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Serological surveillance and *IL-10* genetic variants on anti-HBs titers: Hepatitis B vaccination 20 years after neonatal immunization in Taiwan

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ABSTRACT

Background: The national hepatitis B (HB) vaccination program in Taiwan that began in 1984 has resulted in a significant reduction in the carrier rate among children. However, a significant proportion of Taiwanese neonatal HB immunization recipients have exhibited low anti-HBs titers that fall to non-protective or undetectable levels.

Methods: We recruited 1,677 entering freshman and graduate student participants at a Taiwanese university health center, grouped them into three age groups representing three stages of Taiwan's HB vaccination program, then conducted hepatitis B surface antigen (HBsAg) and antibodies to HBsAg (anti-HBs) serological surveillances for each individual. Univariate and multivariate regression analyses of clinical characteristics and *Interleukin-10* (*IL-10)* genetic variations were also conducted.

Results: A trend toward a decreasing HBsAg carrier rate was observed over the starting dates of the vaccination program (11.7%, 1.6% and 1.7% for age groups 1, 2 and 3, respectively), but we also observed an increasing rate of non-protective anti-HBs titers (15%, 26% and 50.3% for cohorts 1-3, respectively). The percentage of students with non-protective anti-HBs titers increased from 23.1% for students born in 1984, to 25.2% for those born in 1985, to 39.4% for birth-year 1986 students, to 45.7% for birth-year 1987 students, to 56.5% for birth-year 1988 students. The risk for low anti-HBs titers increased concurrently with increases in systolic blood

pressure (BP), the *IL-10* ATA/ACC haplotype, and the *IL-10* ATA present haplotype. Risk for low anti-HBs titers decreased with concurrent decreases in glucose ante cibum (AC, before meals) and the *IL-10* ACC/ACC haplotype.

Conclusions: these results suggest that the genetic determinants may also contribute to variations in anti-HBs titers in immune responses to HB vaccination.

1. Introduction

Over 300 million people worldwide are infected with the hepatitis B virus (HBV), a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1, 2]. Taiwan has one of the highest hepatitis B surface antigen (HBsAg) carrier rates in the world, at times as high as 15-20% [3, 4]. The national hepatitis B (HB) vaccination program that began in 1984 has resulted in a significant reduction in the carrier rate among children [4]. The HB vaccine, which induces protective antibodies against HBsAg, is considered an efficient strategy for controlling HB viral infection and transmission [4-6]. However, between 1% and 10% of all vaccinated individuals fail to produce sufficient levels of protective antibodies,[7, 8] and levels in another segment of the vaccinated population tend to decline to low or virtually undetectable titers as recipients age, resulting in increased risk of HB infection [9, 10].

Studies on genetic variability especially in cytokine and cytokine receptor genes associated with variations in the immune response to childhood vaccination have recently been addressed [11]. Interleukin-10 (IL-10) is a potent cytokine, mainly produced by monocytes, macrophages, T- and B-lymphocytes and exerts pleiotropic effects on immunoregulation and inflammation [12, 13]. Studies on *IL-10* genetic polymorphisms at positions -1082(G/A), -819(C/T), and -592(C/A) generating the ATA, ACC, and GCC haplotypes have revealed an association with IL-10 protein

production [14-17]. The GCC haplotype has been shown to be associated with high IL-10 production, while the ATA haplotype showed lower transcriptional activity than GCC haplotype, producing low levels of IL-10 protein [14]. The -1082 (G/A) polymorphism lies in a putative E-Twenty-Six (ETS) like transcription factor binding site [18], while the -819 (C/T) polymorphism may affect an estrogen receptor element [19]. Similarly, the -592 (C/A) polymorphism locates in a negatively regulatory region and may abolish IL-10 protein production [18, 19]. The transcriptional activity analysis has suggested that these IL-10 promoter genetic variants are important loci. Furthermore, *IL-10* promoter polymorphisms have also been involved in hepatic disease progression and/or antiviral responses [16, 20-22].

In this study, we described the prevalence of HB seromarkers in a group of entering freshman and graduate university students 20 years after the initiation of a national HB vaccination program in Taiwan. The present report was also designed to investigate whether the genetic determinants contribute to variations in anti-HBs titers in immune responses to HB vaccination.

2. Patients and methods

2.1. Hepatitis B (HB) vaccination program

Taiwan's national HB vaccination program began on July 1, 1984 (Figure 1) [23]. In its first year it only covered the newborns of HBsAg-carrier mothers. In July 1986 the program was extended to cover all newborns, and the following July it was extended to all preschool and elementary school students, teenagers, and adults. Since July 1991, the vaccination records of all children entering the first grade must indicate HB vaccinations; those with no or partial records are given vaccinations or boosters at that time. All pregnant Taiwanese women are now screened for HBsAg, and all HBsAg-positive women are further tested for the hepatitis B e antigen (HBeAg). All newborns of high-titer HBsAg carriers or HBeAg-positive mothers are given 0.5 ml (100 IU) of hepatitis B immunoglobulin within 24 hours of birth.

2.2. Study population and design

This study is a cross-sectional seroprevalence survey in September 2005. Study data were collected for new graduate and freshman students entering China Medical University in Taiwan (health screenings are required for all entering students prior to admission). Those willing to participate in the study and who signed informed consent documents were placed in one of three groups according to birth date (Figure 1).

Those with histories of HCV infection, chronic disease, cancer, pregnancy, lactation, excessive alcohol consumption, drug abuse, or immunosuppressant treatment were excluded from the final sample. The final sample consisted of 1,677 students divided into three age groups: those born before June 30, 1984 (age group 1), those born between July 1, 1984 and June 30, 1986 (age group 2), and those born after July 1, 1986 (age group 3). The age group 3 born after 1986 was supposed to be neonatal immunization with HB vaccine at birth and at 1, 2 and 12 months of age according to the HB vaccine policy in Taiwan (Figure 1). Serological measurements focused on HBsAg and anti-HBs titers (Figure 2). Serum anti-HBs titer levels were investigated according to birth year (Figure 3). For those born in 1986, 1987 or 1988, whether clinical characteristics and genetic determinants contribute to variations in anti-HBs titers in immune responses to HB vaccination was considered.

2.3. HB seromarkers and IL-10 genotyping

HBsAg and anti-HBs titers were determined by enzyme immunoassay (Abbott Laboratories, North Chicago, IL). Anti-HBs titers >12 milli international units per milliliter (mIU/mL) were defined as protective and <12 mIU/mL as non-protective. HBsAg-positive individuals were assumed to be HB carriers. Genomic DNA was extracted according to standard protocols (Roche Genomic DNA kit) from participates

who were born after 1986 and were negative for HBsAg. Three biallelic *IL-10* promoter polymorphisms were detected using TaqMan® Genotyping Assays (Applied Biosystems) (Supplemental Table 1).

2.4. Statistical analyses

Unless otherwise indicated, data were expressed as mean ± standard deviation (SD) for the continuous variable. All data were analyzed using Statistical Product and Service Solutions (SPSS) (12.0) for Windows. $\chi 2$ tests were used to determine differences in categorical variables, and odds ratio (OR) and 95% confidence intervals (CI) were calculated for the factors under consideration. We performed forward stepwise multivariate regression analyses to identify factors contributing independently to declining anti-HBs titers. Genotypes were obtained by direct count, followed by allele frequency calculations. In addition to $\chi 2$ tests, *p* values were calculated using the Minitab program; *p* values less than 0.05 were considered statistically significant. Haplotype analyses were performed using Haploview [24].

3. Results

3.1. Hepatitis B (HB) serological data

There were 771 students in age group 1, 246 in group 2, and 660 in group 3. Results from serological tests for HBsAg and anti-HBs titers are shown in Fig. 2. HBsAg carrier rates were 11.7%, 1.6% and 1.7% for groups 1, 2 and 3, respectively ($p<0.001$). Both HBsAg and anti-HBs negative rates were 15.0%, 26.0% and 50.3% ($p<0.001$), and both HBsAg negative and anti-HBs positive rates were 73.3%, 72.4% and 48.0%, also respectively ($p<0.001$).

To identify serum anti-HBs titer trends among seronegative HBsAg students, we assessed anti-HBs titer distributions according to birth year (1980 to 1988) (Figure 3). The percentages of students with non-protective anti-HBs titers (anti-HBs titer $\leqq 12$ mIU/mL) increased from 23.1% for students born in 1984 to 25.2% for those born in 1985 ($p=0.729$), to 39.4% for birth-year 1986 students ($p=0.013$), to 45.7% for birth-year 1987 students ($p<0.001$), to 56.5% for birth-year 1988 students ($p<0.001$). In other words, a decreasing HBsAg carrier rate and an increasing proportion of non-protective anti-HBs titers were observed from older to younger age groups; the increase in non-protective anti-HBs titers was especially pronounced in students born in 1986, 1987 and 1988.

Based on these results, we used univariate and multivariate analyses to examine

whether the clinical characteristics and genetic determinants contribute to variations in anti-HBs titers in immune responses to HB vaccination. In our univariate analyses we categorized clinical characteristics by anti-HBs titer and gender (Table 1). When clinical characteristics were categorized by anti-HBs titer (group A: anti-HBs $\leqq$12 mIU/mL; group B: anti-HBs >12 mIU/mL), statistically significant differences were found for the mean values for systolic BP (p=0.005) and anti-HBs titer (p<0.001). When clinical characteristics were categorized by gender, statistically significant differences were noted for mean values between males and females for all factors except age, diastolic BP, glucose AC, and anti-HBs titer.

3.2. Allele, genotype and haplotype frequencies of IL-10 genetic polymorphisms

To investigate whether the *IL-10* genetic variants contribute to variations in anti-HBs titers in immune responses to HB vaccination, we genotyped *IL-10* genetic polymorphisms in the age group 3, which its vaccine coverage was over 90% [25]. The genotypes of three promoter polymorphisms were identified: -1082A/G, -819T/C and -592A/C (Supplemental Table 1; allele and genotype frequencies are presented in Table 2). As shown, the difference between group A and B polymorphism frequencies at -1082 were not statistically significant, but differences between T/C and A/C change frequencies at positions -819 and -592 in the *IL-10* promoter region were

(p=0.021 and p=0.021, respectively).

Haplotype frequencies were also estimated using these three genetic polymorphisms (Table 2). Six haplotypes were present in both groups A and B. Statistically significant differences were observed between groups A and B in ACC/ACC and ATA/ACC haplotypes. In group A, ACC/ACC genotype frequency was significantly lower (p=0.008; OR=0.36, 95% CI=0.17-0.79) and ATA/ACC frequency was significantly higher (p=0.004; OR=1.77, 95% CI=1.20-2.61). Observed frequencies for individuals carrying the ATA haplotype were 93.2% in group A and 86.4% in group B (p=0.016; OR=2.15, 95% CI=1.14-4.06). Male subgroup analysis results indicate that ATA/ACC haplotype frequencies were 47.9% in group A and 33.7% in group B (p=0.026; OR=1.81, 95% CI=1.07-3.05). Female subgroup analysis results indicate ACC/ACC haplotype frequencies of 2.7% in group A and 11.1% in group B (p=0.016; OR=0.22, 95% CI=0.06-0.83). Also among females, frequencies for individuals carrying the ATA haplotype were 95.6% in group A compared to 86.4% in group B (p=0.022; OR=3.39, 95% CI=1.13-10.19).

The associations between *IL-10* genetic haplotypes and variations in anti-HBs titers in immune responses to HB vaccination were also investigated as shown in Table 3. Individuals carrying the ACC/ACC haplotype (mean 239.99 mIU/mL; 95% CI=95.96-384.01 mIU/mL) had anti-HBs titers at levels that were almost twice as

high as in individuals carrying the ATA/ACC haplotype (mean 102.18 mIU/mL; 95% CI=65.25-139.12 mIU/mL) (Table 3). Individuals carrying the ATA haplotype (mean 122.43 mIU/mL; 95% CI=95.83-149.03 mIU/mL) had anti-HBs titers at levels almost one-half as low as in individuals without it (mean 215.90 mIU/mL; 95% CI=102.08-329.73 mIU/mL) (Table 3).

3.3. Risk factors for declining anti-HBs titers

According to our univariate regression analysis results, statistically significant odds ratios were noted for systolic BP (p=0.005), ACC/ACC (p=0.008), ATA/ACC (p=0.004) and ATA present (p=0.016) (Tables 1 and 2). After adjusting for these possible influence factors, results from a multivariate regression analysis using anti-HBs titers as the dependent variable indicate associations between variations in anti-HBs titers and glucose AC, systolic BP, ACC/ACC, ATA/ACC, and ATA present (Table 4). Specifically, statistically significant correlations were found between increased risk for declining anti-HBs titers and the presence of systolic BP (OR=1.02; p=0.001), ATA/ACC (OR=1.55; p=0.042) and ATA present haplotypes (OR=2.26; p=0.013). That risk decreased with decreasing levels of glucose AC (OR=0.98; p=0.007) and ACC/ACC (OR=0.43; p=0.043).

4. Discussion

This study is a retrospective study. We described the prevalence of HB seromarkers in a group of entering freshman and graduate university students 20 years after the initiation of a national HB vaccination program in Taiwan, and provide specific evidence of declining protective anti-HBs titers in students born after the initiation of a national HB vaccination program in Taiwan. We genotyped *IL-10* genetic polymorphisms in the age group 3, which its vaccine coverage was over 90%. We found that *IL-10* genetic variants were associated with variations in anti-HBs titers. Our results suggest (a) an increased risk for declining anti-HBs titers with increased levels of systolic BP, *IL-10* ATA/ACC haplotype, and *IL-10* ATA present haplotype; and (b) a decreased risk with decreased levels of glucose AC and *IL-10* ACC/ACC haplotype. Combined, these results suggest that the genetic determinants may also contribute to variations in anti-HBs titers in immune responses to HB vaccination.

A decreasing HBsAg carrier rate was observed from older to younger age groups. These statistically significant declines support findings from previous seroepidemiologic studies describing decreases in HBsAg prevalence from a high of 7.7-11.9% among individuals born 1-6 years before the program was implemented, to 3.4-6.3% when only the newborns of HBsAg carrier mothers were immunized, to a low of 0.8-1.7% when all newborns were immunized [10, 23, 26-28]. Before the

vaccination program, mothers carrying HBsAg served as a reservoir for HBV, thus providing the transmission of HBV from one generation to the next. In previous and current reports, in HBV endemic areas, HBV infection occurs mainly during early childhood and mother-to-infant transmission accounts for approximately 50% of the chronic infection cases [29-31]. Nearly 90% of infants born to hepatitis B e Antigen (HBeAg) positive mothers are infected at birth [32]. The similar low HBV carrier rates between group 2 and 3 observed from our study and previous studies suggest that vaccination program in infants of HBsAg carrier mothers including a dose of hepatitis B immune globulin (HBIG) coupled with a multi-dose course of HB vaccine, that is initiated promptly at birth have successfully blocked vertical transmission. And, also, from 1987, all preschoolers, school-age children, teenagers and adults were screened and suggested for vaccination if they did not have enough protective levels of anti-HBs. Therefore, in our comparison of the HBV marker of the students' mothers on groups 1, 2 and 3 (supplementary table 2), we observed that the HBeAg coupled with HBsAg carrier rates were 7.3%, 1.2% and 0.9% for groups 1, 2 and 3, respectively ($p<0.001$). We obtained that a decreasing HBeAg (+) coupled with HBsAg (+) carrier rate was observed from their mothers of older to younger age groups. There were no significant differences among other HBV marker such as HBeAg (-) coupled with HBsAg (+), HBeAg (-) coupled with HBsAg (-), Anti-HBs

(+) and HBeAg (-) coupled with HBsAg (-), Anti-HBs (-) observed from groups 1 and 3. These low HBeAg coupled with HBsAg carrier rates of groups 2 and 3 and their mothers suggest a successful vaccination program announced in Taiwan since 1984.

An increasing proportion of non-protective anti-HBs titers was observed from older to younger age groups. This is a remarkably sharp increase, one giving further support to studies showing an increasing number of adolescents with waning immunity 15 years following their neonatal vaccinations [10, 28, 33]. Several methods have been investigated to overcome the weaker stimulation for antibody production by the use of more immunogenic epitopes and the use of adjuvants [34-39]. The addition of preS1 and preS2 antigen, to the HBsAg provides additional protective immunity. Another method for augmenting the anti-HBV immunity is the use of adjuvants such as vaccine delivery systems and immune-stimulating adjuvants [36, 38, 39].

The male students in the sample had higher levels than female students of BMI, systolic BP, Hb, ALT, AST, creatinine, uric acid, cholesterol, and triglyceride. This is a pattern that has been repeatedly observed among Taiwanese over the past two decades [40, 41]. In addition, overweight college students in the US are also known to have higher levels of BP, fasting insulin, cholesterol, and triglyceride [42]. After adjusting for gender, glucose AC, systolic BP, and other possible confounders, we found that

individuals with decreased glucose AC and increased systolic BP were at higher risk for declining anti-HBs titers.

The role of hepatitis B infection and related serological status in glucose metabolism and increased body weight in humans is currently receiving a considerable amount of research attention, and significant associations between hepatitis B infections and diabetes have been reported for different Asian populations [43, 44]. In addition, adenovirus 36 infections of adipocytes have been found to modulate adipocyte differentiation, leptin production, and glucose metabolism, and to increase the production of IL-6, which is involved in the development of cardiovascular diseases and type 2 diabetes [45, 46]. Details regarding interactions between HB vaccination and glucose metabolism require further clarification.

According to our genetic determinant results, declines in anti-HBs titers increased with increases in *IL-10* ATA/ACC haplotype and *IL-10* ATA present haplotype, and decreased with decreases in *IL-10* ACC/ACC haplotype. IL-10 is an important immuno-modulator that enhances MHC class II antigen expression and antibody production in B cells [13]. It also plays a regulatory role between antigen presenting cells (APCs) and regulatory T cells [47]. *IL-10* promoter haplotypes account for different immune responses against hepatitis viral antigens [22]. Low IL-10 production (ACC haplotype) apparently favors a strong and immediate humoral immune response

to HBsAg, while the presence of the -1082A allele leads to the suppression of anti-HAV production. Our results indicate an association between the *IL-10* haplotypes ACC and ATA and declining anti-HBs titers during the 20 years following neonatal HB vaccination, suggesting that genetic variability in the *IL-10* promoter may play an important role or roles in the regulation of immediate responses to HBsAg and the maintenance of prolonged serum anti-HBs titers.

In conclusion, this study provides further evidence that a significant percentage of Taiwanese who received neonatal vaccinations in the 1980s lack protective anti-HBs. Our data also suggest that the genetic determinants may also contribute to variations in anti-HBs titers in immune responses to HB vaccination.

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Figure Legends

Figure 1. Newly entering students at China Medical University in 2006 who agreed to participate in the study were assigned to one of three age groups: 1, born prior to June 30, 1984 (before Taiwan's hepatitis B vaccination program was initiated); 2, born between July 1, 1984 and June 30, 1986 (when the vaccination program only covered newborns of HBsAg carrier mothers); or 3, born after July 1, 1986 (when all newborns were covered).

Figure 2. Seroprevalence of HBsAg and anti-HBs titers among students in the three age groups. □, seropositive HBsAg; ▩, seronegative HBsAg and seronegative anti-HBs titers; ■, seronegative HBsAg and seropositive anti-HBs titers.

Figure 3. Anti-HBs titer levels among seronegative HBsAg students born between 1980 and 1988.