Original Article

Association of FOXP3 Gene Polymorphism with Chronic Hepatitis B in Chinese Population

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Objective The forkhead transcription factor FOXP3 is a key molecular which can mediate regulatory T cells immune-related inhibitory functions. Increased levels of FOXP3-positive Tregs in peripheral blood have been proved to be associated with a less favorable prognosis in various inflammatory diseases. It is of great interest to investigate the correlation between single nucleotide polymorphism (SNP) of FOXP3 gene and the susceptibility of chronic hepatitis B (CHB).

Methods Two SNPs rs2280883 and rs3761549 of FOXP3 gene in 285 patients with CHB and 295 matched controls were analyzed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF).

Results At rs2280883, there were no significant differences in the distribution of C and T alleles between CHB donors and healthy donors, but at rs3761549, C allele was associated with CHB ($P = 0.001$). Compared with healthy controls, patients with CHB had high frequencies of TT genotype (73.7%) at rs2280883 or CC genotype (73.6%) at rs3761549 of FOXP3 gene. Patients who carried rs2280883 CC genotype [OR 1.744 (95% CI 1.068 - 2.848), $P = 0.011$] or rs3761549 CC genotype [OR 1.633 (95% CI 1.146 - 2.327), $P = 0.0001$] had higher risk of suffering from CHB. Stratified analysis showed that rs3761549 CC genotype was significantly associated with high incidence of HBeAg ($P = 0.019$).

Conclusions These results suggested that FOXP3 gene polymorphism at rs2280883 and rs3761549 might be associated with the increased susceptibility to CHB.

Key words: Hepatitis B, chronic; Gene polymorphism; FOXP3

FOXP3, a forkhead transcription factor family member, is a specific molecular marker of regulatory T cells, which plays an important role in Tregs development and inhibitory function.¹⁻⁴ Increased levels of FOXP3-positive Tregs in peripheral blood and tumor have been reported in patients with various types of cancer, including ovarian,⁵⁻⁶ breast,⁷⁻⁹ hepatocellular carcinoma,¹⁰ and other tumors,¹¹ and the accumulation of Tregs in local lymph nodes or in tumors is associated with a less favorable prognosis.^{5-8,10-12} In China, the vast majority of liver cancer patients is hepatitis B virus carriers who suffered from liver cirrhosis at the terminal stage. Several studies have confirmed that when a number

of pathogenic microorganisms including hepatitis B infected the host, the number of Tregs would increase which could inhibit CD4⁺ T cells and CD8⁺ T cell activation and proliferation and cytokine secretion, thus affect the host's immune response against the occurrence of infection, and lead to chronicity.¹³⁻¹⁶ This phenomenon indicates that FOXP3 gene may play a role in tumor progression and inflammation.

It has been confirmed that the gene polymorphism of FOXP3 is related to the development of autoimmune diseases, such as allergic rhinitis,¹⁷ idiopathic infertility and endometriosis-related infertility.¹⁸ These results showed that polymorphism at rs2280883 of FOXP3 gene might be associated with idiopathic infertility, while polymorphisms at rs3761549 might be related to endometriosis. But whether the FOXP3 gene polymorphism is associated with CHB remains unclear.

According to FOXP3 gene SNP genotype data from the Hapmap phase II + phase III database, two tagSNPs, respectively rs2280883 and rs3761549 were selected for genotyping, which could cover 80% of MAF > 0.1 SNPs. To investigate the correlation between single nucleotide polymorphisms (SNPs)

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of FOXP3 gene and CHB, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF) was applied to acquire the data of FOXP3 gene polymorphism in 285 CHB donors and 295 healthy donors. Here we present data on the association between FOXP3 genetic variation and the susceptibility of CHB in all donors.

PATIENTS AND METHODS

Peripheral blood samples

The study included 285 patients with chronic hepatitis B (who had no evidence of cirrhosis and no antiviral therapy during the six months prior to blood sampling) from Peking University Hepatology Institute (Beijing, China). The peripheral blood samples were obtained from patients with chronic hepatitis B between November 2001 and April 2010. Patients' information such as age, gender, alcohol abuse, AST, ALT, GGT and TBil was recorded in detail in the database (Table 1). There were peripheral blood samples from 295 healthy donors who presented in Peking University People's Hospital. All these healthy donors were selected at random and without virus infection disease. This study was implemented after the approval of Medical Ethics Committee of Peking University People's Hospital. All samples were collected after the written informed consent was obtained from patients according to Declaration of Helsinki.

Biochemical tests

Fasting blood samples collected at each follow-up visit were tested for alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ -glutamyl transpeptidase (GGT) and total bilirubin (TBil) by an automated biochemical analyser 7600 (HITACHI, Japan). Anti-HCV, HBsAg, HBsAb, HBeAg, HBeAb and HBcAb were detected by enzyme immunoassay (Abbott) and anti-HIV by enzyme immunoassay (BioMérieux, Boxtel, Holland).

FOXP3 SNPs genotyping

According to FOXP3 gene SNP genotype data from the Hapmap phase II + phase III database, haplotyping was analyzed with restrictive standards including $r^2 > 0.8$, the minimum allele frequency (MAF) > 0.1 by Haploview 4.2 software. Finally, two tagSNPs, respectively rs2280883 and rs3761549, were selected for genotyping, which could cover 80% of MAF > 0.1 SNPs. DNA from the subjects was extracted from peripheral blood using standard methods. Genotyping was performed by MALDI-TOF for all donors. DNA from the donors was blind coded and tested using a 384 format SpectroCHIPTM microarray. PCR primers and single base extension primers for rs2280883 and rs3761549 sites were designed by Sequenom Assay Design 3.1 software, which were showed in Table 2. Matrix-assisted lasers desorption/ionization time-of-flight mass spectrometer was used for data acquisitions

Table 1. Demographic, clinical and biological characteristics of chronic hepatitis B patients and healthy donors

Characteristics in 2010		CHB donors n = 285	Healthy donors n = 295	P values
Age, n (%)	< 55 yr	187 (65.6%)	189 (64.1%)	0.697
	≥ 55 yr	98 (34.4%)	106 (35.9%)	
Gender, n (%)	Male	186 (65.3%)	174 (59.0%)	0.119
	Female	99 (34.7%)	121 (41.0%)	
Alcohol abuse, n (%)	Absent	30 (10.5%)	27 (9.2%)	0.578
	Present	255 (89.5%)	268 (90.8%)	
ALT ($\bar{x} \pm s$, U/L)		56.0 $\pm$ 30.8	24.0 $\pm$ 6.3	0.008
AST ($\bar{x} \pm s$, U/L)		43.2 $\pm$ 30.6	30.2 $\pm$ 6.4	0.012
GGT ($\bar{x} \pm s$, U/L)		35.9 $\pm$ 27.5	20.2 $\pm$ 14.5	0.011
TBil ($\bar{x} \pm s$, μ mol/L)		20.1 $\pm$ 5.3	13.4 $\pm$ 4.3	0.005

Notes: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ -glutamyl transpeptidase; TBil, total bilirubin.

Table 2. PCR primers and single base extension primers

SNP	PCR primers (5'-3')	Single base extension primers (5'-3')
rs2280883	F: ACGTTGGATGAGATGAAGGAGTTGGGATGG	GACAAGGAAAGGTTGGGAA
	R: ACGTTGGATGTGTCAATACACCCCCAACTG	
rs3761549	F: ACGTTGGATGACCCACAGGTTTCGTTCC	AGTTTCGTTCCGAGAACT
	R: ACGTTGGATGACATCACCTACCACATCCAC	

Notes: F, forward; R, reverse.

from the SpectroCHIPTM. Results were analyzed using Sequenoms MassARRAY RTTM software.

Statistical analysis

Genotype frequencies were obtained by direct counting, and statistical analysis was performed by the chi-square test. Qualitative data were also analyzed by the chi-square test and odds ratio (OR) with 95% confidence intervals (95% CI). *P*-values less than 0.05 were considered statistically significant. The statistical software package we used was SPSS 13.0 for Windows.

RESULTS

Analysis of FOXP3 SNP allele frequency in all donors

As shown in Table 3 and Table 4, there was no significant difference in the distribution of C and T alleles between CHB donors and healthy donors at

rs2280883 ($P = 0.186$). But at rs3761549, C allele frequency in CHB donors was significantly higher than that in healthy donors [OR 1.620 (95% CI 1.223 - 2.146), $P = 0.001$].

Association between FOXP3 genotype and the susceptibility to CHB

The distribution of FOXP3 genotypes at rs2280883 and rs3761549 in all donors was presented in Table 5 and Table 6. There were significant differences in FOXP3 genotypes between CHB donors and healthy donors at both rs2280883 and rs3761549. For rs2280883, CC genotype [OR 1.744 (95% CI 1.068 - 2.848), $P = 0.011$] was associated with higher risk of suffering from CHB than TT or CT variants, and for rs3761549, CC genotype [OR 1.633 (95% CI 1.146 - 2.327), $P = 0.0001$] was strongly associated with higher risk of suffering from CHB than TT or CT variants. This result showed that the FOXP3 genetic variants rs2280883 and

Table 3. Allele frequency at rs2280883 in all donors

Allele	CHB donors, n (%)	Healthy donors, n (%)	OR (95% CI)	<i>P</i> value
rs2280883				0.186
C	122 (21.4)	108 (18.3)	1.215 (0.910 - 1.623)	
T	448 (78.6)	482 (81.7)	0.823 (0.616 - 1.099)	

Table 4. Allele frequency at rs3761549 in all donors

Allele	CHB donors, n (%)	Healthy donors, n (%)	OR (95% CI)	<i>P</i> value
rs3761549				0.001
C	451 (79.4)	345 (70.4)	1.620 (1.223 - 2.146)	
T	117 (20.6)	145 (29.6)	0.617 (0.466 - 0.818)	

Table 5. Association between FOXP3 genotypes at rs2280883 and the susceptibility to CHB

Genotype	CHB donors, n (%) n = 285	Healthy donors, n (%) n = 295	OR (95% CI)	<i>P</i> value
rs2280883				0.011
CC	47 (16.5)	30 (10.2)	1.744 (1.068 - 2.848)	
TT	210 (73.7)	217 (73.6)	1.006 (0.696 - 1.456)	
CT	28 (9.8)	48 (16.3)	0.561 (0.341 - 0.922)	

Table 6. Association between FOXP3 genotypes at rs3761549 and the susceptibility to CHB

Genotype	CHB donors, n (%) n = 285	Healthy donors, n (%) n = 295	OR (95% CI)	<i>P</i> value
rs3761549				0.0001
CC	209 (73.6)	186 (63.1)	1.633 (1.146 - 2.327)	
TT	42 (14.8)	36 (12.2)	1.249 (0.774 - 2.015)	
CT	33 (11.6)	73 (24.7)	0.400 (0.255 - 0.626)	

Table 7. Association between FOXP3 genotypes at rs2280883/rs3761549 and HBeAg of CHB patients

Genotypes	HBeAg positive, n (%) n = 104	HBeAg negative, n (%) n = 181	OR (95% CI)	<i>P</i> values
rs2280883				0.584
CC	19 (18.3)	28 (15.5)	1.221 (0.644 - 2.317)	
TT	77 (74.0)	133 (73.5)	1.029 (0.595 - 1.782)	
CT	8 (7.7)	20 (11.0)	0.671 (0.284 - 1.582)	
rs3761549				0.019
CC	84 (80.8)	122 (67.8)	1.997 (1.119 - 3.564)	
TT	14 (13.5)	28 (15.6)	0.844 (0.423 - 1.688)	
CT	6 (5.8)	30 (16.7)	0.306 (0.123 - 0.763)	

rs3761549 were associated with the susceptibility to chronic hepatitis B.

Stratified analysis on the association between FOXP3 genotypes and clinical pathology of CHB

Since the FOXP3 genetic variants rs2280883 and rs3761549 were strongly associated with the susceptibility to chronic hepatitis B, further analysis was performed to find out the relationship between FOXP3 genotypes and important factor of clinical pathology of CHB, like HBeAg, which were shown in Table 7. At rs2280883, there were no significant differences in the distribution of three genotypes between HBeAg positive and negative CHB donors ($P = 0.584$), while at rs3761549, frequency of CC genotype was significantly higher in HBeAg positive CHB donors than in negative donors [OR 1.997 (95% CI 1.119 - 3.564), $P = 0.019$].

DISCUSSION

It is widely recognized that FOXP3 is a specific marker of CD4⁺CD25⁺FOXP3⁺ Tregs, which can regulate the development and immune suppression function of Tregs.²⁻³ Recently, it has been reported that FOXP3 gene expression was related to inflammatory diseases, such as hepatitis B,^{16,19} hepatitis C,²⁰⁻²¹ acute and chronic allograft rejection.²²⁻²³ Several studies have confirmed that the frequency of Tregs increased when a number of pathogenic microorganisms infected the host, which could inhibit the activation, proliferation and cytokine secretion of CD4⁺ T cells and CD8⁺ T cells, thus affect the host's immune response against the infection, and lead to chronicity.¹³⁻¹⁶ These results suggested that FOXP3 gene might be involved in inflammation.

It is well known that single nucleotide polymorphism within the human genome is most extensive genetic variation. To research SNPs in human genome is of great significance for the study of disease pathogenesis,

diagnosis, treatment and prevention.²⁴ So far, it has been reported that FOXP3 gene polymorphism is associated with a variety of immune-related diseases such as allergic rhinitis,¹⁷ idiopathic infertility and endometriosis-related infertility.¹⁸ These results showed that FOXP3 genetic variants rs2280883 and rs2232368 might be associated with idiopathic infertility, while rs3761549 might be related to endometriosis. But there are no relevant reports on the relationship between FOXP3 gene polymorphism and chronic hepatitis B.

In order to clarify whether the FOXP3 gene polymorphism is associated with chronic hepatitis B, MALDI-TOF was applied to analyze FOXP3 gene polymorphism in CHB donors and healthy donors. The results showed that there were significant differences in FOXP3 genotypes between CHB donors and healthy donors at both rs2280883 and rs3761549. For rs2280883, CC genotype was strongly associated with higher risk of suffering from HCC than TT or CT variants; while for rs3761549, CC genotype was strongly associated with higher risk of suffering from HCC than TT or CT variants. This result showed that the FOXP3 genetic variants rs2280883 and rs3761549 were also associated with the susceptibility to CHB as well as allergic rhinitis and idiopathic infertility.

Since the FOXP3 genetic variants rs2280883 and rs3761549 were strongly associated with the susceptibility to CHB, further analysis was performed to find out the relationship between FOXP3 genotypes and important factor of clinical pathology of CHB, like HBeAg, which were shown in Table 7. Genotypes at rs2280883 were not related to HBeAg, but at rs3761549, CC genotype was significantly associated with HBeAg in CHB donors. These results indicated that genotype at rs3761549 could be a prognostic factor for HBeAg positive CHB occurred within the process of CHB.

Overall, our study showed that FOXP3 gene polymorphism is related to CHB. Polymorphism at rs2280883 and rs3761549 of FOXP3 gene might be

associated with the increased susceptibility to CHB. Therefore, in-depth study should be carried out with more samples and broader population to explain the molecular mechanism of FOXP3 gene in the development of CHB.

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