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Original Article

Association between *CTLA-4* rs231775 polymorphism and hepatocellular carcinoma susceptibility

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Abstract: Aims: Our study aimed to investigate the association of cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) rs231775 polymorphism with hepatocellular carcinoma (HCC) susceptibility. Methods: Genotypes distribution of the control was tested by Hardy-Weinberg Equilibrium (HWE). *CTLA-4* rs231775 polymorphism was analyzed in 80 patients with HCC and 78 healthy controls by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, and the expression level of *CTLA-4* in the serum of all subjects was detected using enzyme linked immunosorbent assay (ELISA) kit. Odd ratio (OR) with 95% confidence interval (CI) were calculated by chi-squared test to determine the correlation of *CTLA-4* rs231775 polymorphism and the risk of HCC. Results: The genotypes frequencies of the control group were in accordance with HWE. The frequencies of genotype AA and allele A in *CTLA-4* rs231775 polymorphism were significantly higher in cases than the control group (AA vs. GG: OR=2.81, *P*=0.043; A vs. G: OR=1.63, *P*=0.022). Meanwhile, the expression level of *CTLA-4* was remarkably higher in cases compared with the controls. The association analysis indicated that AA genotype carriers exhibited highest level of *CTLA-4* (*P*<0.01). Conclusions: The genotype AA and allele A of *CTLA-4* rs231775 polymorphism may have negative effects on HCC by modifying the expression and functions of *CTLA-4*.

Keywords: Hepatocellular carcinoma, *CTLA-4,* polymorphism

Introduction

Liver cancer is one of malignant tumors with the highest incidence all over the world [1]. Cause-of-death statistics of Chinese residents reveal that liver cancer is the second leading cause of death among the malignant tumors. The incidence of hepatocellular carcinoma (HCC) in China is the highest worldwide, and HCC brings serious threat to the health of human beings [2]. However, the pathogenesis of HCC has not been well clarified.

Cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) encoded by *CTLA-4* belongs to the immunoglobulin supergene family. Its gene located on chromosome 2 in q33, which is expressed on the surface of T cells in form of dimers [3]. The function of *CTLA-4* is mainly to transmit inhibitory signals so as to negatively regulate the proliferation of T cells and block the immune responses [4]. Genetic variations or expression disorders of *CTLA-4* may lead to an attenuated inhibitory effect on the activation of T cells, meanwhile autoimmune injury was activated [5, 6]. In recent years, studies concerning the relationship between *CTLA-4* polymorphisms and some diseases have attracted extensive attention. Previous studies have indicated that *CTLA-4* polymorphisms are closely related to various autoimmune diseases such as idiopathic dilated cardiomyopathy (IDCM), lupus erythematosus (LE) and thyroid diseases [7-9]. Just as we have known, *CTLA-4* polymorphism exists a mutation from A to G on exon2 as rs231775, the relationship between *CTLA-4* rs231775 polymorphism with various diseases has been exported, such as gastric cancer [10], colorectal cancer [11], hepatocellular carcinoma and cervical cancer [12]. But the association of *CTLA-4* rs231775 polymorphism with HCC risk is hardly reported.

In present study, a case-control model included 80 cases and 78 controls were conducted. The *CTLA-4* rs231775 polymorphism was checked

Table 1. Expression level of *CTLA-4* in the serum

Group	*CTLA-4* concentration (μg/L)	*P* value
Case group	1.63 ± 0.76	0.022
Control group	0.49 ± 0.18	

by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method. The result about the relevance of *CTLA-4* rs231775 polymorphism with HCC risk provides a theoretical basis for the risk prediction and the treatment of HCC.

Subjects and methods

Research subjects

A case-control study was carried out. 80 patients with HCC by pathological examination were set in case group from Jinan Central Hospital, and 78 individuals who had a health examination in same hospital during the same period had no tumors or genetic diseases belonged to the control group. An age gap of ± 2 years existed among individuals in the two groups. Furthermore, the subjects in two groups had the similar native place, gender and ethnicity, as well as parallel life and eating habits, and had no genetic connections. Our research protocol was supported by Research Ethics Committee of the hospital. Written consents were acquired from every subject.

Sample preparation

5 ml peripheral venous blood was collected from each subject with TRANK 303 heparin vacuum blood collection tube. The expression level of *CTLA-4* in serum was tested by enzyme linked immunosorbent assay (ELISA) kit.

DNA extraction

Genome DNA of peripheral venous blood from all subjects was extracted by using the conventional phenol-chloroform method. The purity and concentration of DNA were tested by an ultraviolet spectrophotometer and then stored at -20°C refrigerator.

Genotyping

PCR primers were designed by Premier 5.0 according to gene sequence of exon 1 in *CTLA-4* gene in Genebank and were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Forward primer: 5'-GCTCTACTTCCTGAAGACCT-3', reverse primer: 5'-AGTCTCACTCACCTTTGCAG-3'. PCR reaction system was a volume of 25 μL solution, which included substrate template DNA (0.5 μg), 1 μL forward primer (0.5 μmol/L), 1 μL reverse primer (0.5 μmol/L), 12.5 μL Master Mix and the corresponding buffer solution. The PCR amplification conditions were as follows: initial denaturation at 95°C for 7 minutes, followed by 35 cycles with 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min and final extension at 72°C for 10 min. The amplification product had a length of 162 bp, and included *CTLA-4* rs231775 polymorphism. 10 μL PCR product was digested by *Bbv I*, and the digested products were checked by 2% polyacrylamide gel electrophoresis (PAGE).

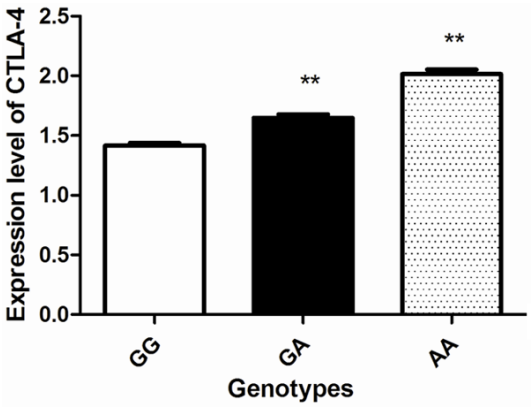

Figure 1. The effects of genotype distribution on the level of *CTLA-4.* **Indicates significant differences.

Statistical analysis

The data analysis was performed with SPSS 18.0 software. The genotypes frequencies of the controls were checked whether the distribution was in accordance with Hardy-Weinberg Equilibrium (HWE). Odds ratio (OR) with 95% confidence interval (CI) were used to evaluate the association of *CTLA-4* rs231775 polymorphism with the risk of HCC, which was calculated by chi-squared test. In present study, the relationship between *CTLA-4* rs231775 polymorphism and the expression of *CTLA-4* in serum was analyzed with linear regression analysis. $P<0.05$ indicates significant difference.

Table 2. The comparison of genotypes and alleles frequencies of *CTLA-4* rs231775 polymorphism in case and control group

CTLA-4 rs231775	Case group (n=80, %)	Control group (n=78, %)	ORs (95% CIs)	*P* value
GG	29 (36.3)	38 (48.7)	1 (Ref.)	-
GA	36 (45.0)	33 (42.3)	1.43 (0.73-2.81)	0.30
AA	15 (18.7)	7 (9.0)	2.81 (1.01-7.78)	0.043
G	94 (58.8)	109 (69.9)	1 (Ref.)	-
A	66 (41.2)	47 (30.1)	1.63 (1.02-2.59)	0.039

Results

HWE examination

The examination result showed that the genotypes frequencies of *CTLA-4* rs231775 polymorphism in control group were satisfied with HWE ($P>0.05$).

Analysis of CTLA-4 rs231775 polymorphism

The digestion results showed AA genotype with a 162 bp fragment, AG genotype with three fragments of 162 bp, 88 bp and 74 bp, and GG genotype with 88 bp and 74 bp fragments.

Analysis of CTLA-4 level in the serum and CTLA-4 rs231775 polymorphism

The *CTLA-4* level in the serum of subjects in the two groups is shown in **Table 1**. The result revealed that the expression level of *CTLA-4* was significantly higher in cases compared with the control group ($P<0.05$). In the same time, we performed an analysis of the relationship between the expression level of *CTLA-4* and genotypes distribution of *CTLA-4* rs231775 polymorphism case group. The result showed that in case group, the expression level of *CTLA-4* was highest in the AA genotype carriers compared with AG and GG ($P<0.01$) (**Figure 1**).

The relationship of CTLA-4 rs231775 polymorphism with HCC susceptibility

We made a comparison in genotypes and alleles frequencies of *CTLA-4* rs231775 polymorphism between the cases and controls. The result demonstrated that frequencies of hemozyous genotype AA and allele A were remarkably higher in case group than controls (18.7% vs. 9.0%, 41.2% vs. 30.1%). We also found that AA genotype and A allele might be risk factors for HCC (AA vs. GG: OR=2.81, 95% CI=1.01-7.78; A vs. G: OR=1.63, 95% CI=1.02-2.59) (**Table 2**).

Discussion

The occurrence and development of HCC is a complex biological process influenced by many factors. The environmental factors are the external causes, whereas genetic susceptibility is an important internal cause and largely determines the risk degree of developing liver cancer among individuals [13, 14]. Human genes have abundant polymorphisms. Genetic mutations may lead to abnormal expressions of corresponding proteins. As a result, each individual may react differently to toxics or carcinogens, and thus may have different liver cancer susceptibility [15, 16]. In recent years, abundant publications have reported genetic variants are related with HCC susceptibility. According to the study of Li et al., genetic variant of *CYP2C19* may be an important risk factor for HCC in China Han population, but not in patients with HBV infection [17]. A meta-analysis from Xiao et al. demonstrated the homozyous null genotypes of glutathioneS-transferasegenetic polymorphisms *GSTM1* and *GSTT1* significantly increased the risk of HCC in Chinese [18].

CTLA-4 is expressed in activated T cells, and is the homologue of CD28 in structure [5]. *CTLA-4* can participate as a negative costimulatory molecule in the activation process of T cells and regulate immune responses of the body [19, 20]. *CTLA-4* polymorphisms can modify the function of *CTLA-4*, and are related to a variety of autoimmune diseases [21-23]. *CTLA-4* rs231775 polymorphism is derived from a Thr to Gly mutations in the signal peptide which may lead to subtle changes in subcellular location of *CTLA-4* mature proteins. In the other hand, the interaction between nascent peptide fragments and molecular chaperones was influenced by genetic variants of *CTLA-4*, which modified the function of *CTLA-4* [24, 25].

Many reports have shown that *CTLA-4* rs231775 polymorphism is correlated with the function of *CTLA-4* [26-28]. The G allele of *CTLA-4* +49G/A polymorphism is associated with the generation process of *CTLA-4* protein by endoplasmic reticulum. The increasing fre-

quency of G allele can reduce the generation and expression of glycosylated proteins in *CTLA-4* +49G/A polymorphism. As a result, the regulation of T cells proliferation is weakened, which will damage the inhibitory function of *CTLA-4* [29].

In present study, the result showed that the case group had an obviously higher frequencies of genotype AA and allele A than the controls, which implied that AA genotype and A allele of *CTLA-4* rs231775 polymorphism might be related to the increasing risk of HCC. In addition, this study also showed that AA genotype had an apparent association with the expression of *CTLA-4* in the serum in cases, suggesting that the *CTLA-4* rs231775 polymorphism might result in disorders of cellular immune function by affecting secreting of *CTLA-4*.

In conclusion, a predicted possible mechanism of *CTLA-4* rs231775 polymorphism for HCC susceptibility may be as follows: the replacement of Thr to Gly in the signal peptide on *CTLA-4* rs231775 polymorphism may change the expression level and function of *CTLA-4* and as a result, the polymorphism affects the individuals susceptibility for HCC. However, our study did not consider effects of the environmental factors on the pathogenesis of HCC and the sample size was relatively small, which may influence the reliability of our results. Therefore, further well-design research with large sample size was required to investigate the issue.

Disclosure of conflict of interest

None.

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