Medicine

Single-Nucleotide Polymorphism of rs7944135 (MPEG1) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study

--Manuscript Draft--

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Abstract:	Background Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment of patients with chronic hepatitis B (CHB) infection. Genetic, age and gender factors were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the SNP had been identified to be associated with HBsAg seroclearance in CHB patients Method Recently, three associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In our current study, these three SNPs were genotyped in 2,565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance. Result We observed that SNP rs7944135 was solely associated with HBsAg seroclearance. Subjects with the AA genotype at rs7944135 macrophage-expressed gene 1 (MPEG1) had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under genotypic model (odds ratio (OR)=1.76. 95% confidence interval (CI)=1.14~2.72, p=.045). Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG+GG of rs7944135 under the recessive model (OR=1.74. 95% CI=1.13~2.66, p=.014). According to the cumulative fraction curve with the log-rank test revealed patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance (p=.039) and HBV DNA undetectable (p=.0074) compared to those with the AG or GG genotype. Conclusion This study examined the associations of three SNPs (rs7944135, rs171941, and

	rs6462008) with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Dear Editor-in-Chief, ***Journal of Medicine***

This is to submit our revised manuscript entitled" Single-Nucleotide Polymorphism of rs7944135 (*MPEG1*) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study" for consideration of publication in the "***Medicine***" journal. Please be informed that this is a revised submission of our manuscript (**MD-D-19-04438**). We are thankful for your kind encouragement regarding to our manuscript. Herewith we are sending our revised manuscript in accordance with the comments given by the reviewer. The revised parts of the manuscript are highlighted in red color.

Lastly, we would like to thank you once again for providing us the opportunity to improve our manuscript. We hope that these revisions are adequate, and that the manuscript is now acceptable for publication in the "***Medicine" journal.***

Sincerely,

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Reviewer Comment:

Reviewer #1: The study re-checked the associations of three SNPs (rs7944135, rs171941, and rs6462008) with HBsAg seroclearance, and identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients. The study only found that patients with chronic hepatitis B with the *MPEG1* AA were more likely to have hepatitis B surface antigen clearance. The study lacks the mechanism related research.

Answer: We sincerely thank the reviewer for the time taken to review our work and the important suggestions given. Our results revealed that the rs7944135 SNP was solely associated with HBsAg seroclearance. It indicated that HBV carriers with the AA genotype of rs7944135 were more susceptible to HBsAg seroclearance compared to those with the AG or GG genotype of rs7944135. As discussed in the discussion part, genotypes of rs7944135 may affect the expression of *MPEG1* and therefore lead to different outcomes of the patients. The paragraph in the discussion part is shown as below: *MPEG1* is a specific gene that was identified in humans and mice in macrophages and has many roles in immune responses [1]. According to HaploReg V4.1, rs7944135 was located at 11q12.1 of *MPEG1,* and it is involved in the changing chromatin status of primary T cells from peripheral blood, which plays a central role in the "primary immune response" of cell-mediated immunity. Furthermore, the *MPEG1* gene has role in immune response through encoding perforin-like protein and recognition of antigen[2]. The perforin-like protein is predicted to be a perforin domain of membrane attack complex (MAC) that helps cytotoxic T cells and natural killer cells kill virus infected cell [3, 4]. In our study, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that rs7944135 AA genotype shows the highest expressed of *MPEG1* gene in compare to GG and AG genotype. It infers that patients with AA genotype

on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the hepatitis B surface Antigen (HBsAg). [line 265-272, page 11]

Reviewer #2: In this manuscript, the authors investigated association between three SNP in *MPEG1* and HBsAg seroclearance in patients with chronic HBV infection. They genotyped these SNPs in 2565 CHB patients with or without HBsAg seroclearance. They found that SNP rs7944135 was associated with HBsAg seroclearance. The patients with AA genotype have higher susceptibility to HBsAg seroclearance and undetectable HBV DNA, comparing with the patients with AG or GG genotypes.

Here are some concerns that need authors to address:

1. I think the therapy (type I interferon? Nucleoside analogues or spontaneous seroclearance?) of patients should be involved in the investigation. Since MPEG1 can be induced by interferon (doi: 10.1159/000345249), it may be a IFN stimulated gene which plays a role in the HBsAg seroclearance induced by type I interferon treatment or spontaneous seroclearance.

Answer: Thanks for your comment, we agree that the therapeutic agent is important for gene response in HBV infection including in HBsAg seroclearance. Interferon plays a critical role in human immune response, it may be induced *MPEG1* response in clearance of HBsAg in serum, and the interferon-induced genes also was reported inhibit HBV replication in transgenic mouse hepatocytes[5]. Clinically, the clearance of HBsAg in CHB as a safe marker for the discontinuation of antiviral treatment with nucleos(t)ide analogues; however, the

limitation of our study that we did not involve the subjects who received antiviral treatment with interferon alfa or nucleoside/nucleotide analogues treatment due to some reasons; treatment for HBV was not reimbursed by the universal national health insurance program in Taiwan until 2003, while the patients in this study were recruited between 1991 and 1992. In addition, at the time of recruitment, the patients were of poor disease awareness and medical accessibility. In previous report, it can be calculated that only 46% of the individuals with chronic HBV infection in Taiwan were aware that they were infected[6] Thus, in this study, the patients had no experience of antiviral treatment. The cohort in this study therefore presents the natural response against HBV without antiviral treatments. However, further studies are required to include antiviral treatments effect into analysis, if the patients had received antiviral treatment.

2.Does SNP rs7944135 affect MPEG1 gene expression? If yes, it will be helpful to explain the molecular mechanism by analyzing MPEG1 expression levels in the patients.

Answer: We thank the reviewer for the suggestions. As shown in Table 4 and Figure 3, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that individuals with AA genotype at rs7944135 show higher expression of *MPEG1* gene comparing to those with GG and AG genotypes. It infers that patients with AA genotype on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the HBsAg. [line 268-272, page 11]

Editorial Formatting Comments

1. Title page: Be sure the title page lists all author names, degrees, and affiliations.

Answer: Thank you for the comments. In this revision, we had an additional author, Wan-Hsuan Chou, who helped revise this manuscript. We have confirmed the authorship list in this revision.

2. Title: Be sure the title includes any specific terms as directed in the reporting guidelines for your type of article (for example, "case report" should be in the title of a CARE-compliant article). The following guidelines specify terms that should be in the title: CARE, CHEERS, CONSORT, PRISMA.

Answer: Thank you for your comments. We have revised the title accordingly.

3. Abstract: Be sure to use a structured abstract, with headings. Use the specific headings listed in the guidelines checklist if your report is based on the CARE, CHEERS, CONSORT, or PRISMA guidelines.

Answer: Thank you for your comments. We have included headings in the abstract.

4. List of abbreviations: If not already included, please include a list of abbreviations used in the text as part of the manuscript text file following the title page. Use abbreviations sparingly in the text, and spell them out the first time you use them. Abbreviations used in tables should be spelled out at the bottom of the table.

Answer: Thank you for your comments. We have added the list of abbreviation.

5. Ethical review, Methods section: If not already included, please state in the Methods section that an ethics committee or institutional review board approved the study, and list the board's name. If ethical approval was waived or not necessary, please state the reason. If the study involves patient consent, state explicitly that informed consent was or was not given, and state the reason if not given.

Answer: Thanks for the comments. We have described institutional review board approved in the method section.

6. Funding/Conflict of Interest information: List any source of funding or anything that could be perceived as a conflict of interest in the Acknowledgments section.

Answer: Thank you for your comments. We have stated the funding and conflict of interest.

7. Acknowledgments: If you list anyone by name in the Acknowledgments section, please confirm that the person gives permission to be named.

Answer: Thank you for your comments. We have confirmed the person who stated in the acknowledgements section.

8. License to Publish: If not already submitted, complete and submit a copy of the Open Access-License to Publish (LTP). The LTP is available to download from the home page of our website, under Forms. Be sure to select the kind of license you would like to use if the paper is accepted. (The different kinds of licenses determine how others can use your work after publication, varying from not restrictive at all to very restrictive.) Fill out all schedules (parts of the form). The corresponding author can sign the form on behalf of all authors; we need only one copy of the form. The form can be filled out and signed electronically, then uploaded as a submission item.

Answer: Thank you for your comments. The corresponding author has confirmed the statement through the electronic system.

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Single-Nucleotide Polymorphism of rs7944135 (*MPEG1*) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study

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Abstract

Introduction: Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment of patients with chronic hepatitis B (CHB) infection. Genetic, age and gender factors were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the SNP had been identified to be associated with HBsAg seroclearance in CHB patients.

Methods: Recently, three associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In our current study, these three SNPs were genotyped in 2,565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance.

Results: We observed that SNP rs7944135 was solely associated with HBsAg seroclearance**.** Subjects with the AA genotype at rs7944135 macrophage-expressed gene 1 (*MPEG1*) had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under genotypic model (odds ratio (OR)=1.76. 95% confidence interval (CI)=1.14~2.72, *p*=.045**)**. Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG+GG of rs7944135 under the recessive model (OR=1.74. 95% CI=1.13~2.66, *p*=.014). According to the cumulative fraction curve with the log-rank test revealed patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance (*p*=.039) and HBV DNA undetectable (*p*=.0074) compared to those with the AG or GG genotype.

Conclusion: This study examined the associations of three SNPs **(**rs7944135, rs171941, and rs6462008**)** with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Abbreviation: CHB=Chronic hepatitis B, CI= confidence interval, GWASs=Genome-wide association studies, HBsAg=Hepatitis B surface antigen, HBV= Hepatitis B virus , *MPEG1*= Macrophage-expressed gene 1, OR=odds ratio, SNPs= Single-nucleotide polymorphisms

Keywords: HBsAg seroclearance; HBV; CHB; rs7944135; single-nucleotide polymorphism

1. Introduction

The hepatitis B virus (HBV) was discovered over five decades ago. Although a prophylactic vaccine has been available, HBV infection still serious problem globally. World Health Organization (WHO, 2018) estimated that there were 257 million new cases and 887,000 deaths from HBV. Most of the deaths were caused by complications, including cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Eventually, the goal of therapy in CHB patients is to alleviate and prevent this complication.

Chronic hepatitis B (CHB) infection can be confirmed by its complicated serological pattern, while hepatitis B surface antigen (HBsAg) was reported to be well-established as a serum marker in the natural history of HBV infection. A low level of the HBsAg is associated with sustained immune control, seroclearance of HBsAg, and a lower risk of HCC [3]. A meta-analysis further confirmed the role of the HBsAg as a predictive marker of hepatitis B, liver cirrhosis, and HCC development, and the rate of spontaneous HBsAg seroclearance [4], an important milestone in the natural history of CHB infection. In addition, the serological profiles of chronic HBV infection also revealed that plasma HBV DNA levels can serve as a marker of disease progress [5]. In general, CHB is defined as the presence of the HBsAg for more than six months after an HBV infection [6] and spontaneous HBsAg seroclearance is defined as the loss of the HBsAg at least six months apart on two occasions and continuing to remain to absent up to the last visit [7]. However, the rate of HBsAg seroclearance in CHB patients is varied globally at an estimated 1%~2% annually [8], at approximately, 0.41% to 1.58% in Europe [9,10], 0.56 to 0.65% in north and south America, respectively [11,12] and 0.12%~2.38% per year in Asian [13], 0.4% per year reported in Korea [14], 2.5% in the Goto Islands of Japan [15] and 1.15% per year was recorded in Taiwan [13].

The clearance of the HBsAg in HBV infection is influenced by many factors, such as genetic and host factors including age, sex, and race. Several studies identified that host genetic polymorphisms may be associated with clinical outcomes of HBV, including HLA DQ (*HLA-DQ*) and DP (*HLA-DP*), [16,17], *IL28B* [18], *HLA-DPB1* [19], *Tumor Necrosis Factor-α* (*TNF-α*) [20], and *Monocyte Chemotactic Protein-1* (*MCP1*) [21]. Some CHB-associated loci, such as *HLA-DPB1*, *HLA-DQA2*, and *HLA-DQB,* also reported by previous GWASs, were identified in a Taiwanese population [22]. However, more the genetic studies still need to be examined in HBV

infection, considering of HBsAg seroclearance as a marker of cure of HBV infection. Recently, Kim *et al.* indicated that three new SNPs (rs7944135, rs171941, and rs6462008) were associated with HBsAg seroclearance in a Korean population. Thus, the purpose of this study was to investigate whether the polymorphisms reported by Korean group are associated with clearance of the HBsAg in Taiwanese HBV patients.

2. Materials and Methods

2.1. Study Subjects

In total, 2565 CHB patients (including 493 with HBsAg seroclearance and 2072 without HBsAg seroclearance) satisfied the inclusion criteria for follow-up of CHB, which were recruited during 1991 to 1992 from seven area in Taiwan (Sanchi, Chutung, Potzu, Kaoshu, Makung, Hushi, and Paisha). All of the study participants were ethnic Chinese (*i.e.*, Taiwanese). All participants in this study provided written informed consent before participation. This project was approved by the ethics committees at Academia Sinica, Taiwan.

2.2. Clinical Evaluation and SNP selection

All patients were tested for hepatitis B or virological markers in the liver, including HBsAg was measured using radioimmunoassay with commercial kits (Abbott Laboratories, North Chicago), HBV DNA were measured by polymerase chain reaction (PCR) using the Cobas Amplicor HBV monitor test kit (Roche Diagnostics, Indianapolis, Ind) and alanine transaminase (ALT) ALT using chemistry autoanalyzer (Model 736, Hitachi, Tokyo, Japan) using commercial reagents (Biomérieux, Marcy L'Etoile, France). HBsAg seroclearance were defined as loss of HBsAg in serum at least six months apart on two occasions and continued to absent up to the last visit. While without HBsAg seroclearance were defined as positive of HBsAg in serum for more than six months apart and continuously detected up to the last visit.

The SNPs **we investigated** in this current study were the replicated study from a GWAS applied in a Korean CHB population reported by TH Kim *et al.* (2018). TH Kim *et al.* (2018) reported **three** SNPs (**rs7944135, rs171941, rs6462008**) associated with seroclearance of the HBsAg in Korean CHB patients. These three SNPs were confirmed to exist in a Taiwanese CHB population.

2.3 DNA Extraction and Genotyping of the three SNPs

DNA was extracted from blood samples and subsequently centrifuged at 3000 rpm for 10 min at 4 ℃ to separate cells and plasma. Specimens were stored below -70 ℃. The buffy coat was isolated from blood samples, and red blood cells (RBCs) were lysed after the addition of RBC lysis buffer.

Three SNPs (rs7944135, rs171941, and rs6462008) were assessed by genotyping. Genotyping assays were performed using a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Polymerase chain reactions (PCRs) were subsequently performed in a 96-well microtiter plate with either an ABI7500 real-time PCR or ABI9700 Thermal Cycler under the following conditions: 10 min of denaturing at 95 ℃, followed by 45 cycles of 15 s of denaturing at 95 ℃ and 30 s for annealing at 60 ℃, with 1 min of a final extension at 60 ℃. Fluorescence signals from amplicons were analyzed using System SDS software vers. 1.2.3(Applied Biosystems, FosterCity, CA).

2.4. SNP annotation data query

Associations between gene expression profiles and the SNPs were confirmed by examining the expression quantitative trait loci (e-QTL) through (GTEx) Portal database (http://www.gtexportal.org/ home/). The GTEx database shows expressions of genes in a variety of tissues.

2.5. Statistical analysis

We performed all analyses using the R environment (https://cran.r-project.org/ and https://www.r-project.org/). We used Student's *t*-test to compare the age, ALT, and follow-up duration between the CHB patients with HBsAg seroclearance and without HBsAg seroclearance groups. We used logistic regression analyses to obtain adjusted OR between the case with HBsAg seroclearance and gender, age, ALT, and follow-up duration. Associations between SNPs and HBsAg seroclearance under genotype and recessive models were assessed using the "SNPassoc" package. The association between (the rs7944135 genotype AA, AG, and GG) and (HBsAg seroclearance and Undetectable of HBV DNA) were also modeled using time to event analysis utilized cumulative fraction curve using cumulative hazard function and log-rank test by

"Survival and survminer" package. While, days between enrollment to undetectable of HBV DNA and occurring HBsAg seroclearance was used at the time scale.

3. Results

3.1. Basic characteristics of chronic HBV-infected patients

We collected data on 2565 chronically HBV-infected subjects, which included 493 CHB with HBsAg sroclearance (361 males and 132 females) and 2072 CHB without HBsAg serclearance (1325 males and 747 females) in this study. The numbers of CHB male patients in the two groups included 361 with HBsAg seroclearance and 1325 without HBsAg seroclearance (p<0.001). Average ages in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 50 and 46.3 years, respectively (p<0.001), with an age range of 30~65 years. Mean of serum ALT values were 17.7 and 16.5 U/L, respectively (p=0.24) (**Table 1**). The lengths of follow up duration in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 6.29 years (6.66 years for male and 5.21 years for females) and 7.79 years (9.17 years for males and 5.40 years for females), respectively. **Table 1** shows that subjects with HBsAg seroclearance were more likely to be older (OR 1.04, 95% (1.03~1.05); p<0.001). In addition, HBsAg seroclearance tended to more likely appeared in males than in females (OR 1.51, 95% (1.21~1.89); P<0.001) even after adjusted for gender, age and ALT. The follow-up duration was significantly shorter (p<0.001) in the patients with HBsAg seroclearance than the group of patients without HBsAg seroclearnce.

3.2. Association of polymorphisms with HBsAg seroclearance

We investigated the association between genetic polymorphisms of three SNPs (rs7944135, rs171941, and rs6462008) and HBsAg seroclearance (**Table 2**). Genotype and recessive models were applied to assess associations of the HBsAg with the three SNPs. Our results revealed that the rs7944135 SNP was solely associated with HBsAg seroclearance. The rs7944135 SNP was found to be significantly associated with HBsAg seroclearance in the genotype and recessive model after adjusted with gender, age and ALT, at p<0.05. This result indicated that HBV carriers with the AA genotype of rs7944135 were more susceptible to HBsAg seroclearance compared to those with the AG or GG genotype of rs7944135. However, the two other SNPs (rs171941, and rs6462008) showed no correlation with HBsAg seroclearance (**Table 2**). A significant association between the rs7944135 AA genotype and HBsAg seroclearance was found 1.76-fold higher susceptibility to clearance of the HBsAg, compared to those with the AG or GG genotype after adjusting for gender, age and ALT, (OR=1.76. 95% (1.14~2.72), p=.045**)**.

Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype of rs7944135 under the recessive model (OR=1.74, 95% (1.13~2.66), *p*=.014).

3.3. The minor Allele frequency of three SNPs in different populations

The **Table 3** shows the minor allele frequency (MAF) of three SNPs (rs7944135, rs171941, and rs6462008) in different populations (*e.g.*, Asian and European), which were these two population were reported higher susceptible to have HBsAg seroclearance in compare to another region worldwide [23]. MAFs of African, American, European and Asian were extracted from the HaploReg browser v4.1 **(**https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php**)**, and MAF of the TWB were adapted from the Taiwan biobank website (https://taiwanview.twbiobank.org.tw/index). **Table 3** showed that our result was close to those in reference to Asian including a Taiwanese population.

3.4. Correlation between *MPEG1* rs7944135 and days of HBsAg seroclearance

In addition, we confirmed the correlation between the time taken to HBsAg seroclearance and the genotype of the *MPEG1* gene SNP rs7944135. Log rank test showed a significant differences in the time taken to occur HBsAg seroclearance among the *MPEG1* gene rs7944135 genotypes of AA, AG, and GG (*p*=.039). The cumulative fraction curve showed a trend of HBV carriers with the AA genotype of rs7944135 indicated higher susceptibility to HBsAg seroclearnce compared to those with the AG or GG genotype (**Figure 1**).

3.5. Correlation between the *MPEG1* rs7944135 genotype and days of HBV DNA undetectable

We further investigated the correlation between the time taken to HBV DNA undetectable and *MPEG1* gene rs7944135 genotypes: AA, AG, and GG. Log rank test showed significant differences among *MPEG1* gene SNP rs7944135 genotypes of AA, AG, and GG in the time taken to HBV DNA undetectable (*p*=.0074). The cumulative fraction curve showed that a trend of the AA genotype of rs7944135 of HBV carriers showed a higher rate to undetected of HBV DNA versus the AG and GG genotypes (**Figure 2**).

3.6. SNP annotation of expression quantitative trait loci (e-QTLs) of rs7944135

To elucidate the association between SNP *rs7944135* and gene expression, we utilized the publicly available databases GTEx Portal (http:// www.gtexportal.org/home/) to obtain the tissue expression quantitative trait loci. As shown in **Table 4** and **Figure 3** the AA genotype of *rs7944135* had highly expressed of *MPEG1* gene when compared to AG and GG genotype.

4. Discussion

Clearance of the HBsAg is the important indicator of recovery from CHB [24]. It was reported that HBsAg seroclearance is associated with the prognosis of CHB [25]. Clearance of the HBsAg in HBV is influenced by many factors, including genetic and host factors such as age, sex, and race [26]. Our approach in this study was to confirm associations the factors of genetic, age, and gender with HBsAg seroclearance. In the current study, we replicated a GWAS study of Korean CHB patients by a candidate gene approach of CHB Taiwan patients to confirm genes associated with HBsAg seroclearance in CHB Taiwanese population. Kim *et al* revealed that three SNPs of rs6462008 located near even-skipped homeobox 1 (*EVX1*), rs171941 located near cardiomyopathy associated 5 (*CMYA5*), and rs7944135 located near *MPEG1* were associated with HBsAg seroclearance in korean population [27]. Recently, we successfully replicated an association of rs7944135 in *MPEG1* with HBsAg seroclearance in a Taiwanese CHB population.

The male patients are mostly more susceptible to the occurrence of HBsAg seroclearance compared to female patients [13,27]. Consistently, our current study showed that male patients tended to be more susceptible than female patients even after adjusted for gender, age, and ALT, which suggests that male patients have stronger immune responses than female patients. Similar to previous studies from Korea and Taiwan [13,28], this reason may due to hormonal involvement in the immune response [29]. The age at HBsAg seroclearance also can be considered a factor. Our study identified that the mean age at HBsAg seroclearance was 50 years. This finding is relatively close to previous studies in Hong Kong (mean, 48.8 years), Korea (mean, 50 years), and Japan (mean, 51 years) [25,30,31].

This study showed that rs7944135 in *MPEG1* is significantly associated with HBsAg seroclearance in a Taiwanese CHB population. We observed that subjects with the AA genotype of *MPEG1* rs7944135 had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype (OR=1.76). In addition, we still found an association in rs7944135 with 1.74-fold increased risk of the AA genotype of HBsAg seroclearance occurring under the recessive model (OR=1.74). Spontaneous HBsAg seroclearance can be predicted through serum levels of the HBsAg and HBV DNA [24,32]. In terms of the time taken to the HBsAg seroclearance and undetected of HBV DNA, results showed that the rs7944135 AA genotype had a higher susceptibility to HBsAg seroclearance and HBV DNA undetectable compared to the AG and GG genotypes. A correlation between HBsAg and HBV DNA in serum was reported by Li

et al. (2007) and revealed that the HBsAg level was not detected during the immune clearance phase as a result of declining HBV DNA levels. In addition, Coffin *et al.* (2019) revealed that with a lower level of the HBsAg, there was a greater likelihood for loss of the HBsAg. The HBsAg at a low level was associated with a lower risk of prognosis of HCC [3]. It is well known that the HBsAg contributes to the immunopathogenesis of persistent HBV infection, and a higher incidence of HBsAg seroclearance was associated with lower HBsAg levels [33].

MPEG1 is a specific gene that was identified in humans and mice in macrophages and has many roles in immune responses [34]. According to HaploReg V4.1, rs7944135 was located at 11q12.1 of *MPEG1*, and it is involved in the changing chromatin status of primary T cells from peripheral blood, which plays a central role in the "primary immune response" of cell-mediated immunity, this may be suggested the role of *MPEG1* in immune clearance of HBsAg in HBV infection. Furthermore, the *MPEG1* gene has role in immune response through encoding perforin-like protein and recognition of antigen[35]. The perforin-like protein is predicted to be a perforin domain of membrane attack complex (MAC) that helps cytotoxic T cells and natural killer cells kill virus infected cell [36,37]. In our study, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that rs7944135 AA genotype shows the highest expressed of *MPEG1* gene in compare to GG and AG genotype. It infers that patients with AA genotype on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the HBsAg.

Additional genes, such as HLA, cytokine tumor necrosis factor (*TNF*)-α, chemokine receptor 5 (*CCR5*) [38], and *MCP1* [21], were found to be associated with the incidence of HBsAg seroclearance through cell-mediated immune responses. Some evidence has been provided that polymorphisms in HLA subtypes are significantly associated with the occurrence of HBsAg seroclearance, such as a polymorphism of *HLA-DP* rs3077 with haplotype GAT having a 2.17-fold association in a Chinese population [39], *HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535 with A alleles in a Japanese population [40], and *HLA-B*4001* in Taiwanese aborigines [41]. There are many polymorphisms of the TNF-α promoter region that were reported to alter TNF-α-associated HBV clearance in many populations [42]. *MCP1* with -2518G>A was associated with HBV clearance in a Korean population [21].

In this study, we investigated the association of individual SNPs with susceptibility to HBsAg seroclearance in Taiwanese CHB patients. We acknowledge that our study needs to be validated by future studies in other population using more samples with multiple polymorphisms. Therefore, this could give enlightenment for the genetic factors that associated in the occurrence of HBsAg seroclearance. However, this study offers the novel finding of a polymorphism of *MPEG1* having a pivotal association with HBsAg seroclearance in Taiwanese CHB patients.

5. Conclusion

Our current study is a replication of an associated study of a SNP of *MPEG1* rs7944135, with susceptibility to HBsAg seroclearance and showed that an HBsAg seroclearance-associated SNP, rs7944135 with the AA genotype, solely has a significant association with the loss of the HBsAg in CHB infection in Taiwanese HBV patients.

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Author Contributions

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Writing – original draft : Lalu Muhammad Irham

Conflicts of Interest

The authors disclose no conflict

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Figure Legends

Figure 1. Cumulative fraction curve of *MPEG1* rs7944135 on hepatitis B surface antigen (HBsAg) seroclearance.

Figure 2. Cumulative fraction curve of the undetected hepatitis B virus (HBV) DNA of HBV patients according to the *MPEG1* rs7944135**.**

Figure 3. *Cis*-expression quantitative trait loci (*cis*-eQTLs) of *MPEG1* rs7944135 (Homo Alt (AA)>Het (AG)>Homo Ref (GG)) in muscle skeletal.

Table:

Table 1. Baseline characteristics of the 2565 hepatitis B virus study participants

Characteristic	With Seroclearance (*N*=493)	Without Seroclerarance (*N*=2072)	*p*-value	Adjusted Odds ratio(95%CI)[c]	*p*-value [c]
Male gender, *n* (%)	361 (73.2)	1325 (63.9)	<0.001[b]*	1.51(1.21~1.89)	< 0.001 *
Mean (SD) age (years)	50±9.74	46.3±9.53	<0.001[a]*	1.04 (1.03~1.05)	< 0.001 *
Age range (years)	30~65	30~65			
ALT (U/L)	17.7±23.8	16.5±19.8	0.24[a]	1.00(0.99~1.00)	0.665
Follow-up duration					
Interval date (years)	6.29± 3.90	7.79±4.48	<0.001[a]*	0.93 (0.91~0.95)	< 0.001 *
Male	6.66±3.76	9.17±3.58			
Female	5.21±4.09	5.40±4.86			

Data are presented as the number, mean ± standard deviation (SD), or median. ALT, alanine aminotransferase (normal range 5~40 U/L). *Significant at $p<0.05$. [a]By Student's *t*-test. [b]By Chi-squared test. [c]Adjusted for gender, age, and ALT.

Table 2. **Associations of *MPEG1* with hepatitis B surface antigen (HBsAg) seroclearance in 2565 hepatitis B virus patients**

SNP	Genotype	With Seroclearance (%) (*N*=493)	Without Seroclearance (%) (*N*=2072)	Genotype		Recessive	
				OR (95% CI)[a]	*p* value[a]	OR (95% CI)[a]	*p* value [a]
rs7944135	AA	33 (6.7)	77 (3.8)	1.76(1.14~2.72)	**0.045***.	1.74(1.13~2.66)	**0.014***.
	AG	148 (30.0)	600 (29.7)	1.05(0.84~1.31)		Reference	
	GG	312 (63.3)	1342 (66.5)	Reference			
rs171941	AA	35 (7.3)	145 (7.2)	0.86(0.69~1.06)	0.349	0.95(0.65~1.41)	0.811
	AG	170 (35.3)	775 (38.4)	0.90(0.60~1.33)		Reference	
	GG	277 (57.5)	1096 (54.4)	Reference			
rs6462008	TT	142 (29.5)	540 (26.9)	0.92(0.69~1.23)	0.519	1.01(0.79~1.28)	0.952
	GT	232 (48.1)	1015 (50.5)	0.87(0.69~1.10)		Reference	
	GG	108 (22.4)	456 (22.7)	Reference			

[a] Adjusted for gender, age and ALT.
the significant *p* value is in **bold** *.
SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 3. Minor allele frequencies of single nucleotide polymorphisms (SNPs) in this study

SNP	Position (hg38) (bp)	Nearest gene	Allele		Minor allele frequencies (MAFs)						
			Major	Minor	EUR	AFR	AMR	ASN	TWB	Ours	HWE
rs7944135	Chr11:59253514	*MPEG1*	G	A	0.49	0.34	0.43	0.26	0.18	0.19	0.81
rs171941	Chr 5:79884303	*CMYA5*	G	A	0.33	0.35	0.45	0.28	0.26	0.26	0.73
rs6462008	Chr 7:27309860	*EVX1*	G	T	0.64	0.71	0.65	0.51	0.48	0.47	0.51

EUR, European; AFR, African; AMR, American; ASN, Asian; TWB, Taiwan Biobank; HWE, *p* value for Hardy-Weinberg equilibrium test in our samples. MAFs of EUR, AFR, AMR and ASN were extracted from the HaploReg browser v4.1 **(https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php)**; MAFs of the TWB were obtained from the Taiwan View website (**https://taiwanview.twbiobank.org.tw/index**).

Table 4. *Cis*-expression of quantitative trait loci (*cis*-eQTL) results of the single-nucleotide polymorphism (SNP) from genotype-tissue expression (GTEx) database.

SNP ID	Gencode ID (ENSG00000-)	Gene symbol	*p*-value	Effect size	Tissue	Actions
rs7944135	197629	*MPEG1*	0.000072	0.11	Muscle - skeletal	AA>AG>GG
	110042	*DTX4*	1.5e-18	-0.33	Nerve-tibial	GG>AG>AA
	110042	*DTX4*	0.000015	-0.15	Muscle - skeletal	GG>AG>AA
	110042	*DTX4*	1.6e-7	-0.22	Lungs	GG>AG>AA
	110042	*DTX4*	0.000059	-0.16	Heart - left ventricle	GG>AG>AA
	110042	*DTX4*	2.7e-12	-0.31	Heart - atrial appendage	GG>AG>AA
	110042	*DTX4*	4.7e-17	-0.38	Esophagus - muscularis	GG>AG>AA
	110042	*DTX4*	5.6e-11	-0.29	Esophagus - mucosa	GG>AG>AA
	110042	*DTX4*	6.0e-9	-0.31	Esophagus - gastroesophageal junction	GG>AG>AA
	110042	*DTX4*	5.9e-9	-0.32	Cells - transformed fibroblasts	GG>AG>AA
	110042	*DTX4*	0.000032	-0.14	Adipose - subcutaneous	GG>AG>AA

Figure Legends

Figure 1. Cumulative fraction curve of *MPEG1* rs7944135 on hepatitis B surface antigen (HBsAg) seroclearance.

Figure 2. Cumulative fraction curve of the undetected hepatitis B virus (HBV) DNA of HBV patients according to the *MPEG1* rs7944135.

Figure 3. *Cis*-expression quantitative trait loci (*cis*-eQTLs) of *MPEG1* rs7944135 (Homo Alt (AA)>Het (AG)>Homo Ref (GG)) in muscle skeletal.

Figure 1.

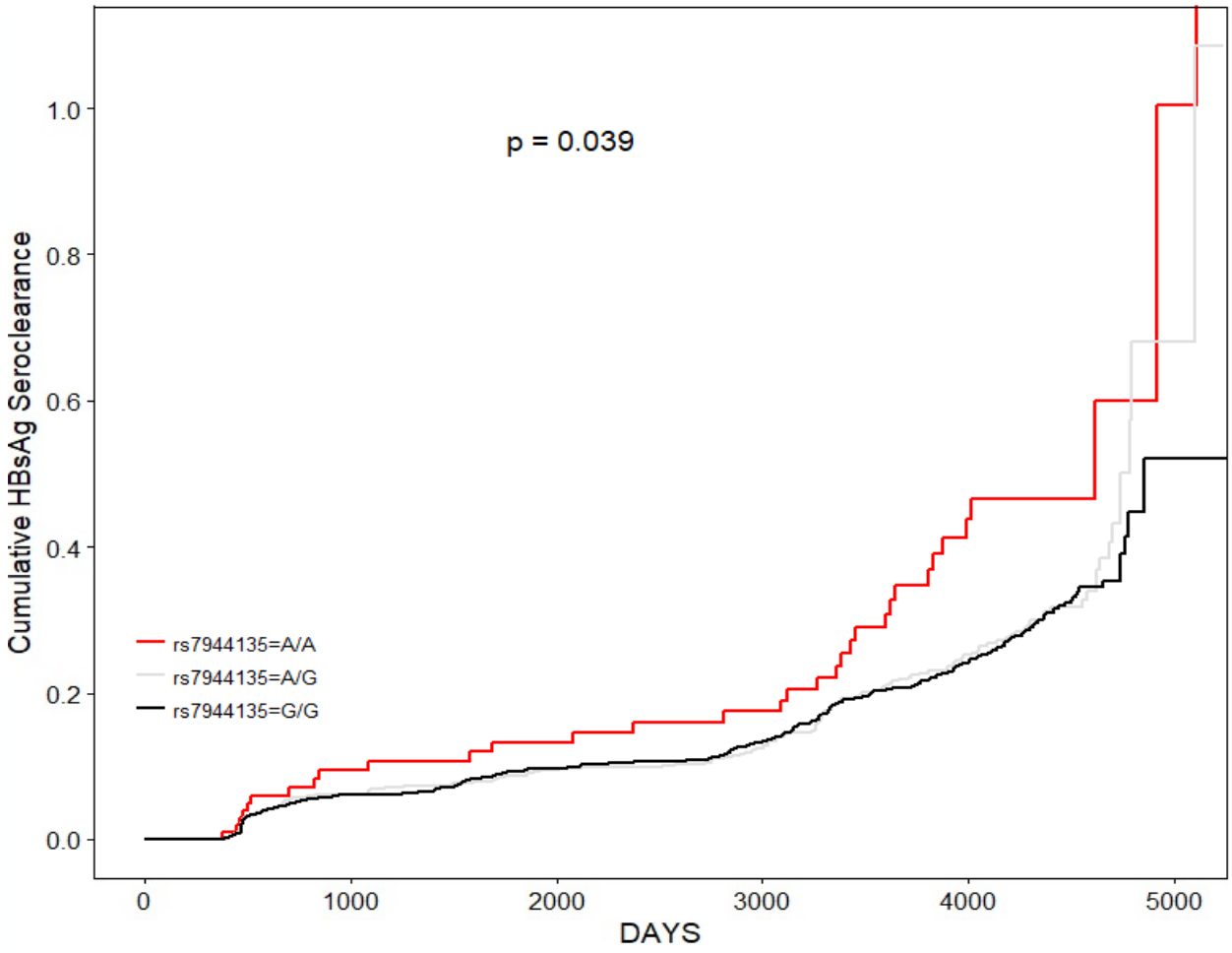

p = 0.039
Cumulative HBsAg Seroclearance
1.0
0.8
0.6
0.4
0.2
0.0
rs7944135=A/A
rs7944135=A/G
rs7944135=G/G
0
1000
2000
3000
4000
5000
DAYS

Figure 2.

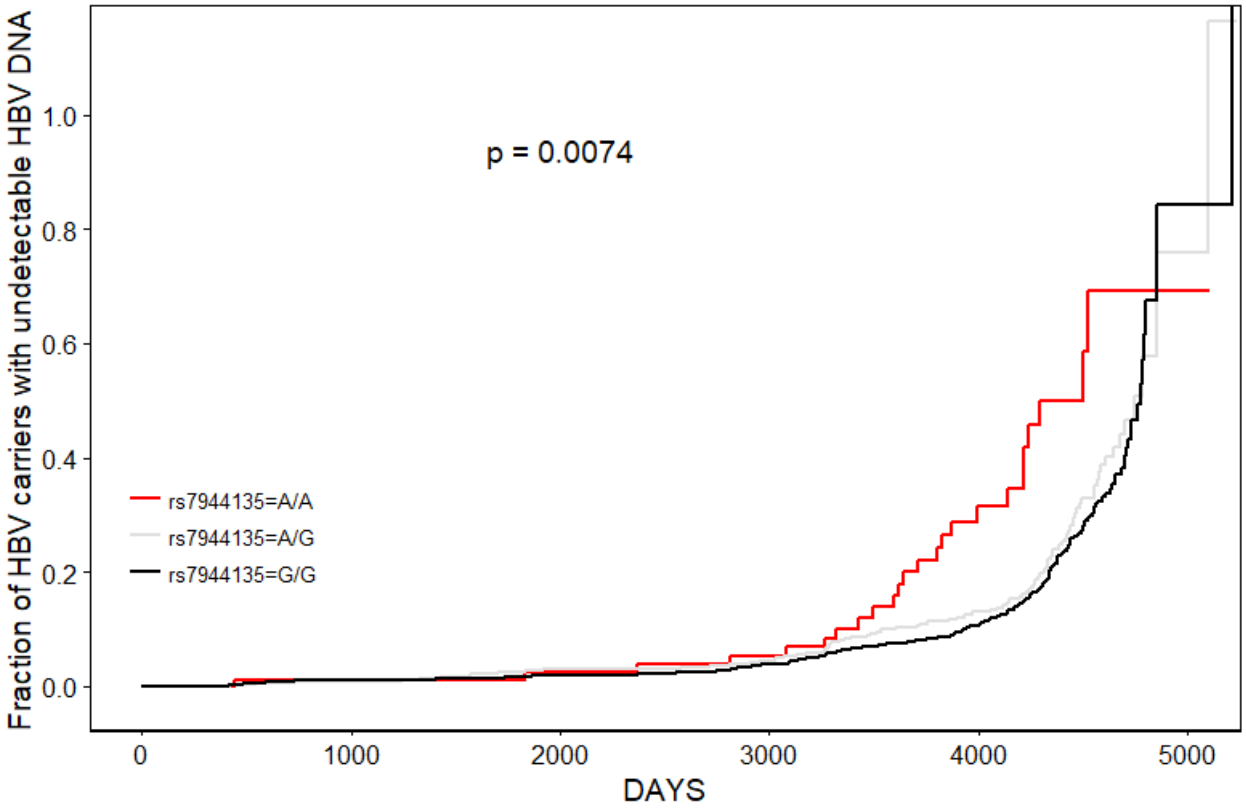
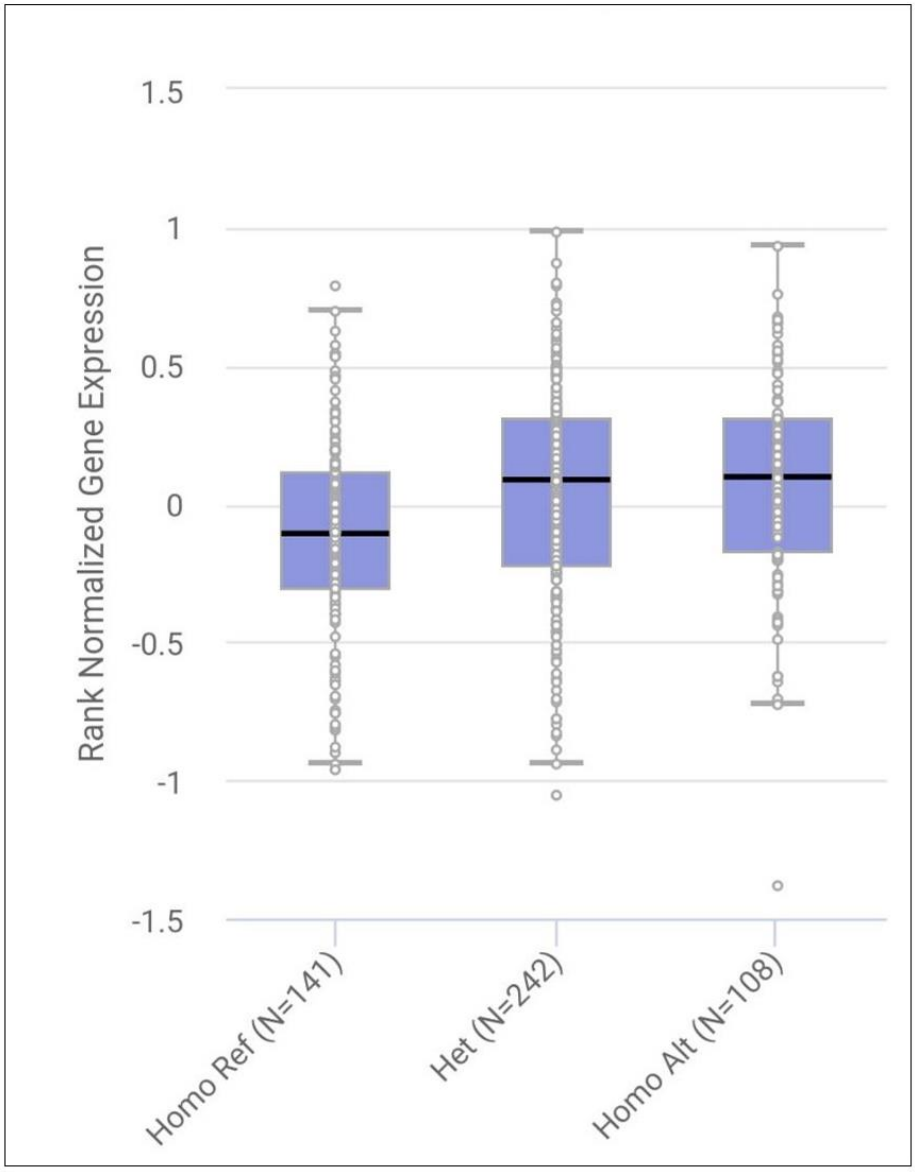

Fraction of HBV carriers with undetectable HBV DNA
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.0074
rs7944135=A/A
rs7944135=A/G
rs7944135=G/G
0
1000
2000
3000
4000
5000
DAYS

Figure 3.


Rank Normalized Gene Expression
1.5
1
0.5
0
-0.5
-1
-1.5
Homo Ref (N=141)
Het (N=242)
Homo Alt (N=108)

STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) ***Cohort study***—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	**Cohort Study 4 and 5**
		(*b*) ***Cohort study***—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4 and 5
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	4
Bias	9	Describe any efforts to address potential sources of bias	-
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	4
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	5 and 6
		(*c*) Explain how missing data were addressed	-
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	-
		(*e*) Describe any sensitivity analyses	-

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	-
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	-
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	-
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	4
		Cross-sectional study—Report numbers of outcome events or summary measures	-
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7 and 8
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	-
Discussion			
Key results	18	Summarise key results with reference to study objectives	10
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	11
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11
Generalisability	21	Discuss the generalisability (external validity) of the study results	10
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Medicine

Single-Nucleotide Polymorphism of rs7944135 (MPEG1) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study

--Manuscript Draft--

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Abstract:	Background Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment of patients with chronic hepatitis B (CHB) infection. Genetic, age and gender factors were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the SNP had been identified to be associated with HBsAg seroclearance in CHB patients Method Recently, three associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In our current study, these three SNPs were genotyped in 2,565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance. Result We observed that SNP rs7944135 was solely associated with HBsAg seroclearance. Subjects with the AA genotype at rs7944135 macrophage-expressed gene 1 (MPEG1) had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under genotypic model (odds ratio (OR)=1.76. 95% confidence interval (CI)=1.14~2.72, p=.045). Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG+GG of rs7944135 under the recessive model (OR=1.74. 95% CI=1.13~2.66, p=.014). According to the cumulative fraction curve with the log-rank test revealed patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance (p=.039) and HBV DNA undetectable (p=.0074) compared to those with the AG or GG genotype. Conclusion This study examined the associations of three SNPs (rs7944135, rs171941, and

	rs6462008) with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Dear Editor-in-Chief, ***Journal of Medicine***

This is to submit our revised manuscript entitled" Single-Nucleotide Polymorphism of rs7944135 (*MPEG1*) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study" for consideration of publication in the "***Medicine***" journal. Please be informed that this is a revised submission of our manuscript (**MD-D-19-04438**). We are thankful for your kind encouragement regarding to our manuscript. Herewith we are sending our revised manuscript in accordance with the comments given by the reviewer. The revised parts of the manuscript are highlighted in red color.

Lastly, we would like to thank you once again for providing us the opportunity to improve our manuscript. We hope that these revisions are adequate, and that the manuscript is now acceptable for publication in the "***Medicine" journal.***

Sincerely,

Wei-Chiao Chang (D.Phil.; Oxon)
Professor, Department of Clinical Pharmacy,
Director, Master Program for Clinical Pharmacogenomics and Pharmacoproteomics,
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Reviewer Comment:

Reviewer #1: The study re-checked the associations of three SNPs (rs7944135, rs171941, and rs6462008) with HBsAg seroclearance, and identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients. The study only found that patients with chronic hepatitis B with the *MPEG1* AA were more likely to have hepatitis B surface antigen clearance. The study lacks the mechanism related research.

Answer: We sincerely thank the reviewer for the time taken to review our work and the important suggestions given. Our results revealed that the rs7944135 SNP was solely associated with HBsAg seroclearance. It indicated that HBV carriers with the AA genotype of rs7944135 were more susceptible to HBsAg seroclearance compared to those with the AG or GG genotype of rs7944135. As discussed in the discussion part, genotypes of rs7944135 may affect the expression of *MPEG1* and therefore lead to different outcomes of the patients. The paragraph in the discussion part is shown as below: *MPEG1* is a specific gene that was identified in humans and mice in macrophages and has many roles in immune responses [1]. According to HaploReg V4.1, rs7944135 was located at 11q12.1 of *MPEG1,* and it is involved in the changing chromatin status of primary T cells from peripheral blood, which plays a central role in the "primary immune response" of cell-mediated immunity. Furthermore, the *MPEG1* gene has role in immune response through encoding perforin-like protein and recognition of antigen[2]. The perforin-like protein is predicted to be a perforin domain of membrane attack complex (MAC) that helps cytotoxic T cells and natural killer cells kill virus infected cell [3, 4]. In our study, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that rs7944135 AA genotype shows the highest expressed of *MPEG1* gene in compare to GG and AG genotype. It infers that patients with AA genotype

on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the hepatitis B surface Antigen (HBsAg). [line 265-272, page 11]

Reviewer #2: In this manuscript, the authors investigated association between three SNP in *MPEG1* and HBsAg seroclearance in patients with chronic HBV infection. They genotyped these SNPs in 2565 CHB patients with or without HBsAg seroclearance. They found that SNP rs7944135 was associated with HBsAg seroclearance. The patients with AA genotype have higher susceptibility to HBsAg seroclearance and undetectable HBV DNA, comparing with the patients with AG or GG genotypes.

Here are some concerns that need authors to address:

1. I think the therapy (type I interferon? Nucleoside analogues or spontaneous seroclearance?) of patients should be involved in the investigation. Since MPEG1 can be induced by interferon (doi: 10.1159/000345249), it may be a IFN stimulated gene which plays a role in the HBsAg seroclearance induced by type I interferon treatment or spontaneous seroclearance.

Answer: Thanks for your comment, we agree that the therapeutic agent is important for gene response in HBV infection including in HBsAg seroclearance. Interferon plays a critical role in human immune response, it may be induced *MPEG1* response in clearance of HBsAg in serum, and the interferon-induced genes also was reported inhibit HBV replication in transgenic mouse hepatocytes[5]. Clinically, the clearance of HBsAg in CHB as a safe marker for the discontinuation of antiviral treatment with nucleos(t)ide analogues; however, the

limitation of our study that we did not involve the subjects who received antiviral treatment with interferon alfa or nucleoside/nucleotide analogues treatment due to some reasons; treatment for HBV was not reimbursed by the universal national health insurance program in Taiwan until 2003, while the patients in this study were recruited between 1991 and 1992. In addition, at the time of recruitment, the patients were of poor disease awareness and medical accessibility. In previous report, it can be calculated that only 46% of the individuals with chronic HBV infection in Taiwan were aware that they were infected[6] Thus, in this study, the patients had no experience of antiviral treatment. The cohort in this study therefore presents the natural response against HBV without antiviral treatments. However, further studies are required to include antiviral treatments effect into analysis, if the patients had received antiviral treatment.

2.Does SNP rs7944135 affect MPEG1 gene expression? If yes, it will be helpful to explain the molecular mechanism by analyzing MPEG1 expression levels in the patients.

Answer: We thank the reviewer for the suggestions. As shown in Table 4 and Figure 3, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that individuals with AA genotype at rs7944135 show higher expression of *MPEG1* gene comparing to those with GG and AG genotypes. It infers that patients with AA genotype on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the HBsAg. [line 268-272, page 11]

Editorial Formatting Comments

1. Title page: Be sure the title page lists all author names, degrees, and affiliations.

Answer: Thank you for the comments. In this revision, we had an additional author, Wan-Hsuan Chou, who helped revise this manuscript. We have confirmed the authorship list in this revision.

2. Title: Be sure the title includes any specific terms as directed in the reporting guidelines for your type of article (for example, "case report" should be in the title of a CARE-compliant article). The following guidelines specify terms that should be in the title: CARE, CHEERS, CONSORT, PRISMA.

Answer: Thank you for your comments. We have revised the title accordingly.

3. Abstract: Be sure to use a structured abstract, with headings. Use the specific headings listed in the guidelines checklist if your report is based on the CARE, CHEERS, CONSORT, or PRISMA guidelines.

Answer: Thank you for your comments. We have included headings in the abstract.

4. List of abbreviations: If not already included, please include a list of abbreviations used in the text as part of the manuscript text file following the title page. Use abbreviations sparingly in the text, and spell them out the first time you use them. Abbreviations used in tables should be spelled out at the bottom of the table.

Answer: Thank you for your comments. We have added the list of abbreviation.

5. Ethical review, Methods section: If not already included, please state in the Methods section that an ethics committee or institutional review board approved the study, and list the board's name. If ethical approval was waived or not necessary, please state the reason. If the study involves patient consent, state explicitly that informed consent was or was not given, and state the reason if not given.

Answer: Thanks for the comments. We have described institutional review board approved in the method section.

6. Funding/Conflict of Interest information: List any source of funding or anything that could be perceived as a conflict of interest in the Acknowledgments section.

Answer: Thank you for your comments. We have stated the funding and conflict of interest.

7. Acknowledgments: If you list anyone by name in the Acknowledgments section, please confirm that the person gives permission to be named.

Answer: Thank you for your comments. We have confirmed the person who stated in the acknowledgements section.

8. License to Publish: If not already submitted, complete and submit a copy of the Open Access-License to Publish (LTP). The LTP is available to download from the home page of our website, under Forms. Be sure to select the kind of license you would like to use if the paper is accepted. (The different kinds of licenses determine how others can use your work after publication, varying from not restrictive at all to very restrictive.) Fill out all schedules (parts of the form). The corresponding author can sign the form on behalf of all authors; we need only one copy of the form. The form can be filled out and signed electronically, then uploaded as a submission item.

Answer: Thank you for your comments. The corresponding author has confirmed the statement through the electronic system.

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Single-Nucleotide Polymorphism of rs7944135 (*MPEG1*) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study

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Abstract

Introduction: Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment of patients with chronic hepatitis B (CHB) infection. Genetic, age and gender factors were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the SNP had been identified to be associated with HBsAg seroclearance in CHB patients.

Methods: Recently, three associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In our current study, these three SNPs were genotyped in 2,565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance.

Results: We observed that SNP rs7944135 was solely associated with HBsAg seroclearance**.** Subjects with the AA genotype at rs7944135 macrophage-expressed gene 1 (*MPEG1*) had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under genotypic model (odds ratio (OR)=1.76. 95% confidence interval (CI)=1.14~2.72, *p*=.045**)**. Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG+GG of rs7944135 under the recessive model (OR=1.74. 95% CI=1.13~2.66, *p*=.014). According to the cumulative fraction curve with the log-rank test revealed patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance (*p*=.039) and HBV DNA undetectable (*p*=.0074) compared to those with the AG or GG genotype.

Conclusion: This study examined the associations of three SNPs **(**rs7944135, rs171941, and rs6462008**)** with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Abbreviation: CHB=Chronic hepatitis B, CI= confidence interval, GWASs=Genome-wide association studies, HBsAg=Hepatitis B surface antigen, HBV= Hepatitis B virus , *MPEG1*= Macrophage-expressed gene 1, OR=odds ratio, SNPs= Single-nucleotide polymorphisms

Keywords: HBsAg seroclearance; HBV; CHB; rs7944135; single-nucleotide polymorphism

1. Introduction

The hepatitis B virus (HBV) was discovered over five decades ago. Although a prophylactic vaccine has been available, HBV infection still serious problem globally. World Health Organization (WHO, 2018) estimated that there were 257 million new cases and 887,000 deaths from HBV. Most of the deaths were caused by complications, including cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Eventually, the goal of therapy in CHB patients is to alleviate and prevent this complication.

Chronic hepatitis B (CHB) infection can be confirmed by its complicated serological pattern, while hepatitis B surface antigen (HBsAg) was reported to be well-established as a serum marker in the natural history of HBV infection. A low level of the HBsAg is associated with sustained immune control, seroclearance of HBsAg, and a lower risk of HCC [3]. A meta-analysis further confirmed the role of the HBsAg as a predictive marker of hepatitis B, liver cirrhosis, and HCC development, and the rate of spontaneous HBsAg seroclearance [4], an important milestone in the natural history of CHB infection. In addition, the serological profiles of chronic HBV infection also revealed that plasma HBV DNA levels can serve as a marker of disease progress [5]. In general, CHB is defined as the presence of the HBsAg for more than six months after an HBV infection [6] and spontaneous HBsAg seroclearance is defined as the loss of the HBsAg at least six months apart on two occasions and continuing to remain to absent up to the last visit [7]. However, the rate of HBsAg seroclearance in CHB patients is varied globally at an estimated 1%~2% annually [8], at approximately, 0.41% to 1.58% in Europe [9,10], 0.56 to 0.65% in north and south America, respectively [11,12] and 0.12%~2.38% per year in Asian [13], 0.4% per year reported in Korea [14], 2.5% in the Goto Islands of Japan [15] and 1.15% per year was recorded in Taiwan [13].

The clearance of the HBsAg in HBV infection is influenced by many factors, such as genetic and host factors including age, sex, and race. Several studies identified that host genetic polymorphisms may be associated with clinical outcomes of HBV, including HLA DQ (*HLA-DQ*) and DP (*HLA-DP*), [16,17], *IL28B* [18], *HLA-DPB1* [19], *Tumor Necrosis Factor-α* (*TNF-α*) [20], and *Monocyte Chemotactic Protein-1* (*MCP1*) [21]. Some CHB-associated loci, such as *HLA-DPB1*, *HLA-DQA2*, and *HLA-DQB,* also reported by previous GWASs, were identified in a Taiwanese population [22]. However, more the genetic studies still need to be examined in HBV

infection, considering of HBsAg seroclearance as a marker of cure of HBV infection. Recently, Kim *et al.* indicated that three new SNPs (rs7944135, rs171941, and rs6462008) were associated with HBsAg seroclearance in a Korean population. Thus, the purpose of this study was to investigate whether the polymorphisms reported by Korean group are associated with clearance of the HBsAg in Taiwanese HBV patients.

2. Materials and Methods

2.1. Study Subjects

In total, 2565 CHB patients (including 493 with HBsAg seroclearance and 2072 without HBsAg seroclearance) satisfied the inclusion criteria for follow-up of CHB, which were recruited during 1991 to 1992 from seven area in Taiwan (Sanchi, Chutung, Potzu, Kaoshu, Makung, Hushi, and Paisha). All of the study participants were ethnic Chinese (*i.e.*, Taiwanese). All participants in this study provided written informed consent before participation. This project was approved by the ethics committees at Academia Sinica, Taiwan.

2.2. Clinical Evaluation and SNP selection

All patients were tested for hepatitis B or virological markers in the liver, including HBsAg was measured using radioimmunoassay with commercial kits (Abbott Laboratories, North Chicago), HBV DNA were measured by polymerase chain reaction (PCR) using the Cobas Amplicor HBV monitor test kit (Roche Diagnostics, Indianapolis, Ind) and alanine transaminase (ALT) ALT using chemistry autoanalyzer (Model 736, Hitachi, Tokyo, Japan) using commercial reagents (Biomérieux, Marcy L'Etoile, France). HBsAg seroclearance were defined as loss of HBsAg in serum at least six months apart on two occasions and continued to absent up to the last visit. While without HBsAg seroclearance were defined as positive of HBsAg in serum for more than six months apart and continuously detected up to the last visit.

The SNPs **we investigated** in this current study were the replicated study from a GWAS applied in a Korean CHB population reported by TH Kim *et al.* (2018). TH Kim *et al.* (2018) reported **three** SNPs (**rs7944135, rs171941, rs6462008**) associated with seroclearance of the HBsAg in Korean CHB patients. These three SNPs were confirmed to exist in a Taiwanese CHB population.

2.3 DNA Extraction and Genotyping of the three SNPs

DNA was extracted from blood samples and subsequently centrifuged at 3000 rpm for 10 min at 4 ℃ to separate cells and plasma. Specimens were stored below -70 ℃. The buffy coat was isolated from blood samples, and red blood cells (RBCs) were lysed after the addition of RBC lysis buffer.

Three SNPs (rs7944135, rs171941, and rs6462008) were assessed by genotyping. Genotyping assays were performed using a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Polymerase chain reactions (PCRs) were subsequently performed in a 96-well microtiter plate with either an ABI7500 real-time PCR or ABI9700 Thermal Cycler under the following conditions: 10 min of denaturing at 95 ℃, followed by 45 cycles of 15 s of denaturing at 95 ℃ and 30 s for annealing at 60 ℃, with 1 min of a final extension at 60 ℃. Fluorescence signals from amplicons were analyzed using System SDS software vers. 1.2.3(Applied Biosystems, FosterCity, CA).

2.4. SNP annotation data query

Associations between gene expression profiles and the SNPs were confirmed by examining the expression quantitative trait loci (e-QTL) through (GTEx) Portal database (http://www.gtexportal.org/ home/). The GTEx database shows expressions of genes in a variety of tissues.

2.5. Statistical analysis

We performed all analyses using the R environment (https://cran.r-project.org/ and https://www.r-project.org/). We used Student's *t*-test to compare the age, ALT, and follow-up duration between the CHB patients with HBsAg seroclearance and without HBsAg seroclearance groups. We used logistic regression analyses to obtain adjusted OR between the case with HBsAg seroclearance and gender, age, ALT, and follow-up duration. Associations between SNPs and HBsAg seroclearance under genotype and recessive models were assessed using the "SNPassoc" package. The association between (the rs7944135 genotype AA, AG, and GG) and (HBsAg seroclearance and Undetectable of HBV DNA) were also modeled using time to event analysis utilized cumulative fraction curve using cumulative hazard function and log-rank test by

"Survival and survminer" package. While, days between enrollment to undetectable of HBV DNA and occurring HBsAg seroclearance was used at the time scale.

3. Results

3.1. Basic characteristics of chronic HBV-infected patients

We collected data on 2565 chronically HBV-infected subjects, which included 493 CHB with HBsAg sroclearance (361 males and 132 females) and 2072 CHB without HBsAg serclearance (1325 males and 747 females) in this study. The numbers of CHB male patients in the two groups included 361 with HBsAg seroclearance and 1325 without HBsAg seroclearance (p<0.001). Average ages in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 50 and 46.3 years, respectively (p<0.001), with an age range of 30~65 years. Mean of serum ALT values were 17.7 and 16.5 U/L, respectively (p=0.24) (**Table 1**). The lengths of follow up duration in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 6.29 years (6.66 years for male and 5.21 years for females) and 7.79 years (9.17 years for males and 5.40 years for females), respectively. **Table 1** shows that subjects with HBsAg seroclearance were more likely to be older (OR 1.04, 95% (1.03~1.05); p<0.001). In addition, HBsAg seroclearance tended to more likely appeared in males than in females (OR 1.51, 95% (1.21~1.89); P<0.001) even after adjusted for gender, age and ALT. The follow-up duration was significantly shorter (p<0.001) in the patients with HBsAg seroclearance than the group of patients without HBsAg seroclearnce.

3.2. Association of polymorphisms with HBsAg seroclearance

We investigated the association between genetic polymorphisms of three SNPs (rs7944135, rs171941, and rs6462008) and HBsAg seroclearance (**Table 2**). Genotype and recessive models were applied to assess associations of the HBsAg with the three SNPs. Our results revealed that the rs7944135 SNP was solely associated with HBsAg seroclearance. The rs7944135 SNP was found to be significantly associated with HBsAg seroclearance in the genotype and recessive model after adjusted with gender, age and ALT, at p<0.05. This result indicated that HBV carriers with the AA genotype of rs7944135 were more susceptible to HBsAg seroclearance compared to those with the AG or GG genotype of rs7944135. However, the two other SNPs (rs171941, and rs6462008) showed no correlation with HBsAg seroclearance (**Table 2**). A significant association between the rs7944135 AA genotype and HBsAg seroclearance was found 1.76-fold higher susceptibility to clearance of the HBsAg, compared to those with the AG or GG genotype after adjusting for gender, age and ALT, (OR=1.76. 95% (1.14~2.72), p=.045**)**.

Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype of rs7944135 under the recessive model (OR=1.74, 95% (1.13~2.66), *p*=.014).

3.3. The minor Allele frequency of three SNPs in different populations

The **Table 3** shows the minor allele frequency (MAF) of three SNPs (rs7944135, rs171941, and rs6462008) in different populations (*e.g.*, Asian and European), which were these two population were reported higher susceptible to have HBsAg seroclearance in compare to another region worldwide [23]. MAFs of African, American, European and Asian were extracted from the HaploReg browser v4.1 **(**https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php**)**, and MAF of the TWB were adapted from the Taiwan biobank website (https://taiwanview.twbiobank.org.tw/index). **Table 3** showed that our result was close to those in reference to Asian including a Taiwanese population.

3.4. Correlation between *MPEG1* rs7944135 and days of HBsAg seroclearance

In addition, we confirmed the correlation between the time taken to HBsAg seroclearance and the genotype of the *MPEG1* gene SNP rs7944135. Log rank test showed a significant differences in the time taken to occur HBsAg seroclearance among the *MPEG1* gene rs7944135 genotypes of AA, AG, and GG (*p*=.039). The cumulative fraction curve showed a trend of HBV carriers with the AA genotype of rs7944135 indicated higher susceptibility to HBsAg seroclearnce compared to those with the AG or GG genotype (**Figure 1**).

3.5. Correlation between the *MPEG1* rs7944135 genotype and days of HBV DNA undetectable

We further investigated the correlation between the time taken to HBV DNA undetectable and *MPEG1* gene rs7944135 genotypes: AA, AG, and GG. Log rank test showed significant differences among *MPEG1* gene SNP rs7944135 genotypes of AA, AG, and GG in the time taken to HBV DNA undetectable (*p*=.0074). The cumulative fraction curve showed that a trend of the AA genotype of rs7944135 of HBV carriers showed a higher rate to undetected of HBV DNA versus the AG and GG genotypes (**Figure 2**).

3.6. SNP annotation of expression quantitative trait loci (e-QTLs) of rs7944135

To elucidate the association between SNP *rs7944135* and gene expression, we utilized the publicly available databases GTEx Portal (http:// www.gtexportal.org/home/) to obtain the tissue expression quantitative trait loci. As shown in **Table 4** and **Figure 3** the AA genotype of *rs7944135* had highly expressed of *MPEG1* gene when compared to AG and GG genotype.

4. Discussion

Clearance of the HBsAg is the important indicator of recovery from CHB [24]. It was reported that HBsAg seroclearance is associated with the prognosis of CHB [25]. Clearance of the HBsAg in HBV is influenced by many factors, including genetic and host factors such as age, sex, and race [26]. Our approach in this study was to confirm associations the factors of genetic, age, and gender with HBsAg seroclearance. In the current study, we replicated a GWAS study of Korean CHB patients by a candidate gene approach of CHB Taiwan patients to confirm genes associated with HBsAg seroclearance in CHB Taiwanese population. Kim *et al* revealed that three SNPs of rs6462008 located near even-skipped homeobox 1 (*EVX1*), rs171941 located near cardiomyopathy associated 5 (*CMYA5*), and rs7944135 located near *MPEG1* were associated with HBsAg seroclearance in korean population [27]. Recently, we successfully replicated an association of rs7944135 in *MPEG1* with HBsAg seroclearance in a Taiwanese CHB population.

The male patients are mostly more susceptible to the occurrence of HBsAg seroclearance compared to female patients [13,27]. Consistently, our current study showed that male patients tended to be more susceptible than female patients even after adjusted for gender, age, and ALT, which suggests that male patients have stronger immune responses than female patients. Similar to previous studies from Korea and Taiwan [13,28], this reason may due to hormonal involvement in the immune response [29]. The age at HBsAg seroclearance also can be considered a factor. Our study identified that the mean age at HBsAg seroclearance was 50 years. This finding is relatively close to previous studies in Hong Kong (mean, 48.8 years), Korea (mean, 50 years), and Japan (mean, 51 years) [25,30,31].

This study showed that rs7944135 in *MPEG1* is significantly associated with HBsAg seroclearance in a Taiwanese CHB population. We observed that subjects with the AA genotype of *MPEG1* rs7944135 had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype (OR=1.76). In addition, we still found an association in rs7944135 with 1.74-fold increased risk of the AA genotype of HBsAg seroclearance occurring under the recessive model (OR=1.74). Spontaneous HBsAg seroclearance can be predicted through serum levels of the HBsAg and HBV DNA [24,32]. In terms of the time taken to the HBsAg seroclearance and undetected of HBV DNA, results showed that the rs7944135 AA genotype had a higher susceptibility to HBsAg seroclearance and HBV DNA undetectable compared to the AG and GG genotypes. A correlation between HBsAg and HBV DNA in serum was reported by Li

et al. (2007) and revealed that the HBsAg level was not detected during the immune clearance phase as a result of declining HBV DNA levels. In addition, Coffin *et al.* (2019) revealed that with a lower level of the HBsAg, there was a greater likelihood for loss of the HBsAg. The HBsAg at a low level was associated with a lower risk of prognosis of HCC [3]. It is well known that the HBsAg contributes to the immunopathogenesis of persistent HBV infection, and a higher incidence of HBsAg seroclearance was associated with lower HBsAg levels [33].

MPEG1 is a specific gene that was identified in humans and mice in macrophages and has many roles in immune responses [34]. According to HaploReg V4.1, rs7944135 was located at 11q12.1 of *MPEG1*, and it is involved in the changing chromatin status of primary T cells from peripheral blood, which plays a central role in the "primary immune response" of cell-mediated immunity, this may be suggested the role of *MPEG1* in immune clearance of HBsAg in HBV infection. Furthermore, the *MPEG1* gene has role in immune response through encoding perforin-like protein and recognition of antigen[35]. The perforin-like protein is predicted to be a perforin domain of membrane attack complex (MAC) that helps cytotoxic T cells and natural killer cells kill virus infected cell [36,37]. In our study, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that rs7944135 AA genotype shows the highest expressed of *MPEG1* gene in compare to GG and AG genotype. It infers that patients with AA genotype on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the HBsAg.

Additional genes, such as HLA, cytokine tumor necrosis factor (*TNF*)-α, chemokine receptor 5 (*CCR5*) [38], and *MCP1* [21], were found to be associated with the incidence of HBsAg seroclearance through cell-mediated immune responses. Some evidence has been provided that polymorphisms in HLA subtypes are significantly associated with the occurrence of HBsAg seroclearance, such as a polymorphism of *HLA-DP* rs3077 with haplotype GAT having a 2.17-fold association in a Chinese population [39], *HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535 with A alleles in a Japanese population [40], and *HLA-B*4001* in Taiwanese aborigines [41]. There are many polymorphisms of the TNF-α promoter region that were reported to alter TNF-α-associated HBV clearance in many populations [42]. *MCP1* with -2518G>A was associated with HBV clearance in a Korean population [21].

In this study, we investigated the association of individual SNPs with susceptibility to HBsAg seroclearance in Taiwanese CHB patients. We acknowledge that our study needs to be validated by future studies in other population using more samples with multiple polymorphisms. Therefore, this could give enlightenment for the genetic factors that associated in the occurrence of HBsAg seroclearance. However, this study offers the novel finding of a polymorphism of *MPEG1* having a pivotal association with HBsAg seroclearance in Taiwanese CHB patients.

5. Conclusion

Our current study is a replication of an associated study of a SNP of *MPEG1* rs7944135, with susceptibility to HBsAg seroclearance and showed that an HBsAg seroclearance-associated SNP, rs7944135 with the AA genotype, solely has a significant association with the loss of the HBsAg in CHB infection in Taiwanese HBV patients.

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Author Contributions

Conceptualization : Lalu Muhammad Irham., Henry Sung-Ching Wong.,Wei-Chiao Chang.

Data Curation : Lalu Muhammad Irham

Formal Analyses :Lalu Muhammad Irham

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Writing – original draft : Lalu Muhammad Irham

Conflicts of Interest

The authors disclose no conflict

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Figure Legends

Figure 1. Cumulative fraction curve of *MPEG1* rs7944135 on hepatitis B surface antigen (HBsAg) seroclearance.

Figure 2. Cumulative fraction curve of the undetected hepatitis B virus (HBV) DNA of HBV patients according to the *MPEG1* rs7944135**.**

Figure 3. *Cis*-expression quantitative trait loci (*cis*-eQTLs) of *MPEG1* rs7944135 (Homo Alt (AA)>Het (AG)>Homo Ref (GG)) in muscle skeletal.

Table 1

Table 1. **Baseline characteristics of the 2565 hepatitis B virus study participants**

Characteristic	With Seroclearance (*N*=493)	Without Seroclerarance (*N*=2072)	*p*-value	Adjusted Odds ratio(95%CI)[c]	*p*-value [c]
Male gender, *n* (%)	361 (73.2)	1325 (63.9)	<0.001[b]*	1.51(1.21~1.89)	< 0.001 *
Mean (SD) age (years)	50±9.74	46.3±9.53	<0.001[a]*	1.04 (1.03~1.05)	< 0.001 *
Age range (years)	30~65	30~65			
ALT (U/L)	17.7±23.8	16.5±19.8	0.24[a]	1.00(0.99~1.00)	0.665
Follow-up duration					
Interval date (years)	6.29± 3.90	7.79±4.48	<0.001[a]*	0.93 (0.91~0.95)	< 0.001 *
Male	6.66±3.76	9.17±3.58			
Female	5.21±4.09	5.40±4.86			

Data are presented as the number, mean ± standard deviation (SD), or median. ALT, alanine aminotransferase (normal range 5~40 U/L). *Significant at $p<0.05$. [a]By Student's *t*-test. [b]By Chi-squared test. [c]Adjusted for gender, age, and ALT.

Table 2

Table 2. **Associations of *MPEG1* with hepatitis B surface antigen seroclearance in 2565 hepatitis B virus patients**

SNP	Genotype	With Seroclearance (%) (*N*=493)	Without Seroclearance (%) (*N*=2072)	Genotype		Recessive	
				OR (95% CI)[a]	*p* value[a]	OR (95% CI)[a]	*p* value [a]
rs7944135	AA	33 (6.7)	77 (3.8)	1.76(1.14~2.72)	**0.045***.	1.74(1.13~2.66)	**0.014***.
	AG	148 (30.0)	600 (29.7)	1.05(0.84~1.31)		Reference	
	GG	312 (63.3)	1342 (66.5)	Reference			
rs171941	AA	35 (7.3)	145 (7.2)	0.86(0.69~1.06)	0.349	0.95(0.65~1.41)	0.811
	AG	170 (35.3)	775 (38.4)	0.90(0.60~1.33)		Reference	
	GG	277 (57.5)	1096 (54.4)	Reference			
rs6462008	TT	142 (29.5)	540 (26.9)	0.92(0.69~1.23)	0.519	1.01(0.79~1.28)	0.952
	GT	232 (48.1)	1015 (50.5)	0.87(0.69~1.10)		Reference	
	GG	108 (22.4)	456 (22.7)	Reference			

[a] Adjusted for gender, age and ALT.
the significant *p* value is in **bold ***.
SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 3. Minor allele frequencies of single nucleotide polymorphisms in this study

SNP	Position (hg38) (bp)	Nearest gene	Allele		Minor allele frequencies (MAFs)						
			Major	Minor	EUR	AFR	AMR	ASN	TWB	Ours	HWE
rs7944135	Chr11:59253514	*MPEG1*	G	A	0.49	0.34	0.43	0.26	0.18	0.19	0.81
rs171941	Chr 5:79884303	*CMYA5*	G	A	0.33	0.35	0.45	0.28	0.26	0.26	0.73
rs6462008	Chr 7:27309860	*EVX1*	G	T	0.64	0.71	0.65	0.51	0.48	0.47	0.51

EUR, European; AFR, African; AMR, American; ASN, Asian; TWB, Taiwan Biobank; HWE, *p* value for Hardy-Weinberg equilibrium test in our samples. MAFs of EUR, AFR, AMR and ASN were extracted from the **HaploReg browser v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php)**; MAFs of the TWB were obtained from the Taiwan View website (**https://taiwanview.twbiobank.org.tw/index**).

Table 4. *Cis*-expression of quantitative trait loci results of the single-nucleotide polymorphism from genotype-tissue expression database.

SNP ID	Gencode ID (ENSG00000-)	Gene symbol	*p*-value	Effect size	Tissue	Actions
rs7944135	197629	*MPEG1*	0.000072	0.11	Muscle - skeletal	AA>AG>GG
	110042	*DTX4*	1.5e-18	-0.33	Nerve-tibial	GG>AG>AA
	110042	*DTX4*	0.000015	-0.15	Muscle - skeletal	GG>AG>AA
	110042	*DTX4*	1.6e-7	-0.22	Lungs	GG>AG>AA
	110042	*DTX4*	0.000059	-0.16	Heart - left ventricle	GG>AG>AA
	110042	*DTX4*	2.7e-12	-0.31	Heart - atrial appendage	GG>AG>AA
	110042	*DTX4*	4.7e-17	-0.38	Esophagus - muscularis	GG>AG>AA
	110042	*DTX4*	5.6e-11	-0.29	Esophagus - mucosa	GG>AG>AA
	110042	*DTX4*	6.0e-9	-0.31	Esophagus - gastroesophageal junction	GG>AG>AA
	110042	*DTX4*	5.9e-9	-0.32	Cells - transformed fibroblasts	GG>AG>AA
	110042	*DTX4*	0.000032	-0.14	Adipose - subcutaneous	GG>AG>AA

SNP, single-nucleotide polymorphism.

Figure 1

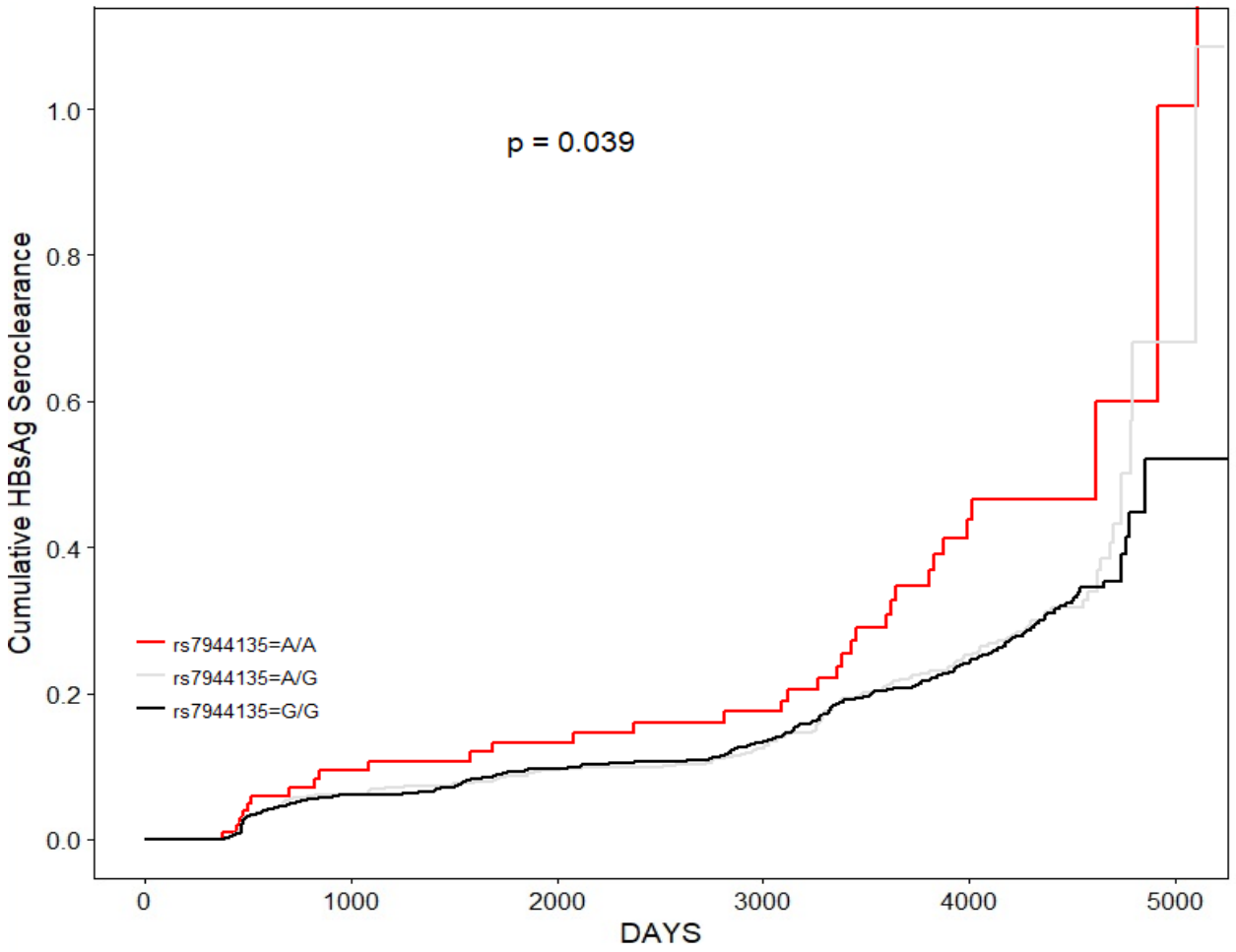


Figure 2

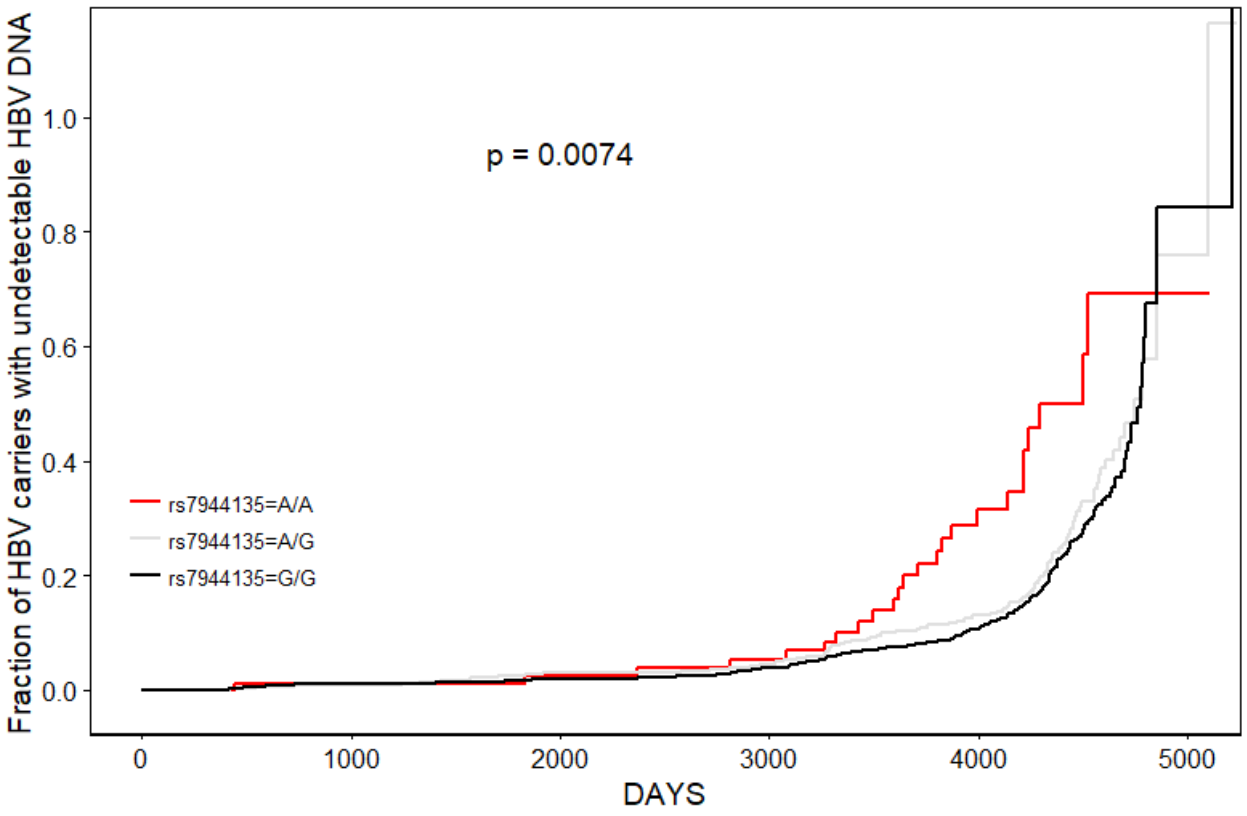
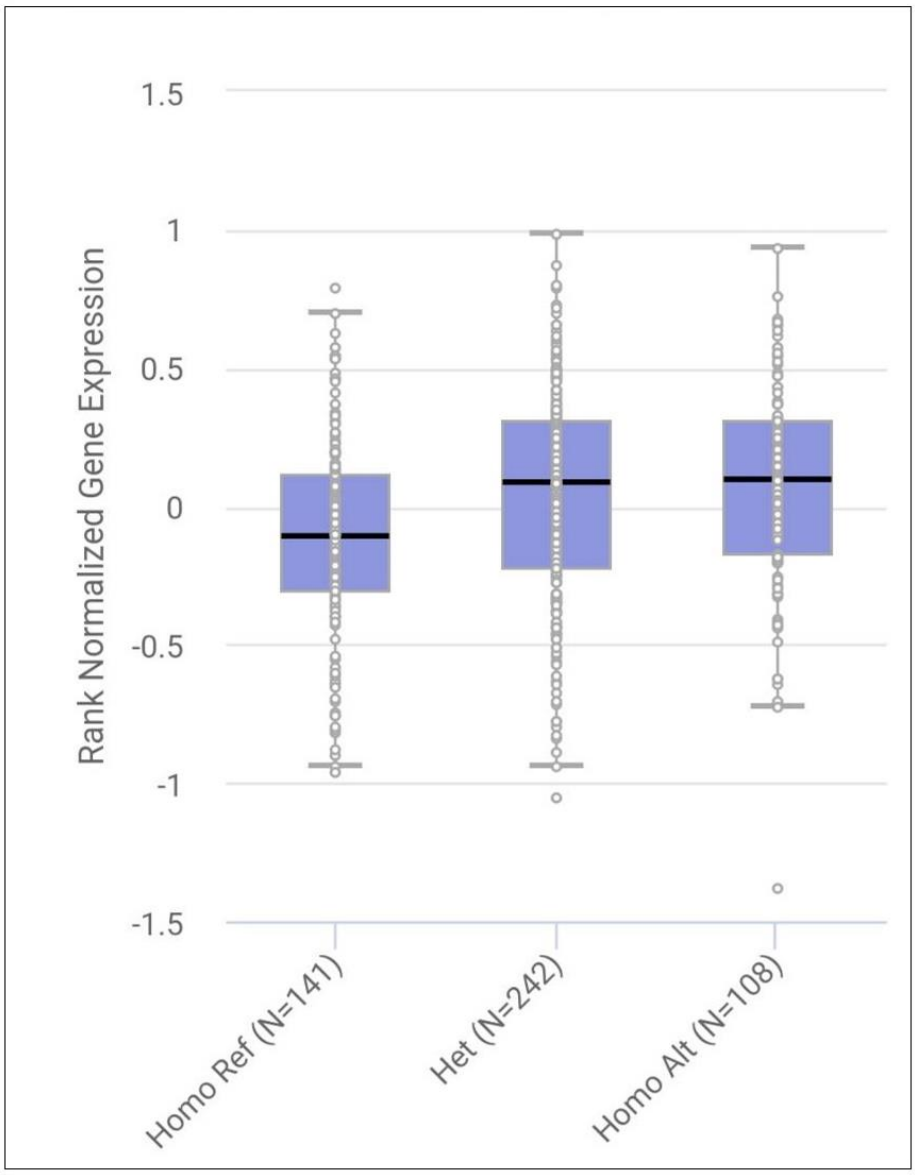


Figure 3



STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) ***Cohort study***—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	**Cohort Study 4 and 5**
		(*b*) ***Cohort study***—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4 and 5
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	4
Bias	9	Describe any efforts to address potential sources of bias	-
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	4
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	5 and 6
		(*c*) Explain how missing data were addressed	-
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	-
		(*e*) Describe any sensitivity analyses	-

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	-
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	-
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	-
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	4
		Cross-sectional study—Report numbers of outcome events or summary measures	-
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7 and 8
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	-
Discussion			
Key results	18	Summarise key results with reference to study objectives	10
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	11
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11
Generalisability	21	Discuss the generalisability (external validity) of the study results	10
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Medicine

Single-Nucleotide Polymorphism of rs7944135 (MPEG1) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study

--Manuscript Draft--

Manuscript Number:	MD-D-19-04438R3
Article Type:	OA: Observational Study (STROBE Compliant)
Section/Category:	4500 Gastroenterology and hepatology
Keywords:	HBsAg seroclearance; HBV; CHB; rs7944135; single-nucleotide polymorphism
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Manuscript Region of Origin:	TAIWAN
Abstract:	Background Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment of patients with chronic hepatitis B (CHB) infection. Genetic, age and gender factors were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the SNP had been identified to be associated with HBsAg seroclearance in CHB patients Method Recently, three associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In our current study, these three SNPs were genotyped in 2,565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance. Result We observed that SNP rs7944135 was solely associated with HBsAg seroclearance. Subjects with the AA genotype at rs7944135 macrophage-expressed gene 1 (MPEG1) had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under genotypic model (odds ratio (OR)=1.76. 95% confidence interval (CI)=1.14~2.72, p=.045). Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG+GG of rs7944135 under the recessive model (OR=1.74. 95% CI=1.13~2.66, p=.014). According to the cumulative fraction curve with the log-rank test revealed patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance (p=.039) and HBV DNA undetectable (p=.0074) compared to those with the AG or GG genotype. Conclusion This study examined the associations of three SNPs (rs7944135, rs171941, and rs6462008) with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Dear Editor-in-Chief, ***Journal of Medicine***

This is to submit our revised manuscript entitled" Single-Nucleotide Polymorphism of rs7944135 (*MPEG1*) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study" for consideration of publication in the "***Medicine***" journal. Please be informed that this is a revised submission of our manuscript (**MD-D-19-04438R2**). We are thankful for your kind encouragement regarding to our manuscript. Herewith we are sending our revised manuscript in accordance with the comments given by the reviewer.

Lastly, we would like to thank you once again for providing us the opportunity to improve our manuscript. We hope that these revisions are adequate, and that the manuscript is now acceptable for publication in the "***Medicine" journal.***

Sincerely,

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Reviewer #1: Although the study examined the different incidence of rs7944135 of 2565 in 493 with HBsAg seroclearance and 2072 without HBsAg seroclearance CHB patients and found that the rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.
As the authors adressed that there were other genes (HLA, TNF-α, CCR5, and MCP1) associated with the incidence of HBsAg seroclearance, which did not examine in the above patients.
Could the authors did some work to exclude the potential impact of the above genes on the HBsAg seroclearance?

Answer: Thanks for reviewer's comments. According to the suggestions, we comprehensively screened the minor allele frequency of the SNPs on the genes including TNF-α, CCR5, and MCP1 from Taiwan Biobank (1517 healthy subjects). The variant of CCR5 Delta32 was reported in the Indian population, however, frequency of this variant is less than 1% [1]. Regarding to the variant of MCP1 (2518G>A), it was identified in the Korean population [2], however, this variant can't be detected in the Taiwanese population. In addition, two variants (rs1800630 and rs1799964) in TNF were reported to associate with HBsAg seroclearance in the Indian population [3]. The minor allele frequency of these two variants in the Taiwanese population are 0.14 and 0.17 respectively. However, these two variants were not the most significant targets from the Korean GWAS report [4]. The aim of this study is to focus on the validation of *MPEG1* from the Korean GWAS results (100 case and 100 control). In this study, we largely increased the sample size to 2565 subjects (493 with HBsAg seroclearance and 2072 without HBsAg seroclearance) and confirmed the critical role of rs7944135 in HBsAg seroclearance.

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Single-Nucleotide Polymorphism of rs7944135 (*MPEG1*) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study

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Abstract

Introduction: Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment of patients with chronic hepatitis B (CHB) infection. Genetic, age and gender factors were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the SNP had been identified to be associated with HBsAg seroclearance in CHB patients.

Methods: Recently, three associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In our current study, these three SNPs were genotyped in 2,565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance.

Results: We observed that SNP rs7944135 was solely associated with HBsAg seroclearance**.** Subjects with the AA genotype at rs7944135 macrophage-expressed gene 1 (*MPEG1*) had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under genotypic model (odds ratio (OR)=1.76. 95% confidence interval (CI)=1.14~2.72, *p*=.045**)**. Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG+GG of rs7944135 under the recessive model (OR=1.74. 95% CI=1.13~2.66, *p*=.014). According to the cumulative fraction curve with the log-rank test revealed patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance (*p*=.039) and HBV DNA undetectable (*p*=.0074) compared to those with the AG or GG genotype.

Conclusion: This study examined the associations of three SNPs **(**rs7944135, rs171941, and rs6462008**)** with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Abbreviation: CHB=Chronic hepatitis B, CI= confidence interval, GWASs=Genome-wide association studies, HBsAg=Hepatitis B surface antigen, HBV= Hepatitis B virus , *MPEG1*= Macrophage-expressed gene 1, OR=odds ratio, SNPs= Single-nucleotide polymorphisms

Keywords: HBsAg seroclearance; HBV; CHB; rs7944135; single-nucleotide polymorphism

1. Introduction

The hepatitis B virus (HBV) was discovered over five decades ago. Although a prophylactic vaccine has been available, HBV infection still serious problem globally. World Health Organization (WHO, 2018) estimated that there were 257 million new cases and 887,000 deaths from HBV. Most of the deaths were caused by complications, including cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Eventually, the goal of therapy in CHB patients is to alleviate and prevent this complication.

Chronic hepatitis B (CHB) infection can be confirmed by its complicated serological pattern, while hepatitis B surface antigen (HBsAg) was reported to be well-established as a serum marker in the natural history of HBV infection. A low level of the HBsAg is associated with sustained immune control, seroclearance of HBsAg, and a lower risk of HCC [3]. A meta-analysis further confirmed the role of the HBsAg as a predictive marker of hepatitis B, liver cirrhosis, and HCC development, and the rate of spontaneous HBsAg seroclearance [4], an important milestone in the natural history of CHB infection. In addition, the serological profiles of chronic HBV infection also revealed that plasma HBV DNA levels can serve as a marker of disease progress [5]. In general, CHB is defined as the presence of the HBsAg for more than six months after an HBV infection [6] and spontaneous HBsAg seroclearance is defined as the loss of the HBsAg at least six months apart on two occasions and continuing to remain to absent up to the last visit [7]. However, the rate of HBsAg seroclearance in CHB patients is varied globally at an estimated 1%~2% annually [8], at approximately, 0.41% to 1.58% in Europe [9,10], 0.56 to 0.65% in north and south America, respectively [11,12] and 0.12%~2.38% per year in Asian [13], 0.4% per year reported in Korea [14], 2.5% in the Goto Islands of Japan [15] and 1.15% per year was recorded in Taiwan [13].

The clearance of the HBsAg in HBV infection is influenced by many factors, such as genetic and host factors including age, sex, and race. Several studies identified that host genetic polymorphisms may be associated with clinical outcomes of HBV, including HLA DQ (*HLA-DQ*) and DP (*HLA-DP*), [16,17], *IL28B* [18], *HLA-DPB1* [19], *Tumor Necrosis Factor-α* (*TNF-α*) [20], and *Monocyte Chemotactic Protein-1* (*MCP1*) [21]. Some CHB-associated loci, such as *HLA-DPB1*, *HLA-DQA2*, and *HLA-DQB,* also reported by previous GWASs, were identified in a Taiwanese population [22]. However, more the genetic studies still need to be examined in HBV

infection, considering of HBsAg seroclearance as a marker of cure of HBV infection. Recently, Kim *et al.* indicated that three new SNPs (rs7944135, rs171941, and rs6462008) were associated with HBsAg seroclearance in a Korean population. Thus, the purpose of this study was to investigate whether the polymorphisms reported by Korean group are associated with clearance of the HBsAg in Taiwanese HBV patients.

2. Materials and Methods

2.1. Study Subjects

In total, 2565 CHB patients (including 493 with HBsAg seroclearance and 2072 without HBsAg seroclearance) satisfied the inclusion criteria for follow-up of CHB, which were recruited during 1991 to 1992 from seven area in Taiwan (Sanchi, Chutung, Potzu, Kaoshu, Makung, Hushi, and Paisha). All of the study participants were ethnic Chinese (*i.e.*, Taiwanese). All participants in this study provided written informed consent before participation. This project was approved by the ethics committees at Academia Sinica, Taiwan.

2.2. Clinical Evaluation and SNP selection

All patients were tested for hepatitis B or virological markers in the liver, including HBsAg was measured using radioimmunoassay with commercial kits (Abbott Laboratories, North Chicago), HBV DNA were measured by polymerase chain reaction (PCR) using the Cobas Amplicor HBV monitor test kit (Roche Diagnostics, Indianapolis, Ind) and alanine transaminase (ALT) ALT using chemistry autoanalyzer (Model 736, Hitachi, Tokyo, Japan) using commercial reagents (Biomérieux, Marcy L'Etoile, France). HBsAg seroclearance were defined as loss of HBsAg in serum at least six months apart on two occasions and continued to absent up to the last visit. While without HBsAg seroclearance were defined as positive of HBsAg in serum for more than six months apart and continuously detected up to the last visit.

The SNPs **we investigated** in this current study were the replicated study from a GWAS applied in a Korean CHB population reported by TH Kim *et al.* (2018). TH Kim *et al.* (2018) reported **three** SNPs (**rs7944135, rs171941, rs6462008**) associated with seroclearance of the HBsAg in Korean CHB patients. These three SNPs were confirmed to exist in a Taiwanese CHB population.

2.3 DNA Extraction and Genotyping of the three SNPs

DNA was extracted from blood samples and subsequently centrifuged at 3000 rpm for 10 min at 4 ℃ to separate cells and plasma. Specimens were stored below -70 ℃. The buffy coat was isolated from blood samples, and red blood cells (RBCs) were lysed after the addition of RBC lysis buffer.

Three SNPs (rs7944135, rs171941, and rs6462008) were assessed by genotyping. Genotyping assays were performed using a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Polymerase chain reactions (PCRs) were subsequently performed in a 96-well microtiter plate with either an ABI7500 real-time PCR or ABI9700 Thermal Cycler under the following conditions: 10 min of denaturing at 95 ℃, followed by 45 cycles of 15 s of denaturing at 95 ℃ and 30 s for annealing at 60 ℃, with 1 min of a final extension at 60 ℃. Fluorescence signals from amplicons were analyzed using System SDS software vers. 1.2.3(Applied Biosystems, FosterCity, CA).

2.4. SNP annotation data query

Associations between gene expression profiles and the SNPs were confirmed by examining the expression quantitative trait loci (e-QTL) through (GTEx) Portal database (http://www.gtexportal.org/ home/). The GTEx database shows expressions of genes in a variety of tissues.

2.5. Statistical analysis

We performed all analyses using the R environment (https://cran.r-project.org/ and https://www.r-project.org/). We used Student's *t*-test to compare the age, ALT, and follow-up duration between the CHB patients with HBsAg seroclearance and without HBsAg seroclearance groups. We used logistic regression analyses to obtain adjusted OR between the case with HBsAg seroclearance and gender, age, ALT, and follow-up duration. Associations between SNPs and HBsAg seroclearance under genotype and recessive models were assessed using the "SNPassoc" package. The association between (the rs7944135 genotype AA, AG, and GG) and (HBsAg seroclearance and Undetectable of HBV DNA) were also modeled using time to event analysis utilized cumulative fraction curve using cumulative hazard function and log-rank test by

"Survival and survminer" package. While, days between enrollment to undetectable of HBV DNA and occurring HBsAg seroclearance was used at the time scale.

3. Results

3.1. Basic characteristics of chronic HBV-infected patients

We collected data on 2565 chronically HBV-infected subjects, which included 493 CHB with HBsAg sroclearance (361 males and 132 females) and 2072 CHB without HBsAg serclearance (1325 males and 747 females) in this study. The numbers of CHB male patients in the two groups included 361 with HBsAg seroclearance and 1325 without HBsAg seroclearance (p<0.001). Average ages in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 50 and 46.3 years, respectively (p<0.001), with an age range of 30~65 years. Mean of serum ALT values were 17.7 and 16.5 U/L, respectively (p=0.24) (**Table 1**). The lengths of follow up duration in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 6.29 years (6.66 years for male and 5.21 years for females) and 7.79 years (9.17 years for males and 5.40 years for females), respectively. **Table 1** shows that subjects with HBsAg seroclearance were more likely to be older (OR 1.04, 95% (1.03~1.05); p<0.001). In addition, HBsAg seroclearance tended to more likely appeared in males than in females (OR 1.51, 95% (1.21~1.89); P<0.001) even after adjusted for gender, age and ALT. The follow-up duration was significantly shorter (p<0.001) in the patients with HBsAg seroclearance than the group of patients without HBsAg seroclearnce.

3.2. Association of polymorphisms with HBsAg seroclearance

We investigated the association between genetic polymorphisms of three SNPs (rs7944135, rs171941, and rs6462008) and HBsAg seroclearance (**Table 2**). Genotype and recessive models were applied to assess associations of the HBsAg with the three SNPs. Our results revealed that the rs7944135 SNP was solely associated with HBsAg seroclearance. The rs7944135 SNP was found to be significantly associated with HBsAg seroclearance in the genotype and recessive model after adjusted with gender, age and ALT, at p<0.05. This result indicated that HBV carriers with the AA genotype of rs7944135 were more susceptible to HBsAg seroclearance compared to those with the AG or GG genotype of rs7944135. However, the two other SNPs (rs171941, and rs6462008) showed no correlation with HBsAg seroclearance (**Table 2**). A significant association between the rs7944135 AA genotype and HBsAg seroclearance was found 1.76-fold higher susceptibility to clearance of the HBsAg, compared to those with the AG or GG genotype after adjusting for gender, age and ALT, (OR=1.76. 95% (1.14~2.72), p=.045**)**.

Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype of rs7944135 under the recessive model (OR=1.74, 95% (1.13~2.66), *p*=.014).

3.3. The minor Allele frequency of three SNPs in different populations

The **Table 3** shows the minor allele frequency (MAF) of three SNPs (rs7944135, rs171941, and rs6462008) in different populations (*e.g.*, Asian and European), which were these two population were reported higher susceptible to have HBsAg seroclearance in compare to another region worldwide [23]. MAFs of African, American, European and Asian were extracted from the HaploReg browser v4.1 **(**https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php**)**, and MAF of the TWB were adapted from the Taiwan biobank website (https://taiwanview.twbiobank.org.tw/index). **Table 3** showed that our result was close to those in reference to Asian including a Taiwanese population.

3.4. Correlation between *MPEG1* rs7944135 and days of HBsAg seroclearance

In addition, we confirmed the correlation between the time taken to HBsAg seroclearance and the genotype of the *MPEG1* gene SNP rs7944135. Log rank test showed a significant differences in the time taken to occur HBsAg seroclearance among the *MPEG1* gene rs7944135 genotypes of AA, AG, and GG (*p*=.039). The cumulative fraction curve showed a trend of HBV carriers with the AA genotype of rs7944135 indicated higher susceptibility to HBsAg seroclearnce compared to those with the AG or GG genotype (**Figure 1**).

3.5. Correlation between the *MPEG1* rs7944135 genotype and days of HBV DNA undetectable

We further investigated the correlation between the time taken to HBV DNA undetectable and *MPEG1* gene rs7944135 genotypes: AA, AG, and GG. Log rank test showed significant differences among *MPEG1* gene SNP rs7944135 genotypes of AA, AG, and GG in the time taken to HBV DNA undetectable (*p*=.0074). The cumulative fraction curve showed that a trend of the AA genotype of rs7944135 of HBV carriers showed a higher rate to undetected of HBV DNA versus the AG and GG genotypes (**Figure 2**).

3.6. SNP annotation of expression quantitative trait loci (e-QTLs) of rs7944135

To elucidate the association between SNP *rs7944135* and gene expression, we utilized the publicly available databases GTEx Portal (http:// www.gtexportal.org/home/) to obtain the tissue expression quantitative trait loci. As shown in **Table 4** and **Figure 3** the AA genotype of *rs7944135* had highly expressed of *MPEG1* gene when compared to AG and GG genotype.

4. Discussion

Clearance of the HBsAg is the important indicator of recovery from CHB [24]. It was reported that HBsAg seroclearance is associated with the prognosis of CHB [25]. Clearance of the HBsAg in HBV is influenced by many factors, including genetic and host factors such as age, sex, and race [26]. Our approach in this study was to confirm associations the factors of genetic, age, and gender with HBsAg seroclearance. In the current study, we replicated a GWAS study of Korean CHB patients by a candidate gene approach of CHB Taiwan patients to confirm genes associated with HBsAg seroclearance in CHB Taiwanese population. Kim *et al* revealed that three SNPs of rs6462008 located near even-skipped homeobox 1 (*EVX1*), rs171941 located near cardiomyopathy associated 5 (*CMYA5*), and rs7944135 located near *MPEG1* were associated with HBsAg seroclearance in korean population [27]. Recently, we successfully replicated an association of rs7944135 in *MPEG1* with HBsAg seroclearance in a Taiwanese CHB population.

The male patients are mostly more susceptible to the occurrence of HBsAg seroclearance compared to female patients [13,27]. Consistently, our current study showed that male patients tended to be more susceptible than female patients even after adjusted for gender, age, and ALT, which suggests that male patients have stronger immune responses than female patients. Similar to previous studies from Korea and Taiwan [13,28], this reason may due to hormonal involvement in the immune response [29]. The age at HBsAg seroclearance also can be considered a factor. Our study identified that the mean age at HBsAg seroclearance was 50 years. This finding is relatively close to previous studies in Hong Kong (mean, 48.8 years), Korea (mean, 50 years), and Japan (mean, 51 years) [25,30,31].

This study showed that rs7944135 in *MPEG1* is significantly associated with HBsAg seroclearance in a Taiwanese CHB population. We observed that subjects with the AA genotype of *MPEG1* rs7944135 had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype (OR=1.76). In addition, we still found an association in rs7944135 with 1.74-fold increased risk of the AA genotype of HBsAg seroclearance occurring under the recessive model (OR=1.74). Spontaneous HBsAg seroclearance can be predicted through serum levels of the HBsAg and HBV DNA [24,32]. In terms of the time taken to the HBsAg seroclearance and undetected of HBV DNA, results showed that the rs7944135 AA genotype had a higher susceptibility to HBsAg seroclearance and HBV DNA undetectable compared to the AG and GG genotypes. A correlation between HBsAg and HBV DNA in serum was reported by Li

et al. (2007) and revealed that the HBsAg level was not detected during the immune clearance phase as a result of declining HBV DNA levels. In addition, Coffin *et al.* (2019) revealed that with a lower level of the HBsAg, there was a greater likelihood for loss of the HBsAg. The HBsAg at a low level was associated with a lower risk of prognosis of HCC [3]. It is well known that the HBsAg contributes to the immunopathogenesis of persistent HBV infection, and a higher incidence of HBsAg seroclearance was associated with lower HBsAg levels [33].

MPEG1 is a specific gene that was identified in humans and mice in macrophages and has many roles in immune responses [34]. According to HaploReg V4.1, rs7944135 was located at 11q12.1 of *MPEG1*, and it is involved in the changing chromatin status of primary T cells from peripheral blood, which plays a central role in the "primary immune response" of cell-mediated immunity, this may be suggested the role of *MPEG1* in immune clearance of HBsAg in HBV infection. Furthermore, the *MPEG1* gene has role in immune response through encoding perforin-like protein and recognition of antigen[35]. The perforin-like protein is predicted to be a perforin domain of membrane attack complex (MAC) that helps cytotoxic T cells and natural killer cells kill virus infected cell [36,37]. In our study, we demonstrated from The Genotype-Tissue Expression (GTEx) portal database that rs7944135 AA genotype shows the highest expressed of *MPEG1* gene in compare to GG and AG genotype. It infers that patients with AA genotype on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the HBsAg.

Additional genes, such as HLA, cytokine tumor necrosis factor (*TNF*)-α, chemokine receptor 5 (*CCR5*) [38], and *MCP1* [21], were found to be associated with the incidence of HBsAg seroclearance through cell-mediated immune responses. Some evidence has been provided that polymorphisms in HLA subtypes are significantly associated with the occurrence of HBsAg seroclearance, such as a polymorphism of *HLA-DP* rs3077 with haplotype GAT having a 2.17-fold association in a Chinese population [39], *HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535 with A alleles in a Japanese population [40], and *HLA-B*4001* in Taiwanese aborigines [41]. There are many polymorphisms of the TNF-α promoter region that were reported to alter TNF-α-associated HBV clearance in many populations [42]. *MCP1* with -2518G>A was associated with HBV clearance in a Korean population [21].

In this study, we investigated the association of individual SNPs with susceptibility to HBsAg seroclearance in Taiwanese CHB patients. We acknowledge that our study needs to be validated by future studies in other population using more samples with multiple polymorphisms. Therefore, this could give enlightenment for the genetic factors that associated in the occurrence of HBsAg seroclearance. However, this study offers the novel finding of a polymorphism of *MPEG1* having a pivotal association with HBsAg seroclearance in Taiwanese CHB patients.

5. Conclusion

Our current study is a replication of an associated study of a SNP of *MPEG1* rs7944135, with susceptibility to HBsAg seroclearance and showed that an HBsAg seroclearance-associated SNP, rs7944135 with the AA genotype, solely has a significant association with the loss of the HBsAg in CHB infection in Taiwanese HBV patients.

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Author Contributions

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Conflicts of Interest

The authors disclose no conflict

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Figure Legends

Figure 1. Cumulative fraction curve of *MPEG1* rs7944135 on hepatitis B surface antigen (HBsAg) seroclearance.

Figure 2. Cumulative fraction curve of the undetected hepatitis B virus (HBV) DNA of HBV patients according to the *MPEG1* rs7944135**.**

Figure 3. *Cis*-expression quantitative trait loci (*cis*-eQTLs) of *MPEG1* rs7944135 (Homo Alt (AA)>Het (AG)>Homo Ref (GG)) in muscle skeletal.

Table 1

Table 1. **Baseline characteristics of the 2565 hepatitis B virus study participants**

Characteristic	With Seroclearance (*N*=493)	Without Seroclerarance (*N*=2072)	*p*-value	Adjusted Odds ratio(95%CI)[c]	*p*-value [c]
Male gender, *n* (%)	361 (73.2)	1325 (63.9)	<0.001[b]*	1.51(1.21~1.89)	< 0.001 *
Mean (SD) age (years)	50±9.74	46.3±9.53	<0.001[a]*	1.04 (1.03~1.05)	< 0.001 *
Age range (years)	30~65	30~65			
ALT (U/L)	17.7±23.8	16.5±19.8	0.24[a]	1.00(0.99~1.00)	0.665
Follow-up duration					
Interval date (years)	6.29± 3.90	7.79±4.48	<0.001[a]*	0.93 (0.91~0.95)	< 0.001 *
Male	6.66±3.76	9.17±3.58			
Female	5.21±4.09	5.40±4.86			

Data are presented as the number, mean ± standard deviation (SD), or median. ALT, alanine aminotransferase (normal range 5~40 U/L). *Significant at $p<0.05$. [a]By Student's *t*-test. [b]By Chi-squared test. [c]Adjusted for gender, age, and ALT.

Table 2

Table 2. **Associations of *MPEG1* with hepatitis B surface antigen seroclearance in 2565 hepatitis B virus patients**

SNP	Genotype	With Seroclearance (%) (*N*=493)	Without Seroclearance (%) (*N*=2072)	Genotype		Recessive	
				OR (95% CI)[a]	*p* value[a]	OR (95% CI)[a]	*p* value [a]
rs7944135	AA	33 (6.7)	77 (3.8)	1.76(1.14~2.72)	**0.045***.	1.74(1.13~2.66)	**0.014***.
	AG	148 (30.0)	600 (29.7)	1.05(0.84~1.31)		Reference	
	GG	312 (63.3)	1342 (66.5)	Reference			
rs171941	AA	35 (7.3)	145 (7.2)	0.86(0.69~1.06)	0.349	0.95(0.65~1.41)	0.811
	AG	170 (35.3)	775 (38.4)	0.90(0.60~1.33)		Reference	
	GG	277 (57.5)	1096 (54.4)	Reference			
rs6462008	TT	142 (29.5)	540 (26.9)	0.92(0.69~1.23)	0.519	1.01(0.79~1.28)	0.952
	GT	232 (48.1)	1015 (50.5)	0.87(0.69~1.10)		Reference	
	GG	108 (22.4)	456 (22.7)	Reference			

[a] Adjusted for gender, age and ALT.
the significant *p* value is in **bold ***.
SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 3. Minor allele frequencies of single nucleotide polymorphisms in this study

SNP	Position (hg38) (bp)	Nearest gene	Allele		Minor allele frequencies (MAFs)						
			Major	Minor	EUR	AFR	AMR	ASN	TWB	Ours	HWE
rs7944135	Chr11:59253514	*MPEG1*	G	A	0.49	0.34	0.43	0.26	0.18	0.19	0.81
rs171941	Chr 5:79884303	*CMYA5*	G	A	0.33	0.35	0.45	0.28	0.26	0.26	0.73
rs6462008	Chr 7:27309860	*EVX1*	G	T	0.64	0.71	0.65	0.51	0.48	0.47	0.51

EUR, European; AFR, African; AMR, American; ASN, Asian; TWB, Taiwan Biobank; HWE, *p* value for Hardy-Weinberg equilibrium test in our samples. MAFs of EUR, AFR, AMR and ASN were extracted from the **HaploReg browser v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php)**; MAFs of the TWB were obtained from the Taiwan View website (**https://taiwanview.twbiobank.org.tw/index**).

Table 4. *Cis*-expression of quantitative trait loci results of the single-nucleotide polymorphism from genotype-tissue expression database.

SNP ID	Gencode ID (ENSG00000-)	Gene symbol	*p*-value	Effect size	Tissue	Actions
rs7944135	197629	*MPEG1*	0.000072	0.11	Muscle - skeletal	AA>AG>GG
	110042	*DTX4*	1.5e-18	-0.33	Nerve-tibial	GG>AG>AA
	110042	*DTX4*	0.000015	-0.15	Muscle - skeletal	GG>AG>AA
	110042	*DTX4*	1.6e-7	-0.22	Lungs	GG>AG>AA
	110042	*DTX4*	0.000059	-0.16	Heart - left ventricle	GG>AG>AA
	110042	*DTX4*	2.7e-12	-0.31	Heart - atrial appendage	GG>AG>AA
	110042	*DTX4*	4.7e-17	-0.38	Esophagus - muscularis	GG>AG>AA
	110042	*DTX4*	5.6e-11	-0.29	Esophagus - mucosa	GG>AG>AA
	110042	*DTX4*	6.0e-9	-0.31	Esophagus - gastroesophageal junction	GG>AG>AA
	110042	*DTX4*	5.9e-9	-0.32	Cells - transformed fibroblasts	GG>AG>AA
	110042	*DTX4*	0.000032	-0.14	Adipose - subcutaneous	GG>AG>AA

SNP, single-nucleotide polymorphism.

Figure 1

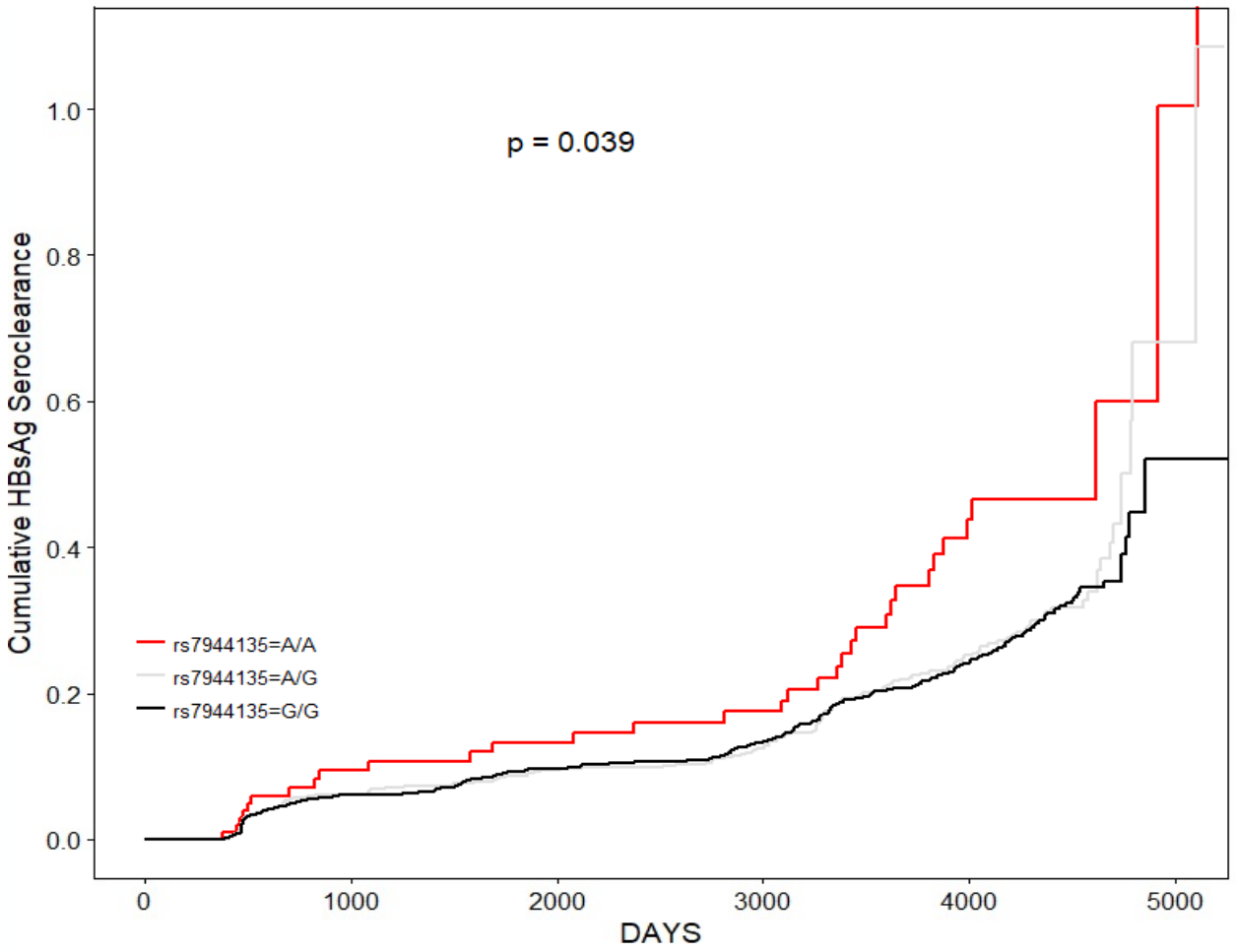


Figure 2

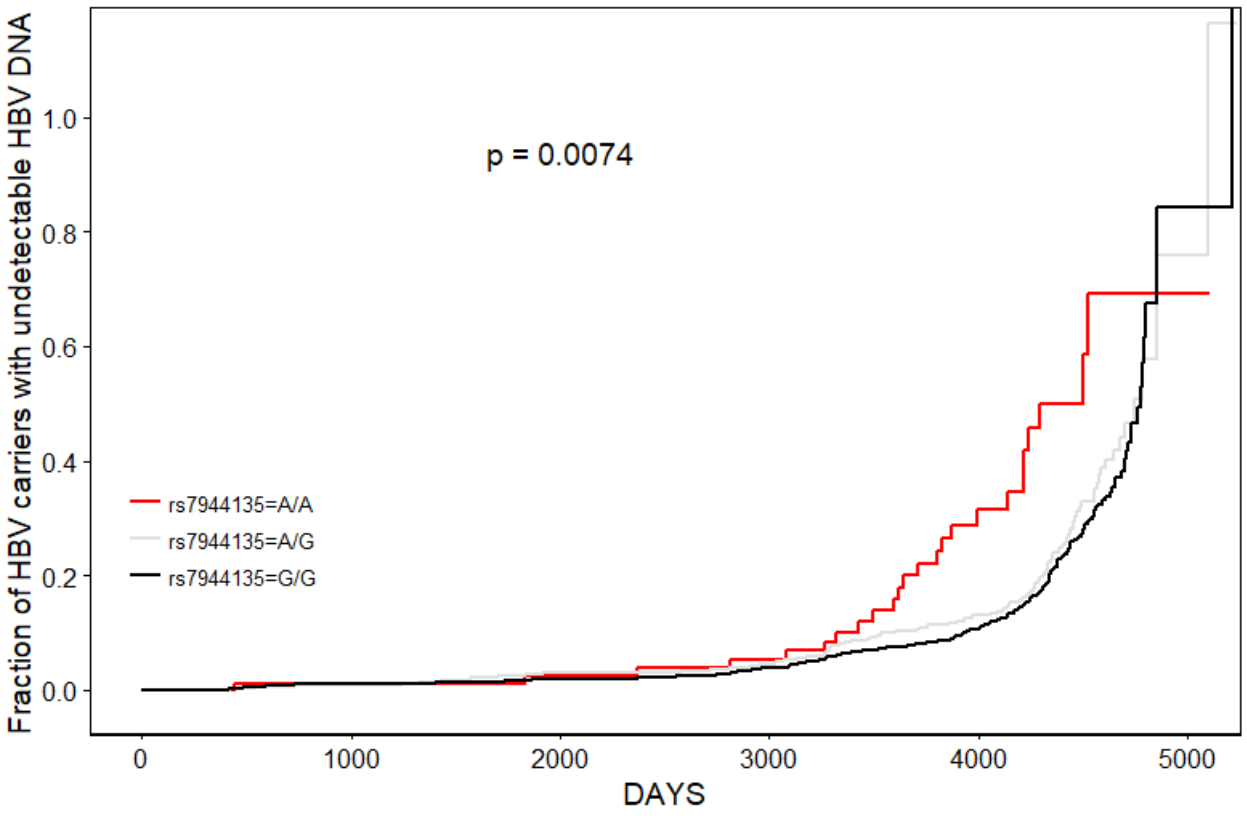
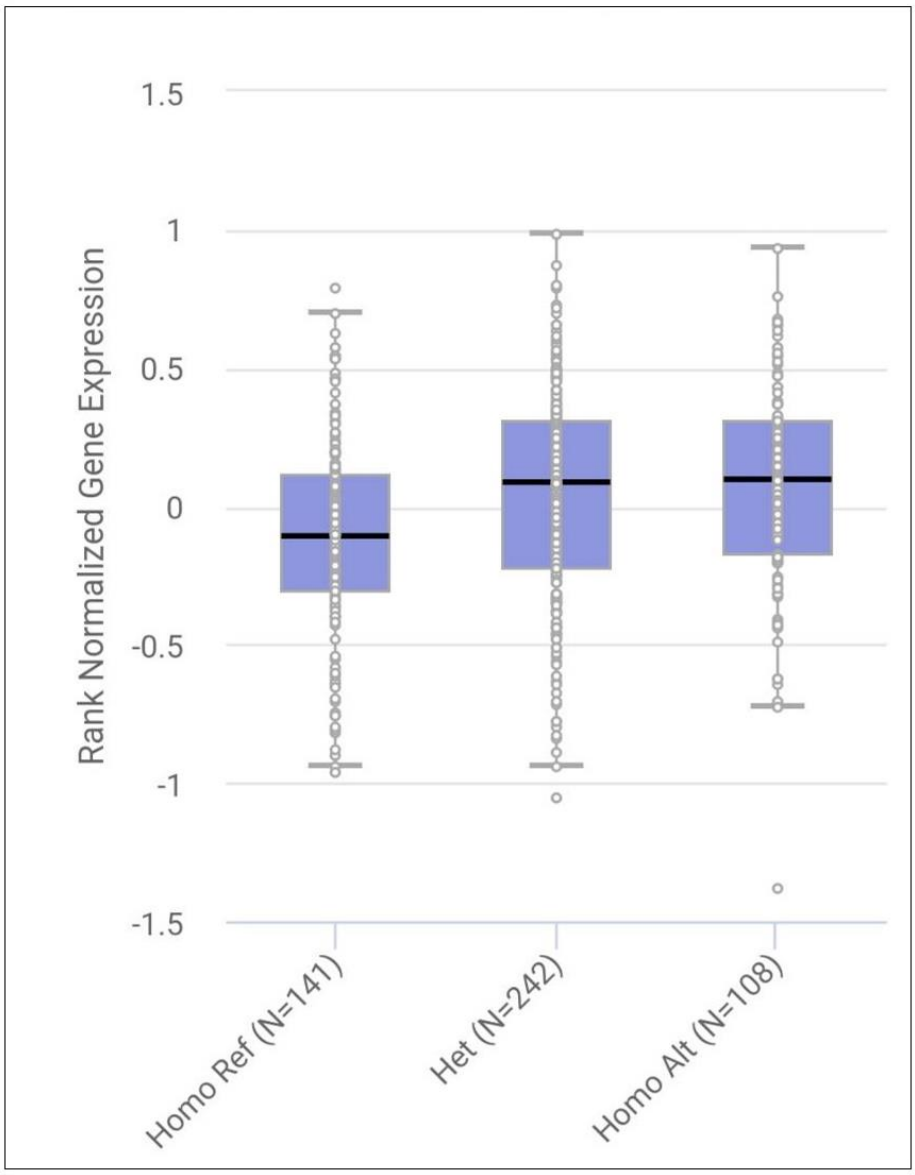


Figure 3



STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) ***Cohort study***—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	**Cohort Study 4 and 5**
		(*b*) ***Cohort study***—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4 and 5
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	4
Bias	9	Describe any efforts to address potential sources of bias	-
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	4
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	5 and 6
		(*c*) Explain how missing data were addressed	-
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	-
		(*e*) Describe any sensitivity analyses	-

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate number of participants with missing data for each variable of interest	-
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	-
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	-
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	4
		Cross-sectional study—Report numbers of outcome events or summary measures	-
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7 and 8
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	-
Discussion			
Key results	18	Summarise key results with reference to study objectives	10
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	11
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11
Generalisability	21	Discuss the generalisability (external validity) of the study results	10
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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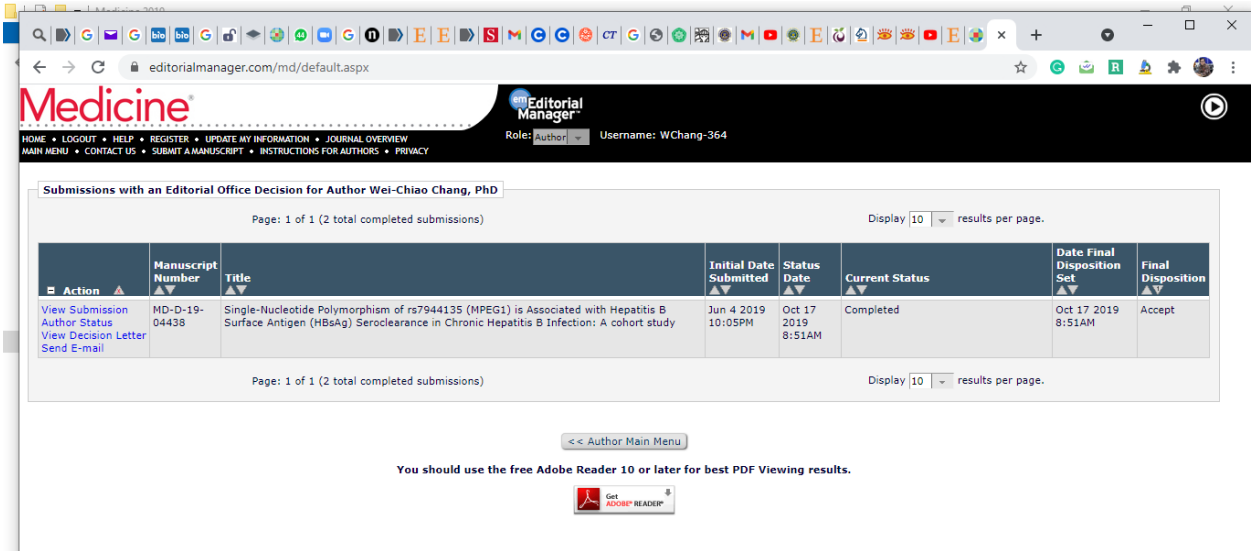

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Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status	Date Final Disposition Set	Final Disposition
View Submission Author Status View Decision Letter Send E-mail	MD-D-19-04438	Single-Nucleotide Polymorphism of rs7944135 (MPEG1) is Associated with Hepatitis B Surface Antigen (HBsAg) Seroclearance in Chronic Hepatitis B Infection: A cohort study	Jun 4 2019 10:05PM	Oct 17 2019 8:51AM	Completed	Oct 17 2019 8:51AM	Accept

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Single-nucleotide polymorphism of rs7944135 (macrophage-expressed gene 1) is associated with hepatitis B surface antigen seroclearance in chronic hepatitis B infection

A cohort study

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Abstract

Clearance of the hepatitis B surface antigen (HBsAg) is the ultimate aim of treatment for patients with chronic hepatitis B (CHB) infection. Genetic, factor age, and gender were reported to be involved in the clearance of HBsAg. However, the rate of HBsAg seroclearance in CHB patients is still low globally and few of the single-nucleotide polymorphism (SNP) had been identified to associated with HBsAg seroclearance in CHB patients.

Recently, 3 associated SNPs (rs7944135, rs171941, and rs6462008) were reported in the clearance of HBsAg in the Korean population. However, these SNPs have not been investigated in the CHB Taiwanese population. In present study, these 3 SNPs were genotyped in 2565 Taiwanese CHB patients including 493 CHB patients with HBsAg seroclearance and 2072 without HBsAg seroclearance.

We observed that SNP rs7944135 was solely associated with HBsAg seroclearance. Subjects with the AA genotype at rs7944135 of macrophage-expressed gene 1 had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype under the genotypic model (odds ratio [OR] = 1.76. 95% confidence interval [CI] = 1.14–2.72, $P = .045$). Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype compared to AG + GG of rs7944135 under the recessive model (OR = 1.74. 95% CI = 1.13–2.66, $P = .014$). According to the cumulative fraction curve with the log-rank test revealed that patients with the AA genotype of rs7944135 showed higher susceptibility to occur HBsAg seroclearance ($P = .039$) and HBV DNA undetectable ($P = .0074$) compared to those with the AG or GG genotype.

This study examined the associations of 3 SNPs (rs7944135, rs171941, and rs6462008) with HBsAg seroclearance, and we identified that rs7944135 is solely associated with HBsAg seroclearance in Taiwanese CHB patients.

Abbreviations: CHB = chronic hepatitis B, CI = confidence interval, GWASs = genome-wide association studies, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, *MPEG1* = macrophage-expressed gene 1, OR = odds ratio, SNPs = single-nucleotide polymorphisms.

Keywords: CHB, HBsAg seroclearance, HBV, rs7944135, single-nucleotide polymorphism

1. Introduction

The hepatitis B virus (HBV) was discovered over 5 decades ago. Although a prophylactic vaccine has been available, HBV infection is still a serious problem globally. World Health Organization (2018) estimated that there were 257 million new cases and 887,000 deaths from HBV. Most of the deaths were caused by

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complications, including cirrhosis and hepatocellular carcinoma (HCC).[1,2] Eventually, the goal of therapy in CHB patients is to alleviate and prevent this complication.

Chronic hepatitis B (CHB) infection can be confirmed by its complicated serological pattern, while hepatitis B surface antigen (HBsAg) was reported to be well-established as a serum marker in the natural history of HBV infection. A low level of the HBsAg is associated with sustained immune control, seroclearance of HBsAg, and a lower risk of HCC.[3] A meta-analysis study further confirmed the role of the HBsAg as a predictive marker of hepatitis B, liver cirrhosis, and HCC development, and the rate of spontaneous HBsAg seroclearance,[4] an important milestone in the natural history of CHB infection. In addition, the serological profiles of chronic HBV infection also revealed that plasma HBV DNA levels can serve as a marker of disease progress.[5] In general, CHB is defined as the presence of the HBsAg for more than 6 months after an HBV infection [6] and spontaneous HBsAg seroclearance is defined as the loss of the HBsAg at least 6 months apart on 2 occasions and continuing to remain to absent up to the last visit.[7] However, the rate of HBsAg seroclearance in CHB patients is varied globally at an estimated 1% to 2% annually,[8] at approximately, 0.41% to 1.58% in Europe,[9,10] 0.56 to 0.65% in north and south America, respectively[11,12] and 0.12% to 2.38% Asian,[13] 0.4% per year reported in Korea,[14] 2.5% in the Goto Islands of Japan,[15] and 1.15% per year was recorded in Taiwan.[13]

The clearance of the HBsAg in HBV infection is influenced by many factors, such as genetic and host factors including age, sex, and race. Several studies identified that host genetic polymorphisms may be associated with clinical outcomes of HBV, including HLA DQ (*HLA-DQ*) and DP (*HLA-DP*),[16,17]*IL28B*,[18]*HLA-DPB1*,[19] tumor necrosis factor-α (*TNF-*α),[20] and monocyte chemotactic protein-1 (*MCP1*).[21] Some CHB-associated loci, such as *HLA-DPB1*, *HLA-DQA2*, and *HLA-DQB,* also reported by previous genome-wide association studies (GWASs), were identified in a Taiwanese population.[22] However, more the genetic studies still need to be examined in HBV infection, considering of HBsAg seroclearance as a marker of cure of HBV infection. Recently, Kim et al indicated that three new SNPs (rs7944135, rs171941, and rs6462008) were associated with HBsAg seroclearance in a Korean population. Thus, the purpose of this study was to investigate whether the polymorphisms reported by the Korean group are associated with clearance of the HBsAg in Taiwanese HBV patients.

2. Materials and methods

2.1. Study subjects

In total, 2565 CHB patients (including 493 with HBsAg seroclearance and 2072 without HBsAg seroclearance) satisfied the inclusion criteria for follow-up of CHB, which were recruited during 1991 to 1992 from 7 area in Taiwan (Sanchi, Chutung, Potzu, Kaoshu, Makung, Hushi, and Paisha). All of the study participants were ethnic Chinese (ie, Taiwanese). All participants in this study provided written informed consent before participation. This project was approved by the ethics committees at Academia Sinica, Taiwan.

2.2. Clinical evaluation and SNP selection

All patients were tested for hepatitis B or virological markers in the liver, including HBsAg was measured using radioimmunoassay with commercial kits (Abbott Laboratories, North Chicago), HBV DNA were measured by polymerase chain reaction (PCR) using the Cobas Amplicor HBV monitor test kit (Roche Diagnostics, Indianapolis, IN) and alanine transaminase (ALT) ALT using chemistry autoanalyzer (Model 736; Hitachi, Tokyo, Japan) using commercial reagents (Biomérieux, Marcy L'Etoile, France). HBsAg seroclearance were defined as loss of HBsAg in serum at least 6 months apart on 2 occasions and continued to absent up to the last visit. While without HBsAg seroclearance were defined as positive of HBsAg in serum for more than 6 months apart and continuously detected up to the last visit.

The SNPs we investigated in this present study were the replicated study from a GWAS applied in a Korean CHB population reported by Kim et al. Kim et al reported 3 SNPs (rs7944135, rs171941, rs6462008) associated with seroclearance of the HBsAg in Korean CHB patients. These 3 SNPs were confirmed to exist in a Taiwanese CHB population.

2.3. DNA extraction and genotyping of the 3 SNPs

DNA was extracted from blood samples and subsequently centrifuged at 3000 rpm for 10 minutes at 4°C to separate cells and plasma. Specimens were stored below −70°C. The buffy coat was isolated from blood samples, and red blood cells (RBCs) were lysed after the addition of RBC lysis buffer.

Three SNPs (rs7944135, rs171941, and rs6462008) were assessed by genotyping. Genotyping assays were performed using a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA). PCRs were subsequently performed in a 96-well microtiter plate with either an ABI7500 real-time PCR or ABI9700 Thermal Cycler under the following conditions: 10 minutes of denaturing at 95°C, followed by 45 cycles of 15 seconds of denaturing at 95°C and 30 seconds for annealing at 60°C, with 1 minutes of a final extension at 60°C. Fluorescence signals from amplicons were analyzed using System SDS software vers. 1.2.3 (Applied Biosystems).

2.4. SNP annotation data query

Associations between gene expression profiles and the SNPs were confirmed by examining the expression quantitative trait loci (e-QTL) through (genotype-tissue expression [GTEx]) Portal database (http://www.gtexportal.org/home/). The GTEx database shows expressions of genes in a variety of tissues.

2.5. Statistical analysis

We performed all analyses using the R environment (https://cran.r-project.org/ and https://www.r-project.org/). We used Student *t* test to compare the age, ALT, and follow-up duration between the CHB patients with HBsAg seroclearance and without HBsAg seroclearance groups. We used logistic regression analyses to obtain adjusted OR between the case with HBsAg seroclearance and gender, age, ALT, and follow-up duration. Associations between SNPs and HBsAg seroclearance under genotype and recessive models were assessed using the "SNPassoc" package. The association between (the rs7944135 genotype AA, AG, and GG) and (HBsAg seroclearance and undetectable of HBV DNA) were also modeled using time to event analysis utilized cumulative fraction curve using cumulative hazard function and log-rank test by "Survival and survminer" package. While, days between enrollment to undetectable of HBV DNA and occurring HBsAg seroclearance was used at the time scale.

Table 1

Baseline characteristics of the 2565 hepatitis B virus study participants.

Characteristic	With seroclearance (N = 493)	Without seroclerarance (N = 2072)	*P*-value	Adjusted odds ratio (95%CI)[§]	*P*-value[§]
Male gender, n (%)	361 (73.2)	1325 (63.9)	<.001[*,‡]	1.51 (1.21–1.89)	<.001[*]
Mean (SD) age, yr	50 ± 9.74	46.3 ± 9.53	<.001[*,†]	1.04 (1.03–1.05)	<.001[*]
Age range, yr	30–65	30–65			
ALT, U/L	17.7 ± 23.8	16.5 ± 19.8	.24[†]	1.00 (0.99–1.00)	.665
Follow-up duration			<.001[*,†]	0.93 (0.91–0.95)	<.001[*]
Interval date, yr	6.29 ± 3.90	7.79 ± 4.48			
Male	6.66 ± 3.76	9.17 ± 3.58			
Female	5.21 ± 4.09	5.40 ± 4.86			

Data are presented as the number, mean ± SD, or median. ALT (normal range 5–40 U/L).
ALT = alanine aminotransferase, CI = confidence interval, SD = standard deviation.
[*] Significant at $P < .05$.
[†] By Student *t* test.
[‡] By Chi-squared test.
[§] Adjusted for gender, age, and ALT.

3. Results

3.1. Basic characteristics of chronic HBV-infected patients

We collected data on 2565 chronically HBV-infected subjects, including 493 CHB with HBsAg sroclearance (361 males and 132 females) and 2072 CHB without HBsAg serclearance (1325 males and 747 females) in this study. The numbers of CHB male patients in the 2 groups included 361 with HBsAg seroclearance and 1325 without HBsAg seroclearance ($P < .001$). Average ages in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 50 and 46.3 years, respectively ($P < .001$), with an age range of 30 to 65 years. Mean of serum ALT values were 17.7 and 16.5 U/L, respectively ($P = .24$) (Table 1). The lengths of follow up duration in the CHB patients with HBsAg seroclearance and without HBsAg seroclearance group were 6.29 years (6.66 years for male and 5.21 years for females) and 7.79 years (9.17 years for males and 5.40 years for females), respectively. Table 1 shows that subjects with HBsAg seroclearance were more likely to be older (odds ratio [OR] 1.04, 95% [1.03–1.05]; $P < .001$). In addition, HBsAg seroclearance tended to more likely appeared in males than in females (OR 1.51, 95% [1.21–1.89]; $P < .001$) even after adjusted for gender, age and ALT. The follow-up duration was significantly shorter ($P < .001$) in the patients with HBsAg seroclearance than the group of patients without HBsAg seroclearnce.

3.2. Association of polymorphisms with HBsAg seroclearance

We investigated the association between genetic polymorphisms of 3 SNPs (rs7944135, rs171941, and rs6462008) and HBsAg seroclearance (Table 2). Genotype and recessive models were applied to assess associations of the HBsAg with the 3 SNPs. Our results revealed that the rs7944135 SNP was solely associated with HBsAg seroclearance. The rs7944135 SNP was found to be significantly associated with HBsAg seroclearance in the genotype and recessive model after adjusted with gender, age, and ALT, at $P < .05$. This result indicated that HBV carriers with the AA genotype of rs7944135 were more susceptible to HBsAg seroclearance compared to those with the AG or GG genotype of rs7944135. However, the 2 other SNPs (rs171941, and rs6462008) showed no correlation with HBsAg seroclearance (Table 2). A significant association between the rs7944135 AA genotype and HBsAg seroclearance was found 1.76-fold higher susceptibility to clearance of the HBsAg, compared to those with the AG or GG genotype after adjusting for gender, age and ALT, (OR = 1.76. 95% [1.14–2.72], $P = .045$). Furthermore, we found a 1.74-fold increased risk of acquiring HBsAg seroclearance associated with the AA genotype of rs7944135 under the recessive model (OR = 1.74, 95% [1.13–2.66], $P = .014$).

Table 2

Associations of *MPEG1* with hepatitis B surface antigen seroclearance in 2565 hepatitis B virus patients.

SNP	Genotype	With seroclearance (%) (N = 493)	Without seroclearance (%) (N = 2072)	Genotype		Recessive	
				OR (95% CI)[†]	*P*-value[†]	OR (95% CI)[†]	*P*-value[†]
rs7944135	AA	33 (6.7)	77 (3.8)	1.76 (1.14–2.72)	**.045**[*]	1.74 (1.13–2.66)	**.014**[*]
	AG	148 (30.0)	600 (29.7)	1.05 (0.84–1.31)		Reference	
	GG	312 (63.3)	1342 (66.5)	Reference			
rs171941	AA	35 (7.3)	145 (7.2)	0.86 (0.69–1.06)	.349	0.95 (0.65–1.41)	.811
	AG	170 (35.3)	775 (38.4)	0.90 (0.60–1.33)		Reference	
	GG	277 (57.5)	1096 (54.4)	Reference			
rs6462008	TT	142 (29.5)	540 (26.9)	0.92 (0.69–1.23)	.519	1.01 (0.79–1.28)	.952
	GT	232 (48.1)	1015 (50.5)	0.87 (0.69–1.10)		Reference	
	GG	108 (22.4)	456 (22.7)	Reference			

CI = confidence interval, OR = odds ratio, SNP = single-nucleotide polymorphism.
[*] The significant *P*-value is in **bold**.
[†] Adjusted for gender, age and ALT.

Table 3

Minor allele frequencies of single nucleotide polymorphisms in this study.

SNP	Position (hg38) (bp)	Nearest gene	Allele		Minor allele frequencies (MAFs)						
			Major	Minor	EUR	AFR	AMR	ASN	TWB	Ours	HWE
rs7944135	Chr11:59253514	*MPEG1*	G	A	0.49	0.34	0.43	0.26	0.18	0.19	0.81
rs171941	Chr 5:79884303	*CMYA5*	G	A	0.33	0.35	0.45	0.28	0.26	0.26	0.73
rs6462008	Chr 7:27309860	*EVX1*	G	T	0.64	0.71	0.65	0.51	0.48	0.47	0.51

MAFs of EUR, AFR, AMR, and ASN were extracted from the HaploReg browser v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php); MAFs of the TWB were obtained from the Taiwan View website (https://taiwanview.twbiobank.org.tw/index).

AFR = African, AMR = American, ASN = Asian, *CMYA5* = cardiomyopathy associated 5, EUR = European, *EVX1* = even-skipped homeobox 1, HWE = *P*-value for Hardy–Weinberg equilibrium test in our samples, *MPEG1* = macrophage-expressed gene 1, TWB = Taiwan Biobank.

3.3. The minor allele frequency of 3 SNPs in different populations

The Table 3 shows the minor allele frequency (MAF) of 3 SNPs (rs7944135, rs171941, and rs6462008) in different populations (eg, Asian and European), which were these 2 population were reported higher susceptible to have HBsAg seroclearance in compare to another region worldwide.[23] MAFs of African, American, European, and Asian were extracted from the HaploReg browser v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php), and MAF of the Taiwan Biobank (TWB) were adapted from the TWB website (https://taiwanview.twbiobank.org.tw/index). Table 3 showed that our result was close to those in reference to Asian including a Taiwan biobank (TWB) population.

3.4. Correlation between macrophage-expressed gene 1 rs7944135 and days of HBsAg seroclearance

In addition, we confirmed the correlation between the time taken to HBsAg seroclearance and the genotype of the macrophage-expressed gene 1 (*MPEG1*) gene SNP rs7944135. Log-rank test showed significant differences in the time taken to occur HBsAg seroclearance among the *MPEG1* gene rs7944135 genotypes of AA, AG, and GG ($P = .039$). The cumulative fraction curve showed a trend of HBV carriers with the AA genotype of rs7944135 indicating higher susceptibility to HBsAg seroclearnce compared to those with the AG or GG genotype (Fig. 1).

3.5. Correlation between the MPEG1 rs7944135 genotype and days of HBV DNA undetectable

We further investigated the correlation between the time taken to HBV DNA undetectable and *MPEG1* gene rs7944135 genotypes: AA, AG, and GG. Log-rank test showed significant differences among *MPEG1* gene SNP rs7944135 genotypes of AA, AG, and GG in the time taken to HBV DNA undetectable ($P = .0074$). The cumulative fraction curve showed that a trend of the AA genotype of rs7944135 of HBV carriers had a higher rate to undetected of HBV DNA versus the AG and GG genotypes (Fig. 2).

3.6. SNP annotation of e-QTLs of rs7944135

To elucidate the association between SNP *rs7944135* and gene expression, we used the publicly available databases GTEx Portal (http://www.gtexportal.org/home/) to obtain the tissue e-QTLs. As shown in Table 4 and Figure 3 the AA genotype of *rs7944135* had highly expressed of *MPEG1* gene if compared to AG and GG genotype.

4. Discussion

Clearance of the HBsAg is an important indicator of recovery from CHB.[24] It was reported that HBsAg seroclearance is associated

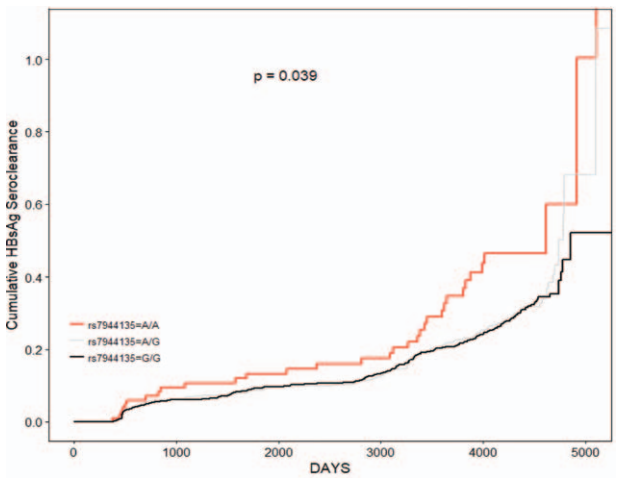

Figure 1. Cumulative fraction curve of *MPEG1* rs7944135 on HBsAg seroclearance. HBsAg = , hepatitis B surface antigen, *MPEG1* = macrophage-expressed gene 1.

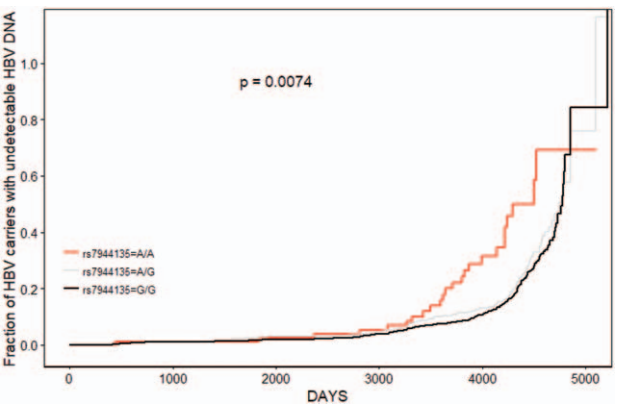

Figure 2. Cumulative fraction curve of the undetected HBV DNA of HBV patients according to the *MPEG1* rs7944135. HBV = hepatitis B virus, *MPEG1* = macrophage-expressed gene 1.

Table 4

***Cis*-expression of quantitative trait loci results of the single-nucleotide polymorphism from genotype-tissue expression database.**

SNP ID	Gencode ID (ENSG00000-)	Gene symbol	*P*-value	Effect size	Tissue	Actions
rs7944135	197629	*MPEG1*	.000072	0.11	Muscle-skeletal	AA>AG>GG
	110042	*DTX4*	1.5e−18	−0.33	Nerve-tibial	GG>AG>AA
	110042	*DTX4*	.000015	−0.15	Muscle-skeletal	GG>AG>AA
	110042	*DTX4*	1.6e−7	−0.22	Lungs	GG>AG>AA
	110042	*DTX4*	.000059	−0.16	Heart-left ventricle	GG>AG>AA
	110042	*DTX4*	2.7e−12	−0.31	Heart-atrial appendage	GG>AG>AA
	110042	*DTX4*	4.7e−17	−0.38	Esophagus-muscularis	GG>AG>AA
	110042	*DTX4*	5.6e−11	−0.29	Esophagus-mucosa	GG>AG>AA
	110042	*DTX4*	6.0e−9	−0.31	Esophagus-gastroesophageal junction	GG>AG>AA
	110042	*DTX4*	5.9e−9	−0.32	Cells-transformed fibroblasts	GG>AG>AA
	110042	*DTX4*	.000032	−0.14	Adipose-subcutaneous	GG>AG>AA

SNP = single-nucleotide polymorphism.

with the prognosis of CHB.[25] Clearance of the HBsAg in HBV is influenced by many factors, including genetic and host factors such as age, sex, and race.[26] Our approach in this study was to confirm associations the factors of genetic, age, and gender with HBsAg seroclearance. In the present study, we replicated a GWAS study of Korean CHB patients by a candidate gene approach of CHB Taiwan patients to confirm genes associated with HBsAg seroclearance in CHB Taiwanese population. Kim et al revealed that 3 SNPs of rs6462008 located near even-skipped homeobox 1, rs6462008 located near even-skipped homeobox 1 (*EVX1*), and rs171941 located near cardiomyopathy associated 5 (*CMYA5*), and near *MPEG1* were associated with HBsAg seroclearance in the korean population.[27] in this study, we successfully replicated an association of rs7944135 in *MPEG1* with HBsAg seroclearance in a Taiwanese CHB population.

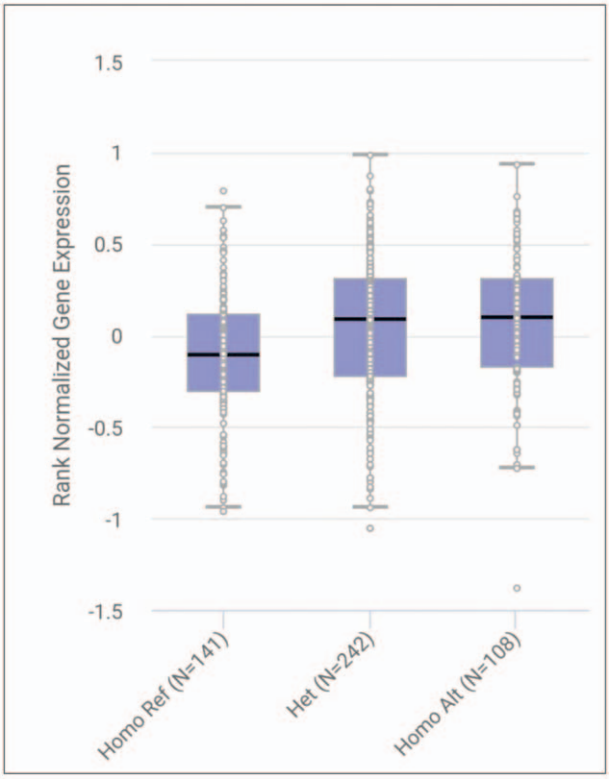

Figure 3. *Cis*-eQTLs of *MPEG1* rs7944135 (Homo Alt (AA)>Het (AG)>Homo Ref (GG)) in muscle-skeletal. *Cis*-eQTLs = *cis*-expression quantitative trait loci, *MPEG1* = macrophage-expressed gene 1.

The male patients are mostly more susceptible to the occurrence of HBsAg seroclearance compared to female patients.[13,27] Consistently, our present study showed that male patients tended to be more susceptible than female patients even after adjusted for gender, age, and ALT, Similar to previous studies from Korea and Taiwan,[13,28] this reason may due to hormonal involvement in the immune response.[29] The age at HBsAg seroclearance also can be considered as a factor. Our study identified that the mean age at HBsAg seroclearance was 50 years. This finding is relatively close to previous studies in Hong Kong (mean, 48.8 years), Korea (mean, 50 years), and Japan (mean, 51 years).[25,30,31]

This study showed that rs7944135 in *MPEG1* is significantly associated with HBsAg seroclearance in a Taiwanese CHB population. We observed that subjects with the AA genotype of *MPEG1* rs7944135 had a higher susceptibility to HBsAg clearance, compared to those with the AG or GG genotype (OR = 1.76). In addition, we still found an association in rs7944135 with 1.74-fold increased risk of the AA genotype of HBsAg seroclearance occurring under the recessive model (OR = 1.74). Spontaneous HBsAg seroclearance can be predicted through serum levels of the HBsAg and HBV DNA.[24,32] In terms of the time taken to the HBsAg seroclearance and undetected of HBV DNA, results showed that the rs7944135 AA genotype had a higher susceptibility to HBsAg seroclearance and HBV DNA undetectable compared to the AG and GG genotypes. A correlation between HBsAg and HBV DNA in serum was reported by Liu et al and revealed that the HBsAg level was not detected during the immune clearance phase as a result of declining HBV DNA levels. In addition, Coffin et al revealed that with a lower level of the HBsAg, there was a greater likelihood for loss of the HBsAg. The HBsAg at a low level was associated with a lower risk of prognosis of HCC.[3] It is well known that the HBsAg contributes to the immunopathogenesis of persistent HBV infection, and a higher incidence of HBsAg seroclearance was associated with lower HBsAg levels.[33]

MPEG1 is a specific gene that was identified in human and mice in macrophages and has many roles in immune responses.[34] According to HaploReg V4.1, rs7944135 was located at 11q12.1 of *MPEG1*, and was involved in the changing chromatin status of primary T cells from peripheral blood, which plays a central role in the "primary immune response" of

cell-mediated immunity, the Information may suggest the role of *MPEG1* in immune clearance of HBsAg in HBV infection. Furthermore, the *MPEG1* gene has a role in immune response through encoding perforin-like protein and recognition of antigen.[35] The perforin-like protein is predicted to be a perforin domain of membrane attack complex that helps cytotoxic T cells and natural killer cells kill virus infected cell.[36,37] In our study, we demonstrated from The GTEx portal database that rs7944135 AA genotype shows the highest expressed of *MPEG1* gene in compare to GG and AG genotype. It infers that patients with AA genotype on rs7944135 could have higher expression of *MPEG1* and lead to higher immune response against HBV, and results in seroclearance of the HBsAg.

Additional genes, such as HLA, cytokine *TNF*-α, chemokine receptor 5,[38] and *MCP1*,[21] were found to be associated with the incidence of HBsAg seroclearance through cell-mediated immune responses. Some evidence has been provided that polymorphisms in HLA subtypes are significantly associated with the occurrence of HBsAg seroclearance, such as a polymorphism of *HLA-DP* rs3077 with haplotype GAT having a 2.17-fold association in a Chinese population,[39]*HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535 with A alleles in a Japanese population,[40] and *HLA-B*4001* in Taiwanese population.[41] There are many polymorphisms of the TNF-α promoter region that were reported to alter TNF-α-associated HBV clearance in many populations.[42]*MCP1* with -2518G>A was associated with HBV clearance in a Korean population.[21]

In this study, we investigated the association of individual SNPs with susceptibility to HBsAg seroclearance in Taiwanese CHB patients. We acknowledge that our study needs to be validated by future studies in other population using more samples, this study offers the findings that polymorphism of MPEG1 have a pivotal association with HBsAg seroclearance in Taiwanese CHB patients.

5. Conclusion

Our present study is a replication of study for a SNP of MPEG1 rs7944135, with susceptibility to HBsAg seroclearance. Our study showed that an HBsAg seroclearance-associated SNP, rs7944135 with AA genotype, solely has a significant association with the loss of the HBsAg in CHB infection in Taiwanese HBV patients.

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