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Research Article

Correlation of Foxp3 Gene Expression with Serum Alanine Transaminase (ALT) Levels and Hepatitis B Viral Load in Cirrhosis and Hepatocellular Carcinoma (HCC) Patients

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Abstract

Background: Foxp3 T_{reg} can inhibit activation, proliferation and effector functions of several kinds of other immune cells, including CD4+ and CD8+ T cells, natural killer (NK) and NKT cells and dendritic cells (DC). As such, Foxp3 T_{reg} cells are widely considered to be the principal mediator of dominant self-tolerance immune homeostasis. In HBV infection, Foxp3 T_{reg}cells have been associated with the incidence and extent of liver damage. It has been recognized that higher ALT levels was a risk predictor for more active immune response against HBV and more extensive hepatocyte damage. The extent of hepatocyte necrosis and the degree of ALT elevation do not always correlate; HBV DNA could be a important factor than ALT level for the subsequent development of cirrhosis and HCC. This study was designed to assess the correlation of Foxp3 gene expression with HBV-DNA and serum Alanine transaminase (ALT) in patients with cirrhosis and HCC.

Methods: The study was conducted among 60 patients. The study population were divided into four groups (15 in each groups)-HBV positive cirrhosis, HBV negative cirrhosis, HBV positive HCC and HBV negative HCC. Expression of Foxp3 gene was observed using real time PCR. Foxp3 gene expressions in the above mentioned groups were correlated with serum ALT level and HBV viral load.

Result: Foxp3 gene was significantly higher in HBV-positive patients with HCC than HBV-positive cirrhosis. Similarly, the expression of Foxp3 was significantly higher in HBV-positive HCC than HBV-negative HCC patients. However, the expression of Foxp3 was increased in HBV-positive cirrhosis in comparison with HBV-negative cirrhosis. Foxp3 gene expression in liver was not correlated with the serum levels of ALT in any of the study groups. HBV- DNA load also did not correlated with Foxp3 gene expression in HBV positive HCC and HBV positive cirrhosis patients.

Conclusion: This study shows that there was no significant change with the expression of Foxp3 gene in any of the study groups with ALT level or viral load, though differential expression of Foxp3 gene were observed in cirrhosis and HCC patients.

Keywords: Foxp3; ALT; HBV-DNA; Liver Cirrhosis; Hepatocellular Carcinoma

Introduction

Hepatocellular carcinoma (HCC), which consists predominantly of primary liver cancer, is the fifth most common malignancy in men and the eighth one in women worldwide. The number of new cases of HCC is about 564 000 per year [1]. Cirrhosis and virus infection, such as hepatitis B virus (HBV) are the major known risk factors for HCC [2,3]. HCC has a poor prognosis and a low survival rate in the majority of patients [4]. To improve the treatment of HCC will require a better understanding of the biological development and molecular events in the immune system of HCC.

Forkhead box protein 3 (Foxp3) is a member of the forkhead/winged-helix family of transcriptional regulators and is highly conserved in normal cells. The full-length protein contains 431 amino acids. Foxp3 is considered to be an important gene of thymically derived and naturally occurring regulatory T cells (Tregs) [5]. Mutations in human Foxp3 are associated with immune diseases, such as multi-organ autoimmune disorder, immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX) [6], in which T_{regs} from affected patients are greatly reduced in number and suppressive activity [7-9]. A high prevalence of T_{regs} is thought to be an unfavorable prognostic indicator for HCC [10].

Foxp3 Treg can inhibit activation, proliferation and effector functions of several kinds of other immune cells, including CD4+ and CD8+ T cells, natural killer (NK) and NKT cells and dendritic cells (DC). As such, Foxp3 T_{regs} cells are widely considered to be the principal mediator of dominant self-tolerance immune homeostasis7. In HBV infection, Foxp3 T_{regs} cells have been associated with the incidence and extent of liver damage [11]. As increase level of ALT indicates the liver injury (cirrhosis) and increase HBV-DNA indicates the replication status of hepatits B virus during HCC, the

purpose of this study was to assess the correlation of Foxp3 gene expression with HBV-DNA and ALT in patients with cirrhosis and HCC.

Materials and Methods

Patient selection and Sample collection:

The study participants were divided into four groups (15 in each groups), namely HBV positive cirrhosis and HBV positive HCC as disease groups and HBV negative cirrhosis and HBV negative HCC as control groups. On the basis of Clinically (ascitis, jaundice, oedema, palmer erythema and varicial bleeding), biochemically (HbsAg status, Anti HBc total, serum ALT) and sonographycally diagnosed Cirrhosis and histopathologically diagnosed HCC patients were selected as patients groups. Control groups were recruited in this study who were HBsAg and Anti HBc (total) negative but had cirrhosis and HCC. After obtaining informed written consent, ultrasound guided Fine Needle Aspiration Cytology (FNAC) was performed at the Department of Hepatology of BSMMU by trained Hepatologists. The FNAC samples were collected in RB (RNA binding) buffer solution for extraction of RNA using Total RNA mini kit (Geneaid, Taipe, Taiwan). All the samples were transported under appropriate conditions to the Molecular Laboratory of the Department of Virology, BSMMU, for further procedures. Five ml of blood was collected from each patient and kept at -700C for ALT assay and HBV-DNA quantitation. ALT measurement and HBV-DNA quantitation were performed at the Department of Biochemistry and Virology respectively.

Serum ALT and HBV-DNA measurement

The levels of serum ALT were measured biochemically using an automated analyzer (ALTI Flex® reagent cartridge,) by Dimension EXL USA, at the Department of Bio-chemistry, BSMMU.

Five µl of serum were used to detect HBV-DNA of each patient. At first 5 µl lysis buffer was added to PCR tube, then patient's serum was added. After 10 minutes of incubation, 40 µl of master mix was added in the PCR tube. Finally total 50 µl of reaction volume was used for detection of HBV-DNA. PCR was performed with initial denaturation at 95˚c for 15 min, followed by denaturation at 95˚c for 15 sec, annealing at 57˚c for 30 sec and lastly, extention at 72˚C for 30 sec. Fluorescence signal were measured during 45 PCR cycles. Serum HBV DNA was measured using Single step Hepatitis B Viral DNA Quantitative Fluorescence Diagnostic Kit (Genebio HBV DNA quantitation Kit, USA) at the Department of Virology, BSMMU.

RNA extraction and real-time PCR

Total 200 µl RNA was extracted from FNAC sample using Total RNA mini kit (tissue) (Geneaid, Taipe, Taiwan) according to the manufacturer's instructions. The complementary deoxyribonucleic acid (cDNA) was synthesized using cDNA synthesis Kit (Solis-biodyne, Tartu, Estonia). A total of 5µl of cDNA were used for real-time Polymerase chain reaction (PCR). The selected gene expression was analyzed using Step One PCR (Applied Biosystem, USA) using 5 x HOT FIREPol Evagreen® qPCR Mixplus (ROX) (Solis-Biodyne, Tartu, Estonia). The primer sequences were used. The following primers were used (5'-3') Foxp3 sense: CACAACATGCGACCCCCTTTCACC; Foxp3 antisense: AGGTTGTGGCGGATGGCGTTCTTC; GAPDH sense: 5´-ATCCCATCACCATCTTCCAG-3´; GAPDH antisense: 5´-ATGAGTCCTTCCACGATACC-3´. Glyceral dehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control to normalize the PCR reaction of selected genes. PCR was performed with initial denaturation at 95˚c for 15 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for GAPDH, 60˚C for Foxp3 for 30 sec, and lastly, extention at 72˚C for 30 sec. Fluorescent signal reporter generated in qPCR were measured.

Statistical analysis

Comparisons between HBV positive cirrhosis and HCC and HBV positive HCC and HBV negative HCC were performed using Wilcoxon Rank Sum (Mann-Whitney) test. Two-sample t-test was performed between HBV positive cirrhosis and HBV negative cirrhosis. The correlation of Foxp3 gene expression with the ALT level and the viral load were analyzed using the Pearson correlation (r) test. Data were analysed with Statistical Program for Social Science (SPSS) version 16.0 software (SPAA Inc, USA). p value <0.05 was considered statistically significant and p value <0.001 as highly significant.

Results

A total of 60 patients were included in this study, of whom 49 (81.66%) were males and 11 (18.33%) were females. The mean ± SD of ALT levels of HBV positive HCC, HBV negative HCC, HBV positive cirrhosis and HBV negative cirrhosis were 57.20 ± 19.93 IU/L, 52.27 ± 13.13 IU/L, 62.07 ± 45 IU/L and 48 ± 12.0 IU/L respectively. The mean viral load of HBV positive HCC was 4.02 ± 1.37 and HBV positive cirrhosis was 3.52 ± 1.53 [$\log_{10}$(copies/ml)], and this difference was statistically significant (p value <0.01).The most common complaints among patients were ascitis (65%), palmer erythema 75%, oedema 66.6% and variceal bleeding in 50% in HBV positive cirrhosis.

Comparison between HBV positive HCC and HBV negative HCC showed that Foxp3 (P<0.001) was significantly increased in HBV positive HCC (Table 2). The up regulation of Foxp3 expression was 14.48 fold in HBV positive HCC patients when compared with HBV negative HCC patients 1.00 fold (not shown in Table 2). Foxp3 was significantly increased (P <0.001) in HBV positive HCC patients than HBV positive cirrhosis patients when compared between these two groups (Table 3). The up regulation of Foxp3 expression were 6.32 fold in HBV positive HCC when compared with HBV positive cirrhosis where these was 1.00 fold (not shown in Table 3).

However, comparison between HBV positive cirrhosis and HBV negative cirrhosis showed that Foxp3 (P=0.0004) was significantly increased in HBV positive cirrhosis (Figure 1). The up regulation of Foxp3 gene expression was 3.81 fold in HBV positive cirrhosis when compared with HBV negative cirrhosis which was 1.00 fold (Figure 1).

Foxp3 gene expression in hepatocytes was positively correlated with the serum levels of ALT (r=-0.022, p>0.05) in HBV positive HCC patients and HBV negative cirrhosis (r= .235, p>0.05) but negatively correlated with HBV negative HCC and HBV positive cirrhosis (r= -.263, p>0.05; r=-.069, p>0.05). patients. A negative relationship was observed between Foxp3 gene expression and

Variables	Catagories	HBV positive HCC	HBV negative HCC	HBV positive Cirrhosis	HBV negative Cirrhosis
Age	mean ± SD	52.40 ± 9.2	48.27 ± 12.4	49.40 ± 7.4	45.40 ± 12.2
Sex	Male (n=49) %	12(80)	13(87)	13(87)	11(74)
	Female (n=11) %	3(20)	2(13)	2(13)	4(26)
ALT	mean ± SD IU/L <40 (n=12) >40 (n=44)	 31.25 ± 4.74 66.63 ± 13.23	 36.75 ± 3.15 73.45 ± 46.77	 36.50 ± 2.73 58.00 ± 9.87	 33.00 ± 4.53 56.00 ± 7.7
HBV DNA*	mean ± SD $Log_{10\ copies/ml}$	4.02 ± 1.37	-	3.52 ± 1.53	-
Clinical features**	Ascities (n=20)	2(10)	1(5)	13(65)	4(20)
	Jaundice (n=18)	7(39)	5(28)	3(17)	3917)
	Oedema (n=12)	-	-	8(66.6)	4(33.3)
	Palmer erythema (n=4)	-	-	3(75)	1(25)
	Varicial bleeding (n=16)	2(12.5)	-	8(50)	6(37.5)

Table 1: Comparison of variables among study groups.

The number within parentheses indicates percentage. * p<0.05 is significant. ** some had combined feature

Gene	Group	Sample no	Rank-sum	expected	P-value
Foxp3	HBV positive HCC	15	345	232.5	P<0.001
	HBV negative HCC	15	120	232.5	

Table 2: Relative expression of the examined genes among HBV positive HCC and HBV negative HCC.

Two-sample Wilcoxon rank-sum (Mann-whitney) test.

Gene	Group	Sample no	Rank-sum	expected	P-value
Foxp3	HBV positive HCC	15	336	232.5	P<0.001
	HBV positive Cirrhosis	15	129	232.5	

Table 3: Relative expression of the studied genes among HBV positive HCC and cirrhosis.

Two-sample Wilcoxon rank-sum (Mann-whitney) test.

Variables	Catagories	HBV positive HCC(n=15)	HBV negative HCC(n=15)	HBV positive Cirrhosis(n=15)	HBV negative Cirrhosis(n=15)
Foxp3	Foxp3 with ALT levels	.022	-.263	-.069	.235
	p value	.950	.435	.840	.486
	Foxp3 with HBV-DNA	-.387		-.263	
	p value*	.268		.409	

Table 4: Correlation of expression of Foxp3 gene in liver with serum ALT levels and HBV-DNA in different groups.

*Pearson correlation test were done where p value, p<0.05 was considered to be significant.

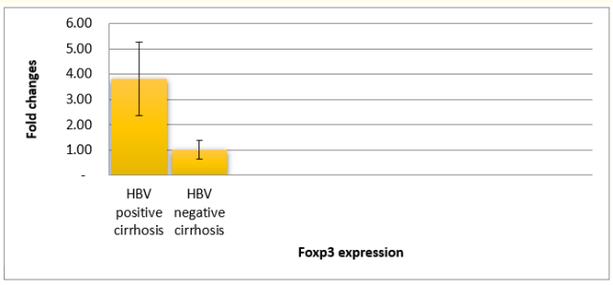

Figure 1: Comparison Foxp3 expression among HBV positive cirrhosis and HBV negative cirrhosis.

Discussion

In this study, Foxp3 genes were selected to elucidate differences in gene expression which may be useful to understand the molecular pathogenesis and develop specific markers at different stages of viral hepatitis B, especially cirrhosis and HCC. Although the factors and molecular events associated with the progression of HCC are complex and not established, Foxp3 has been shown to play an important role in T_{reg} in HCC invation [1,10,12].

A specific molecular marker of regulatory T cells, Foxp3, exerts great influence on the development and function of regulatory T_{reg} cells. The increased levels of regulatory T_{reg} cells, are also associated with increased HBV-DNA levels, and depletion of regulatory T_{reg} cells results in increased HBV-specific CD4+ and CD8+ T-cell proliferation and IFN production.

In the present study, Foxp3 gene expression was higher in HBV positive HCC than HBV negative HCC. The findings of higher number of T_{reg} cells in patients with HCC have implication both in the pathogenesis of tumor and in the design of immunotherapy against it. The elimination of CD+CD25+ T cells may enhance tumor immunity when combined with the current attempts to augment immunogenicity of tumor cells [13].

Previous study shows that, there was significant association between Foxp3 expression and HBsAg concentration, but not ALT. These results suggested that Foxp3 was involved in the persistent presence of HBV in CHB patients, but not directly involved in liver injury [14].

Conclusion

The present study concluded that there were variations in the expression of Foxp3 genes among cirrhosis and HCC patients with or without HBV. Foxp3 gene was more upregulated in HBV positive HCC patients. These particular gene may be responsible for the molecular pathogenesis and clinical outcome of HBV positive cirrhosis and HCC patients. Thus, the Foxp3 genes may be used as a marker for cirrhosis and HCC. However, these aspects need to be evaluated further by studies among larger numbers of cirrhosis and HCC patients.

The present study has some limitations. The sample size of cirrhotic and HCC subjects was smaller, stages of liver damage were not biopsy proven and the serum level of Foxp3 gene were not measured. However, the study result raises the possibility of undertaking further studies involving larger cohorts of cirrhotics and HCC of both HBV and non viral etiology.

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