

ORIGINAL**Influence of single nucleotide polymorphisms of cytokine genes on anti-HBs antibody production after hepatitis B vaccination in a Japanese young adult population**Nobuyasu YUKIMASA^{a)}, Shoichi SATO^{b)}, Wataru OBOSHI^{c)}, Toru WATANABE^{a)}, and Ryuichi UZAWA^{d)}^{a)}Graduate School of Health Sciences, Kagawa Prefectural University of Health Sciences, Takamatsu, Japan, ^{b)}Clinical Laboratory, Chiba Emergency Medical Center, ^{c)}Department of Medical Technology, Kagawa Prefectural University of Health Sciences, ^{d)}Uzawa Clinic

Abstract : Hepatitis B (HB) vaccination is one of the most efficient tools to prevent the transmission of the virus. Considerable variability exists in HB vaccine responses, with 5-10% of healthy Japanese adults demonstrating no response following a standard vaccination. Recently, polymorphisms of immune-regulatory genes, such as cytokine genes, have been reported to influence the immune response to HB vaccine. The aim of this study was to investigate the underlying mechanisms of the genetic association between several cytokine gene polymorphisms and the immune response to HB vaccination in a Japanese population. One hundred and twenty three vaccinated young adults were classified according to the level of antibody-titer (anti-HBs). Single nucleotide polymorphism typing for IFN- γ (+874, 3'-UTR), IL-10 (-591, -819, -1082), and TNF- α (-308, -857), was accomplished using the PCR-RFLP or SSP-PCR method. The TNF- α (-857) CC type and the IL-10 (-1082) AG type were present more frequently in the low titer group than in the high titer group. The TNF- α (-857) CC type was found to be significantly associated with low response of serum anti-HBs. The anti-HBs antibody was not readily produced in the IL-10 (-1082) AG and TNF- α (-857) CC haplotype. Conversely, the antibody was readily produced in the IL-10 (-1082) AA and TNF- α (-857) CC haplotype, and the IL-10 (-1082) AA and TNF- α (-857) CT haplotype, suggesting a high likelihood of the IL-10 (-1082) AG type to be included in the low anti-HBs group, and high anti-HBs antibody production in those with the TNF- α (-857) CT type. These SNPs may produce ethnically-specific differences in the immune response to HB vaccine in the Japanese population. *J. Med. Invest.* 63 : 256-261, August, 2016

Keywords : hepatitis B vaccination, anti-HBs titers, cytokine gene polymorphisms, interleukin (IL)-10, tumor necrosis factor (TNF)- α

INTRODUCTION

The hepatitis B virus (HBV) affects approximately 350 million people worldwide and causes a broad range of pathologies, including chronic hepatitis B, hepatic cirrhosis, and hepatocellular carcinoma (1). The HB vaccine, using recombinant HBV surface protein antigen (HBsAg), is administered as a preventative measure to health care workers with a high occupational risk of infection (2, 3). However, individual differences in the immune response capability to HB vaccination in healthy adults has been reported, with no immunological response, resulting in insufficient production of neutralizing antibody, found in 5-10% of those vaccinated (4, 5). Both exogenous and endogenous factors have been reported to underlie this phenomenon (6, 7). Exogenous factors include : stress, nutritional condition, cigarette smoking, and other infections ; and endogenous factors include : sex, age, obesity, and genetic factors. Indeed, investigation of HB vaccine-specific immune response in twins revealed that genetic factors accounted for approximately 60% of the immune response to the vaccine (8). The influence of the human leukocyte antigen (HLA) type, involved in antigen presentation, has been identified as a major genetic factor, and many studies have reported the association between HLA type and immune response to HB vaccine (4, 5, 9, 10). Cytokine gene polymorphisms are also strongly suspected to influence immune response to the

vaccine due to their immunoregulatory functions (8). Indeed, HLA type and cytokine gene polymorphisms have been identified as independent factors (11). Cytokines function in various immune responses, including antigen recognition, processing, and presentation, antibody production and class switch, and regulation of cytokine interaction. The association of interleukin (IL)-1 β , IL-10, tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β , IL-13, and interferon (IFN)- γ gene polymorphisms with immunological unresponsiveness to HB vaccination have been reported mainly in western subjects (12-16).

A consensus has not yet been reached regarding these findings, and there has been no study on genetic factors involved in the immune response to HB vaccine in Japanese subjects. Therefore, the aim of the present study, we investigated the association between anti-HBs antibody production after HB vaccination and single nucleotide polymorphisms (SNPs) of putative cytokine genes in Japanese subjects. Since our preliminary study was not suggested this association regarding SNPs in IL-1 β , IL-13, and TGF- β , we focused on putative effect of cytokine genes on antibody production, TNF- α , IL-10, and IFN- γ .

MATERIALS AND METHODS

The subjects were selected to minimize the influence of age, and thus elucidate genetic factors of HB vaccine immune response in a Japanese population. The subjects were 123 healthy, non-obese, non-smoking, fourth-year student volunteers of the Laboratory of Science, Saitama Prefectural University School of Health, and the Department of Medical Technology, Kagawa Prefectural University

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of Health Sciences (26 males and 97 females ; age from 21 to 24 years old ; body mass index : BMI < 25.0 kg/m²). The objective of the study was explained to participants, and consent for study cooperation was obtained. This study was approved by the Ethics Committees of Saitama Prefectural University and Kagawa Prefectural University of Health Sciences, and written consent was obtained from all participating subjects in accordance with the Declaration of Helsinki.

The primary objective of the HB vaccination for the subjects was to prepare them for clinical practice. They received the first, second, and third administrations of yeast-derived recombinant HB vaccine (Bimmugen ; The Chemo-Sero-Therapeutic Research Inst.) in May, June, and November of their third year in school, respectively. Blood samples for evaluation of the anti-HBs antibody level were collected in May of their fourth year ; thus, the antibody level was measured approximately 6 months after the third vaccination. Samples were collected in blood sampling tubes with EDTA-2Na, plasma was separated by centrifugation, and plasma HBs antibody was quantified by chemiluminescence enzyme immunoassay (CLEIA ; Fuji Rebio Inc.). When the antibody level reached 1,000 mIU/ml or higher, the upper limit of the sample was determined using an enzyme linked immunosorbent assay (ELISA ; Siemens Healthcare Japan Co., Ltd.). Genomic DNA was extracted from buffy-coat white blood cells using a DNA extraction kit (QIAamp DNA Blood mini kit ; QIAGEN Co., Ltd.).

SNPs of cytokine genes were detected by sequence-specific primer (SSP)-polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism (RFLP) analysis. The target cytokine gene SNPs, PCR primer base sequences, and detection method are shown in Table 1. The PCR reaction consisted of 100 ng of genomic DNA as a template, 1×PCR buffer (1.5 mM MgCl₂), 200 μM dNTPs, 0.8 μM of each primer, 1U *TaKaRa Ex Taq* DNA polymerase (TaKaRa Bio Inc.), and molecular grade water to a final volume of 25 μl. All cycling conditions of PCR were as follows : an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec denaturation, 55-64°C for 30 sec of primer annealing, 72°C for 30 sec extension, and a final extension step of 7 min at 72°C in the last cycle. Amplified PCR products or restriction fragment patterns were detected by 5% polyacrylamide gel electrophoresis.

Subjects were divided based on their anti-HBs antibody level into low (< 10 mIU/ml), middle (10-100 mIU/ml), and high (> 100

mIU/ml) groups, and the detection frequencies of the SNP types of the target genes were compared among the groups. The frequencies of SNP genotypes and independence of each antibody level group were analyzed using Fisher's exact test. The Hardy-Weinberg equilibrium (HWE) test for assessing the SNP genotype frequency among subjects was conducted using a HWE calculator including an analysis for ascertainment bias software (17). To confirm the individual factors, standardized residual analysis of SNP genotypes was performed among three antibody level groups. Results were expressed as the median values of anti-HBs antibody. The anti-HBs antibody levels were normalized by logarithmic transformation, differences among polymorphic groups were compared using Kruskal-Wallis tests, and Mann-Whitney tests were used to analyze differences between two polymorphic subgroups. Multiple regression analyses were performed in order to ascertain involvement in antibody production. StatFlex ver.6 (ArTec Co., Ltd.) software was used to perform statistical analyses. *P* values (significance probability) < 0.05 were considered as significant. Linkage disequilibrium (LD) statistics were calculated using Haploview 4.2 (18).

RESULTS

Investigation of the influence of sex revealed that antibody levels tended to be lower in male subjects (mean values of anti-HBs : male ; 405.9±113.2(SE) mIU/ml, female ; 479.9±175.1(SE) mIU/ml), but were not significantly different (*P*=0.11). Differences in the genotype frequency and antibody level were observed in IL-10 (-1082 ; rs1800629) and TNF-α (-857 ; rs1799724) (*P*< 0.05). In the other gene SNP types, a small, but non-significant, difference in frequency was noted in IL-10 (-819 ; rs1800871) (*P*=0.055). All target SNP genotypes met the Hardy-Weinberg equilibrium principle [Table 2]. When associations were investigated using standardized residual analysis, a significantly lower frequency was observed with the IL-10 (-1082) AA type (*P*< 0.01), and significantly higher frequency was observed with the IL-10 (-1082) AG type (*P*< 0.05) in low antibody level group. A significantly higher frequency was observed with the IL-10 (-819) CT type (*P*< 0.05) in low antibody level group. In addition, a significantly higher frequency was observed with the TNF-α (-857) CC type (*P*< 0.05) in low and middle antibody level groups.

Table 1 Target cytokine gene SNPs, PCR primers and detection methods.

genes	position	rs	primers	method (enzyme)
IL-10	-1082	1800896	Sense(A) : 5'-actactaaggcttcttgggaa-3' Sense(G) : 5'-ctactaaggcttcttgggag-3' Antisense : 5'-cagtgccaactgagaatttgg-3'	SSP-PCR
IL-10	-819	1800871	Sense : 5'-tcaacttctccaccatc-3' Antisense : 5'-gtgctcaccatgaccctac-3'	PCR-RFLP (<i>Msi</i> I)
IL-10	-592	1800872	Sense : 5'-ggtgagcactacctgactagc-3' Antisense : 5'-cctaggtcacagtgcactgg-3'	PCR-RFLP (<i>Rsa</i> I)
TNF-α	-857	1799724	Sense : 5'-aagtcgagatggggaccgccgtaaa-3' Antisense : 5'-ccccagtggtggccatactctt-3'	PCR-RFLP (<i>Hinc</i> II)
TNF-α	-308	1800629	Sense : 5'-aggcaataggtttgagggccat-3' Antisense : 5'-ggcgggatttggaaagt-3'	PCR-RFLP (<i>Nco</i> I)
IFN-γ	+874	2430561	Sense(A) : 5'-ttctacaacacaaatcaaatca-3' Sense(T) : 5'-ttctacaacacaaatcaaatct-3' Antisense : 5'-tcaacaaagctgactactcca-3'	SSP-PCR
IFN-γ	3'-UTR	2069727	Sense(C) : 5'-gggagagaggaagattctgaaac-3' Sense(T) : 5'-gggagaggaagattctgaaat-3' Antisense : 5'-tttggcttctgccttt-3'	SSP-PCR

UTR : untranslated region, SSP-PCR : sequence-specific primer-polymerase chain reaction, RFLP : restriction fragment length polymorphism

Table 2 Association of IL-10, TNF- α , and IFN- γ SNPs genotype frequencies in vaccinated students with outcomes of anti-HBs titer groups, and the Hardy-Weinberg equilibrium (HWE) test.

		Anti-HBsAb (mIU/ml)			P value	HWE P value
		Low-responder	Middle-responder	High-responder		
		< 10.0 n=26	10-100 n=35	> 100 n=62		
IL-10 (-1082) : rs1800896 ancestral allele : A	AA	14	30	49	0.016	0.123
	AG	12	5	13		
	GG	0	0	0		
IL-10 (-819) : rs180087 ancestral allele : C	CC	2	1	10	0.055	0.347
	CA	18	17	26		
	AA	6	17	26		
IL-10 (-592) : rs1800872 ancestral allele : C	CC	2	1	8	0.141	0.110
	CA	18	17	29		
	AA	6	17	25		
TNF- α (-857) : rs1799724 ancestral allele : C	CC	22	28	37	0.013	0.057
	CT	4	6	26		
	TT	0	0	0		
IFN- γ (3'-UTR) : 2069727 ancestral allele : A	AA	20	25	43	0.836	0.066
	AG	6	10	19		
	GG	0	0	0		
IFN- γ (+874) : rs2430561 ancestral allele : A	AA	21	28	50	0.780	0.728
	AT	5	6	12		
	TT	0	1	0		

Significant associations ($P < 0.05$) are in **bold**.

The median anti-HBs antibody level was compared among the SNP types of each cytokine gene, and was found to be lower in the IL-10 (-1082) AG than AA type, but the difference was not significant [Figure 1-a]. In the IL-10 (-819) CT type, a significant difference in the mean antibody level was observed compared with those in the other types (CC/TT) ($P < 0.05$) [Figure 1-b]. The anti-HBs antibody production level was significantly lower in the TNF- α (-857) CC than CT type ($P < 0.05$) [Figure 1-c]. The TNF- α (-308) GA type was detected in only 2 subjects in the present study ; however, their anti-HBs antibody levels of 1.1 and 55.9 mIU/ml were lower than the mean level (465.0 mIU/ml) of the GG type. No other

investigated SNP type influenced the anti-HBs antibody production level (data not shown).

Based on multiple regression analyses with the low antibody level group (< 10 mIU/ml), the anti-HBs antibody was not readily produced in the IL-10 (-1082) AG and TNF- α (-857) CC haplotype ($P < 0.05$). Conversely, the antibody was readily produced in the IL-10 (-1082) AA and TNF- α (-857) CC haplotype, and the IL-10 (-1082) AA and TNF- α (-857) CT haplotype ($P < 0.05$) [Figure 2]. Table 3 shows multiple regression analysis data for the IL-10 (-1082) AG type ($P < 0.05$) and TNF- α (-857) CT type ($P < 0.01$).

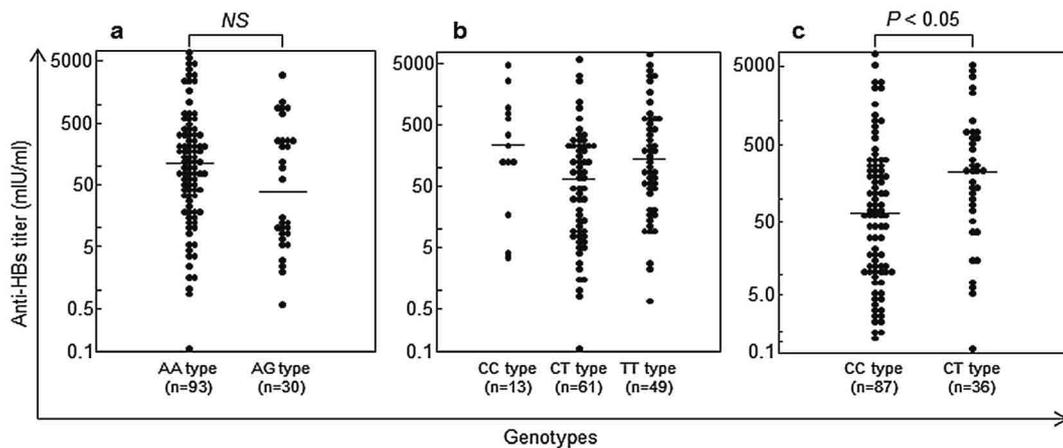


Figure 1. Distribution of anti-HBs antibody titers. a : Distribution of anti-HBs antibody titers in IL-10 (-1082) AA and AG types(Mann-Whitney test). b : Distribution of anti-HBs antibody titers in IL-10 (-819) CC, CT, and TT types(Kruskal-Wallis test ; $P < 0.05$). c : Distribution of anti-HBs antibody titers in TNF- α (-857) CC and CT types(Mann-Whitney test). In all the figures, central horizontal lines show the median. NS : non-significant.

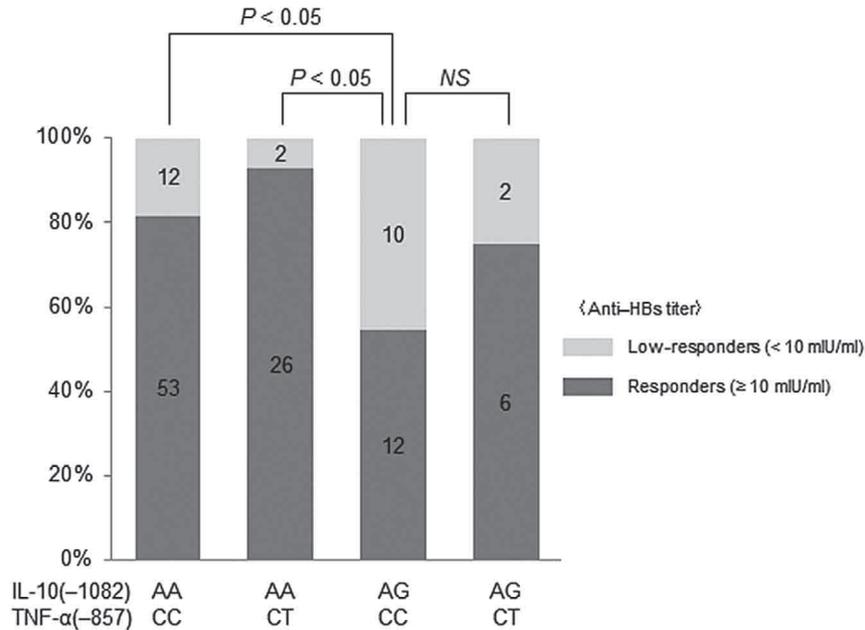


Figure 2. The ratio of low-responders and responders in IL-10(-1082) and TNF-α (-857) genotypes. NS : non-significant. (Multiple regression analysis)

Table 3 Results of multiple regression analysis for the IL-10 (-1082) AG and TNF-α (-857) CT types.

	β	SE(β)	T value	P value
Intercept	2.26	0.09		
IL-10(-1082) AG type	-0.33	0.16	2.04	0.043
TNF-α(-857) CT type	0.40	0.15	2.63	0.010

β : Standardized partial regression coefficient (β-weight).

SE(β) : Standard error of the β-weight. T value : significance of the β-weight.

Significant associations (P < 0.05) are in **bold**.

Haplotype analysis of IL-10 SNPs showed the linkage disequilibrium between IL-10 (-819) and IL-10 (-592) had values of $D' = 0.926$, $r^2 = 0.841$, and $LOD = 34.7$, whereas IL-10 (-1082) and IL-10 (-819) had values of $D' = 0.349$, $r^2 = 0.031$, $LOD = 0.61$, and IL-10 (-1082) and IL-10 (-592) had values of $D' = 0.448$, $r^2 = 0.053$, $LOD = 1.05$.

DISCUSSION

It has been reported that the functions of type 1 (Th1) and 2 (Th2) helper T cells cytokines were altered in HB vaccine non-responders (19). Hyodo *et al.* (20) reported that stimulation using HBV core antigen increases production of the Th2-related cytokine, IL-10, and is involved in persistent HBV infection. Miyazoe *et al.* (21) reported that the IL-10 (-592) CC type is involved in elimination of HBV. Furthermore, Rahman *et al.* (22) reported that HBs antigen-induced IFN-γ production tended to be low in peripheral blood mononuclear cells in HB vaccine non-responders. In a study on cytokine-dependent antiviral action of CD4-positive T cells in the treatment of chronic hepatitis B patients with HB vaccine, HBV-DNA was significantly decreased in patients with high levels of IFN-γ and TNF-α production. These findings reveal that HB vaccine does not induce CD8-positive T cell proliferation, i.e., HB vaccine induces IFN-γ and TNF-α production by stimulation of Th1, and exhibits antiviral action by reducing HBV-DNA by

antibody production (23).

It has been reported that IFN-γ production by peripheral blood mononuclear cells in response to HBs antigen stimulation was decreased in HB vaccine non-responders (22). We investigated IFN-γ SNPs rs2430561 (+874) and rs2069727 (3'-untranslated region ; UTR), which are likely to influence IFN-γ production level and has been suggested to be involved in the 3' transcriptional regulatory region of the IFN-γ gene in the immune response to HB vaccine (16) ; however, no involvement of these SNPs in the anti-HBs antibody production level was observed. The IFN-γ signal may influence the immune response to HB vaccine by the previous our study (24), thus, it is necessary to investigate factors that may influence anti-HBs antibody production by regulating IFN-γ production.

SNPs present in the IL-10 gene promoter region have been suggested to be involved in the immune response to HB vaccine, and a study on the association between the IL-10 SNPs and response to HB vaccine suggested involvement of IL-10 (-1082) (13). We also observed a similar tendency ; the frequency of the IL-10 (-1082) AG type was significantly higher in the low antibody level group, suggesting its association with the level of immune response to the vaccine. Höhler *et al.* (13) reported that the Ets-binding sequence is present in the IL-10 (-1082) A/G region, and that the major allele, A, has a stronger binding ability to the inhibitory transcription regulator PU.1 compared with that of the minor allele, G, resulting in inhibition of IL-10 expression. They also reported that

the ACC haplotype, comprised of 3 sites in the IL-10 promoter region (-1082/-819/-592), decreases IL-10 gene transcription activity and interferes with IFN- γ inhibition by IL-10, promoting anti-HBs antibody production. We evaluated the value of D' , r^2 , and LOD of the haplotypes. The results of linkage disequilibrium analysis in Japanese subjects reveal IL-10 (-819) was in strong linkage disequilibrium with IL-10 (-592), suggesting their presence on the same haplotype block; however IL-10 (-1082) and IL-10 (-819), IL-10 (-1082), and IL-10 (-592) were not present on the same haplotype block. These findings suggest that the response to HB vaccine can be estimated based on solely on the IL-10 (-1082) in Japanese subjects. We hypothesize that this SNP influences transcription regulation and changes the IL-10 gene expression level, influencing anti-HBs antibody production and altering responsiveness to the vaccine. Höhler *et al.* (13) suggested that because IL-10 is a Th1 cell-inhibitory cytokine, a pathway that changes the IL-10 expression level may influence HBs antibody production. Our study found a tendency for a low anti-HBs antibody level in the IL-10 (-819) CT type, suggesting that IL-10 plays an important role in the immune response to HB vaccine.

An association between TNF- α (-308) and unresponsiveness to HB vaccine has previously been reported (14). The TNF- α (-308) GA type was found to influence TNF- α gene expression, reducing the anti-HBs antibody level. Kroeger *et al.* (25) reported that the TNF- α (-308) minor allele A increases TNF- α gene transcription activity. However, the frequency of the TNF- α (-308) allele A is very low in the Japanese population, and the GA type was detected in only 2 subjects in the present study. A low anti-HBs antibody level in these 2 subjects suggests that this allele may be able to influence on the immune response to HB vaccine. In our study, the antibody level was significantly higher in the hetero CT type than in the major homo CC type of the TNF- α (-857) C/T polymorphism of the TNF- α gene transcription regulatory region. This SNP is also a polymorphism in the gene promoter region and may influence the gene expression level. Qiu *et al.* (26) reported that the TNF- α (-857) CT type of the TNF- α gene promoter region changes the TNF- α gene expression level and influences the progression of hepatitis B pathology. The authors suggested that HB vaccination promotes anti-HBs antibody production in TNF- α (-857) CT type patients via high TNF- α expression, resulting in resistance to the progression of hepatitis B. A similar finding was reported by Kim *et al.* (27) in a study involving hepatitis B patients in Korea. These findings provide support for the involvement of TNF- α in anti-HBs antibody production after HB vaccination, and indicate the need for further detailed investigation of the influence of racial differences in genetic factors. Our findings are the first report of the association between TNF- α (-857) CT type and immune response to HB vaccine.

Inheritance of the IL-10 (-1082) and TNF- α (-857) types are genetic factors influencing HBs antibody production in the Japanese population. Further studies with a greater number of subjects may contribute to a better understanding of the influence of cytokine gene polymorphisms and expression of cytokines on the humoral immune response to HBsAg vaccination. Well-designed investigations with representing different ethnicities are warranted to confirm our findings.

DISCLOSURE

The authors report no potential conflicts of interest.

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