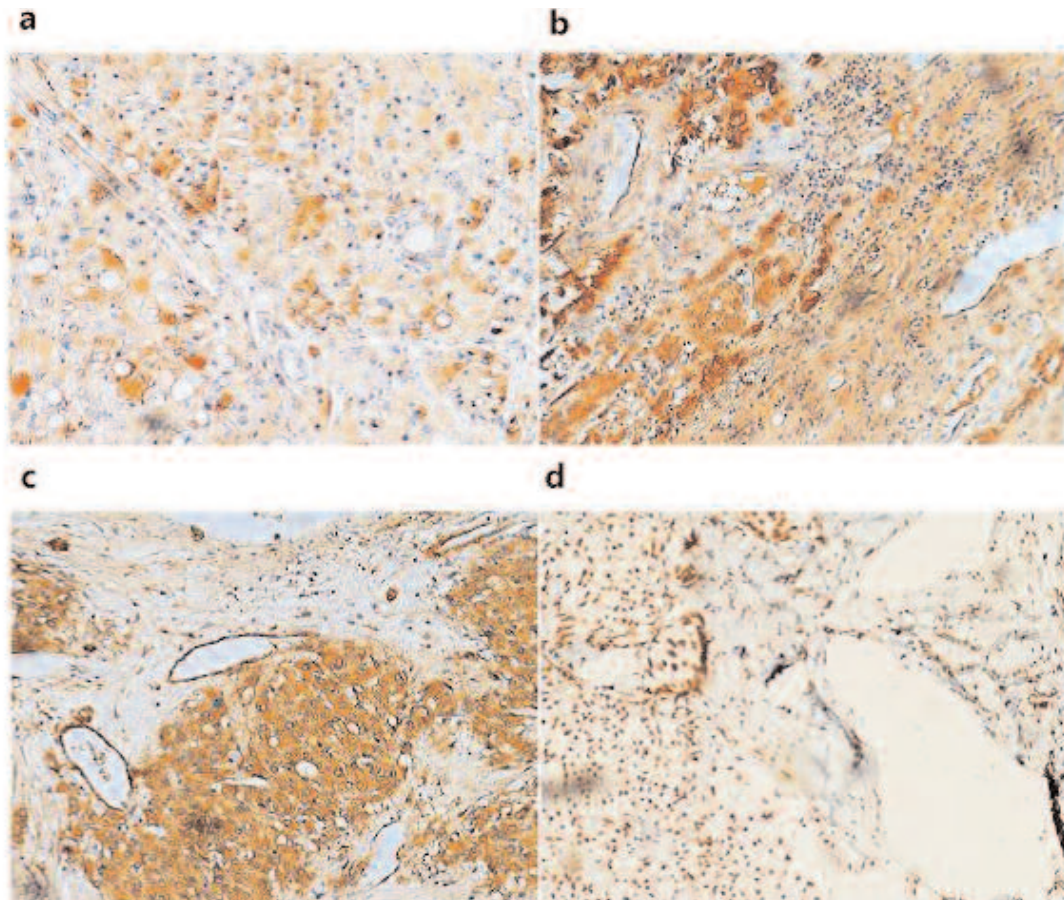


Figure 1. The positive expression of PD-L1 in hepatocellular carcinoma tissues, para carcinoma, portal area and adipose tissue. **A**, Hepatocellular carcinoma; **B**, Cancer adjacent connective tissue; **C**, Department of irrigation area; **D**, Adipose tissue. H&E, 200 \times .

addition, PD-L1 is expressed in the liver tissue adjacent to carcinoma portal exchange and tumor, and PD-L1 expression can be found on lymphocytes and endothelial cells of hepatocellular carcinoma (Figure 1).

The Expression of PD-L1 in Hepatocellular Carcinoma and its Relationship to Clinical Pathology

The expression of PD-L1 is related to HBV infection and portal vein thrombosis, but not related to the age and sex, tumor size, TNM stage, differentiation and serum AFP levels of patients (Table I). Among patients with the infection of HBV positive, the positive rate of PD-L1 (85.71%) is significantly higher than that of HBV negative patients (56.25%) ($p=0.004$, Pearson χ^2 test). In patients without portal vein thrombosis, the positive rate of PD-L1 (89.29%) is significantly higher than that of patients with portal vein thrombosis (68.18%) ($p=0.003$, Pearson χ^2 test).

Relationship Among the Expression of PD-L1 and APE1 and P53

Among 128 primary liver cancer patients, 86 cases are APE1 positive, 42 cases are negative, and the positive rate is 67.19%. The expression of P53 is positive in 78 cases, 50 cases are negative, and the positive rate of expression is 60.94% (Figure 2). The expression levels of PD-L1, APE1 are closely related to P53 expression level (Table II). The expression of PD-L1 is negatively correlated with P53 expression (correlation coefficient -0.227 , $p=0.010$); Expression of APE1 is positively correlated with P53 expression (correlation coefficient 0.189 , $p=0.032$).

Survival Analysis

Overall survival of patients with the high expression of PD-L1 is significantly longer than that of patients with low expression (4 months vs. 11 months, $p=0.001$; Log Rank test; Table III;

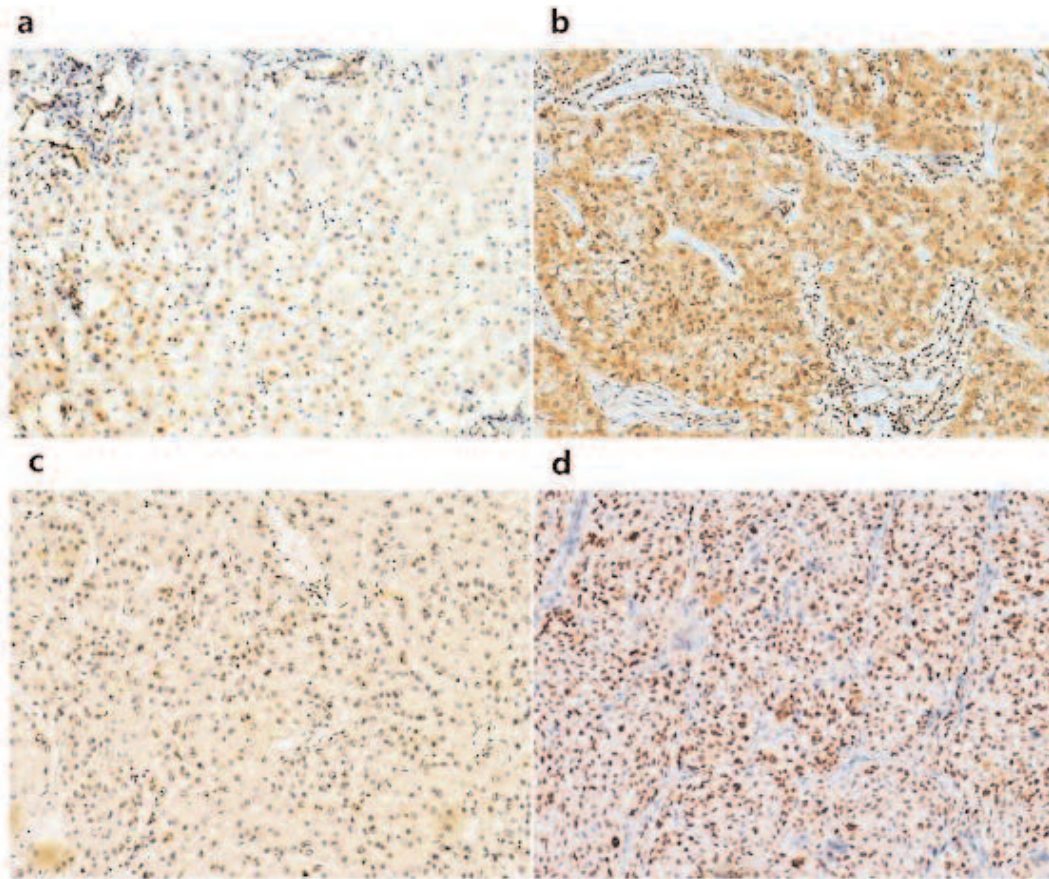


Figure 2. **A**, APE1 negative expression in hepatocellular carcinoma tissues; **B**, PE1 positive expression in hepatocellular carcinoma tissues; **C**, p53 negative expression in hepatocellular carcinoma tissues; **D**, p53 positive expression in hepatocellular carcinoma tissues. H&E, 200×.

Table II. Analysis of the relationship among the expression of PD-L1, APE1 and P53.

		APE1	PDL1	P53
APE1	Pearson Correlation	1	-0.058	0.189
	P (2-tailed)		0.512	0.032
	N	128	128	128
PDL1	Pearson Correlation	-0.058	1	-0.227
	P (2-tailed)	0.512		0.010
	N	128	128	128
P53	Pearson Correlation	0.189	-0.227	1
	P(2-tailed)	0.032	0.010	
	N	128	128	128

Pearson correlation coefficient analysis.

Figure 3a), and the risk coefficient (HR) is 0.583 (95% CI 0.369-0.961). The expression of APE1 and P53 protein is not found to have a relationship with the postoperative survival period of primary hepatocellular carcinoma of patients

(Table III; Figure 3b-c). Overall survival of patients with positive and negative expression of APE1 are respectively ten and seven months ($p=0.162$; Log Rank test). Overall survival of patients with positive and negative expression of

Table III. The correlation between PD-L1, APE1, P53 and survival period.

Protein expression	n (%)	Overall survival (OS) month	P ¹	HR (95% CI) 2	P ²
PD-L1					
Positive	105 (82.03)	11			
Negative	23 (17.97)	4	0.001	0.472 (0.296-0.755)	0.002
APE1					
Positive	86 (67.19)	10			
Negative	42 (32.81)	7	0.162	0.771 (0.526-1.130)	0.182
p53					
Positive	78 (60.94)	8			
Negative	50 (39.06)	8	0.341	0.843 (0.584-1.216)	0.362

¹Log-rank test; ²Single factor Cox regression model.

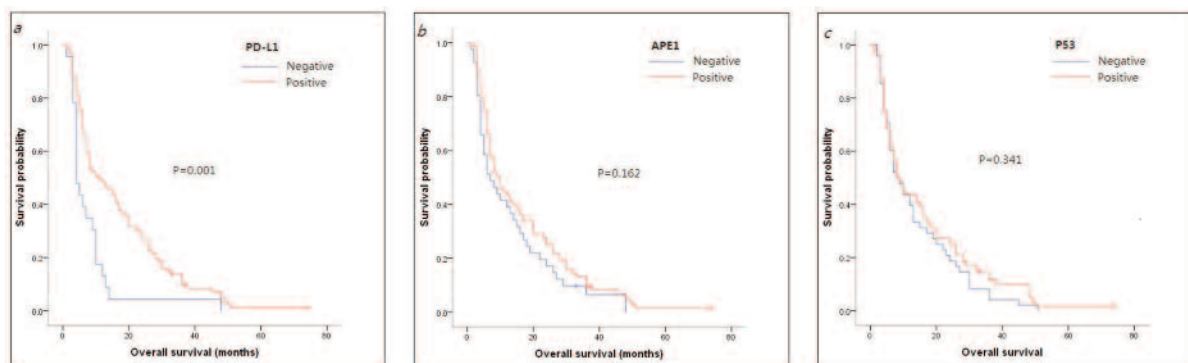
P53 protein are both eight months ($p=0.341$; Log Rank test). After that multivariate analysis of Cox regression (forward LR method) adjusts sex, age, degree of differentiation, tumor size, clinical staging, HBV infection status, portal vein thrombosis and serum AFP levels, find that age (HR 0.615, 95% CI 0.419-0.902, $p=0.013$), clinical staging (HR 1.679, 95% CI 1.065-2.646, $p=0.026$), portal vein thrombosis (HR 1.565, 95% CI 1.024-2.393, $p=0.039$) and the expression level of PD-L1 (HR 0.514, 95% CI 0.315-0.839, $p=0.008$) are independent prognostic factors of primary hepatocellular carcinoma.

Discussion

Through the analysis of the PD-L1 expression in primary hepatocellular carcinoma, its effects on the prognosis, and its relationship with the APE1 and P53 expression. This study finds that high expression of the PD-L1 protein in patients with HBV-positive infection and without portal

vein thrombosis is negatively correlated with P53 protein expression. The prognostic factors of primary hepatocellular carcinoma, the OS of patients with high expression of PD-L1 is significantly longer than patients with low expression. The APE1 protein expression is positively correlated with the P53 protein expression. The APE1 and P53 protein has not been found to be the prognostic factor of liver cancers. This result suggests that there may be some connection among the PD-L1, APE1 and P53, but there is no study about the mechanism of these three expressions yet. This study is the first to report that the expression level of PD-L1 in primary hepatocellular carcinoma may affect the prognosis and has a negative correlation with P53 protein.

The PD-L1 is a transmembrane glycoprotein of the B7 family, encoding 290 amino acids, there are IgV and IgC domain in its extracellular^{24,25}. It is located in the chromosome9p24.2, whose expression is more widely than other members of the family²⁶. The PD-L1 binding to

**Figure 3.** The correlation between PD-L1, APE1, P53 and survival period.

its receptor PD-1 can activate T cells negative regulation cytokine secretion and proliferation of the T cells⁵. Many studies have shown that the cancer cells themselves can up-regulate inhibitor molecules PD-L1 to make tumor escape from host immune^{27,28}. The high expression of PD-L1 in the tumor is related to immune escape mechanism, resulting in enhanced activity of Treg and antitumor T cell anergy, which often indicates a poor prognosis¹⁴. However, the PD-L1 intracellular signaling pathway is not clear. Some studies have considered that its expression requires activation of transcription 3 (STAT3) combined with the CD274 promoter, but the expression induced by IFN- γ requires MAPK and JAK/STAT signaling pathway mediated. The inhibition of certain proteins in the signal path above can make the expression of PD-L1 decrease, and it is expected to become the target of antitumor therapy²⁹. Recently, that anti-PD-1 (BMS-936558) and anti-PD-L1 (BMS936559) monoclonal antibody enhance T cell-mediated antitumor effect has obtained the treatment response in early clinical trials³⁰. Our study shows that in the patients with HBV-positive infection, the positive rate of PD-L1 is higher and is related to the fact that the pathogen may through the PD-1 signal form continuously chronic infection³¹. This would suggest that the PD-L1 can also be used as one of the influencing factors of liver cancer, and cause us to think about whether the PD-L1 can be used as an indicator of primary liver cancer risk. At the same time, our results suggest that the high expression of the PD-L1 in hepatocellular carcinoma tissues and longer survival of patients with high expression can be used as prognostic factors of primary liver cancer. However, previous studies have found that high expression of PD-L1 and PD-L2 in tumor cells of some patients with hepatocellular carcinoma, increases significantly. The disease-free and total survival rate are both significantly lower than the patients with PD-L negative or low expression³².

APE1 has been proved to activate transcription factors to play an important physiological role through dependent or independent pathway by the redox^{33,34}. These transcription factors include (such as AP-1, NF- κ B, p53, HIF) and are widespread in various tissues and are tissue specific (such as PEBP-2, Pax-5, Pax-8, TTF-1). They control the different cell biological processes such as differentiation, proliferation and apoptosis. APE1 maintains reduction activity status of these transcription factors in cellular, so

as to control the cell cycle proliferation and apoptosis³⁵. The level of APE1 expression significantly increases under oxidative stress³⁶. Our study shows that in primary hepatocellular carcinoma, APE1 expression is significantly high; whose positive rate is 67.19%. The expression of APE1 is not found to be related to the survival period of primary hepatocellular carcinoma.

The P53 gene is an important apoptosis gene and plays an important role in the maintenance of cell growth and reproduction. The APE1 activates the p53 by the redox and non-redox dependent manner to promote transcription activation of a large number of the p53 target gene, thereby, promoting p53 dependent apoptosis³⁷. Previous studies have found ectopic expression of APE1/p53+ HCC high degree of malignancy, suggesting that ectopic expression of APE1 and P53 mutation may have synergistic effects on the process of tumor occurrence and development³⁸. Our study confirm that the expression of P53 gene has a correlation with the expression of the PD-L1 and APE1 protein in primary hepatocellular carcinoma. The expression of P53 is negatively correlated with that of the PD-L1, and positively correlated with the expression of APE1. But it has not been found that the APE1 expression has a correlation with the PD-L1 expression in hepatocellular carcinoma tissue and we need to do further research to elucidate the specific mechanism.

Conclusions

The high expression of PD-L1 in primary hepatocellular carcinoma and the better prognosis of patients with high expression have an important guiding significance for clinical practice. The regulation of the PD-1 pathway in immune therapy is promising. There is a correlation between the expression of PD-L1 and P53; however, we need further researches to elucidate the specific mechanism.

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