Research Article

Comprehensive investigation of cytokine- and immune-related gene variants in HBV-associated hepatocellular carcinoma patients

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Host genotype may be closely related to the different outcomes of Hepatitis B virus (HBV) infection. To identify the association of variants and HBV infection, we comprehensively investigated the cytokine- and immune-related gene mutations in patients with HBV-associated hepatocellular carcinoma (HBV-HCC). Fifty-three HBV-HCC patients, 53 self-healing cases (SH) with HBV infection history and 53 healthy controls (HCs) were recruited, the whole exon region of 404 genes were sequenced at >900× depth. Comprehensive variants and gene levels were compared between HCC and HC, and HCC and SH. Thirty-nine variants (adjusted \( P < 0.0001 \), Fisher’s exact test) and 11 genes (adjusted \( P < 0.0001 \), optimal unified approach for rare variant association test (SKAT-O) gene level test) were strongly associated with HBV-HCC. Thirty-four variants were from eight human leukocyte antigen (HLA) genes that were previously reported to be associated with HBV-HCC. The novelities of our study are: five variants (rs579876, rs579877, rs368692979, NM_145007:c.*131, *130delITG, NM_139165:exon5:c.623-2->TT) from three genes (REAT1E, NOD-like receptor (NLR) protein 11 (NLRP11), hydroxy-carboxylic acid receptor 2 (HCAR2)) were found strongly associated with HBV-HCC. We found 39 different variants in 11 genes that were significantly related to HBV-HCC. Five of them were new findings. Our data implied that chronic hepatitis B patients who carry these variants are at a high risk of developing HCC.

Introduction

Hepatitis B virus (HBV) affected more than 248 million individuals worldwide [1] and caused deaths of 50000–1.2 million each year [2]. The HBV infection outcomes including self-healing and persistent infection, are associated with viral factors, environmental factors, and host genetic factors [3]. Host immune responses, such as virus recognition, antigen processing and presentation, as well as immune regulation are associated with pathogen infection and clearance [4]. The innate immunity plays important roles in immunopathology and treatment of HBV infection [5]. Toll-like receptors (TLRs) and innate immune sensors of pathogen serve as immune regulators of both innate and adaptive immune responses [6]. Recent study showed that young mice with TLR4 mutation exhibit rapid HBV clearance [7]. NOD-like receptor (NLR), another immune sensor can sense and react to viral infection through the inflammasomes [8,9]. The adaptive immunity genes such as
human leukocyte antigen (HLA), have been widely studied in the HBV infection by genome-wide association study (GWAS) [10-14]. Polymorphisms of some cytokine genes are also associated with the outcomes of patients with HBV infection [15-18]. With the advances in high-throughput sequencing (HTS), some rare variants have been found and associated with HBV infection. To identify the variants associated with outcomes of patients with HBV infection, we systematically sorted and sequenced 404 genes related to innate and adaptive immunity, cytokine and cytokine receptors, and immune regulator genes in Chinese population infected with/without HBV infection. The identification of variants associated with the outcome of patients with HBV infection helps physicians treat the patients more specifically.

**Materials and methods**

**Study subjects and sample collections**

Three groups were included in the present study: hepatocellular carcinoma (HCC) group individuals who had HBV infection history and self-healed (self-healing (SH)) and healthy controls (HCs). The participants were from the First, Second, Third, and Fourth Hospital of Hebei Medical University (Shijiazhuang, China) and the Fifth Hospital of Shijiazhuang (Shijiazhuang, China) between January 2015 and January 2016. HCC diagnosis was according to the guidelines of the America Association for the Study of Liver Diseases (AASLD) [19]. HCC patients were seropositive for HBsAg or had HBV infection history. Self-healing HBV patients had no previous HBV immunization and the liver enzymes were normal, seropositive for HBsAb and HBeAb, and seronegative for HBsAg, HBeAg, and HBV DNA. HCs had normal liver enzymes, seronegative for HBCab, HBeAb, HBeAg, HBsAb, and HBsAg, no previous HBV immunization, and no endocrine, cardiovascular, and kidney diseases, and other liver diseases history. All participants had written informed consents. The protocol of the present study was in accordance with the Declaration of Helsinki, and was approved by the Human Ethnic Committee of the Second Hospital of Hebei Medical University. Five milliliters of whole blood were drawn and stored at −80°C until use. DNA was extracted from whole blood samples using an Invitrogen PureLink Genomic DNA Mini Kit (Thermo Fisher, Foster City, CA, U.S.A.).

**Cytokines and immune genes search and gene panel design**

The cytokines and cytokine receptors, TLRs, NLRs, HLA family genes, T-cell activation, and co-stimulation genes, natural killer cell target genes, G-protein-coupled receptor genes such as free fatty acid receptor (FFAR), hydroxy-carboxylic acid receptor (HCAR) family genes were sorted according to the HUGO Gene Nomenclature Committee (HGNC) database [20]. TargetSeq™ (iGeneTech, Beijing, China), an RNA probe based liquid phase chips were used to capture the whole exome region of selected genes.

**Targeted region sequencing**

The concentration of DNA was determined using a Qubit dsDNA BR Assay Kit (Thermo Fisher, Foster City, CA, U.S.A.). Two microliters of sample DNA were used for sequencing. Using an automated SPRiWorks System (Beckman Coulter, San Jose, CA, U.S.A.), the 200–500 bp size libraries were constructed with a TrueSeq DNA Sample Preparation Kit (Illumina, San Diego, CA, U.S.A.). Then the small (200–500 bp) size libraries were further used for capturing using TargetSeq™ liquid chip capture sequencing kits (iGeneTech, Beijing, China) according to the manufacturer's instructions. In short, before the hybrid capture, small size libraries were mixed with Hybridization Block (iGeneTech, Beijing, China) to avoid any repeating sequences to form hybrid itself. And then, melting the Hybridization Buffer at room temperature and preheating Hybridization Buffer in 65°C water bath. After the solution completely dissolved, 20 μl Hybridization Buffer was mixed with 20 μl small-size library in a PCR tube, and 5 μl RNase block (Thermo Fisher, Foster City, CA, U.S.A.) was mixed with ssRNA probe to prevent probe degradation. The purpose of liquid phase hybrid was to capture objective DNA fragments by ssRNA probe complementary pairing. The Hybridization capture was performed in ABI 2720 PCR (Thermo Fisher, Foster City, CA, U.S.A.) at 65°C overnight for incubation. After hybridization, the DNA:RNA hybrid was enriched by biotin-labeled magnetic beads (Thermo Fisher, Foster City, CA, U.S.A.). Finally, the targetted sequences were amplified in ABI 2720 PCR. PCR parameters were: 95°C for 4 min, 98°C for 20 s, 65°C for 30 s, 16 cycles, 72°C for 30 s, 72°C for 5 min, and 12°C hold. PCR reagents were KAPA Taq PCR Kits (KAPA Biosystems, Boston, Massachusetts, U.S.A.) and Nextflex primers were synthesized by Invitrogen (Thermo Fisher, Foster City, CA, U.S.A.). The quality of amplified library was determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.), and the library DNA concentration was again determined by Qubit dsDNA BR assay kit according to the manufacturer's instructions. Libraries with good quality and DNA concentration >3 ng/μl were sequenced using an Illumina Hiseq X-ten sequencer (Illumina, San Diego, CA, U.S.A.).
Table 1 Demographics

|                     | HCC     | HC       | SH       |
|---------------------|---------|----------|----------|
| Gender, male/female | 45/8    | 26/27    | 30/23    |
| Age, M (Q)          | 56.0 (11.50) | 34.0 (16.0) | 55.0 (19.0) |
| Drinking, no/yes    | 40/13   | 46/7     | 43/10    |
| Smoking, no/yes     | 41/12   | 46/7     | 41/12    |
| HBV DNA, M (Q)      | 3200 (30842) | -        | -        |
| HbsAg. (+/-)        | 41/12   | 0/53     | 0/53     |
| HbeAg. (+/-)        | 17/36   | 0/53     | 0/53     |
| Anti-Hbe, (+/-)     | 7/11    | -        | -        |
| ALT, M (Q)          | 38.0 (88.8) | -        | -        |
| AST, M (Q)          | 58.0 (72.5) | -        | -        |
| AFP, M (Q)          | 161.2 (1070.0) | -        | -        |

Abbreviations: AFP, α-fetoprotein; ALT, Alanine aminotransferase; Anti-Hbe, antibody to HBeAg; AST, aspartate aminotransferase; HbeAg, HBV E antigen; HbsAg, HBV surface antigen; M, mean; Q, quartile.

Bioinformatics analyses

Data QC

The adaptor sequences and raw reads with low quality reads were filtered using Trimmmomatic software [21]. The adaptor sequences were GATCGGAAGAGCACACGTCT and AGATCGGAAGAGCGTCGTAGGAAACAGGTGT, the criterion of low-quality sequence was the quality value of no more than Q20 or the accuracy of no more than 99%. The lengths should be longer than 40 bp after removing the base that does not meet the criteria. Finally fastqc software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) was used to measure data quality to make sure that 95% of the remaining reads or clean reads with quality were more than Q30.

Variants calling

The clean reads were aligned to the reference human genome (February 2009, hg19, GRCh37, downloaded from UCSC) by BWA MEM software [22] to generate BAM files. In order to improve the accuracy, the samtools [23] and picard software (http://broadinstitute.github.io/picard/) were used to remove PCR repetitive sequence. The Genome Analysis Toolkit (GATK) [24] was used to detect the variants such as SNPs and InDels in BAM files. Finally, ANNOVAR software [25] was used to annotate the variants.

Functional effect of variants

Phenolyzer [26] was used to predict the association between the genotype and phenotype of HBV infection.

Association testing

Both single-variant and gene-based tests were performed. For single-variant based test, Fisher’s exact test was used to compare the different variants between HCC/control groups or subgroup, the false discovery rate (FDR) adjusted P-value <0.001 was considered significant. For gene-based tests, optimal unified approach for rare variant association test (SKAT-O) [27], a rare-variant association test used in small-sample case-control genome study, was used to assess excess-risk mutations in HCC/controls and subgroups. Default setting was used and significance were defined at FDR adjusted P-value <0.0001.

Results

Study subjects

A total of 53 HCC patients, 53 SH cases with previous HBV infection and 53 HCs were recruited, the clinical characteristics and statistics of study subjects were listed in Table 1.

Targeted gene investigations

A total of 404 genes or coding region of ~500 kilo (K) bp, were sorted according to HGNC database, including the whole cytokine and receptor family genes, some innate immunity and adaptive immunity related genes which reported as pathogen sensors (Table 2). The cytokine and receptor family genes were: 43 interleukin (IL) family genes, 42 IL receptor genes, 21 interferon (IFN) genes, 5 IFN receptor genes, 45 chemokine ligand (CCL) genes, 24 chemokine
Table 2 Summary of genes investigated in the study

| Group                        | Subgroup and gene numbers                                                                 |
|------------------------------|-------------------------------------------------------------------------------------------|
| Cytokines and receptors      | ILs and receptors, n=85; IFNs and receptors, n=26; chemokines and receptors, n=69;        |
|                              | tumor necrosis factor and receptors, n=47                                                 |
| Innate immunity              | TLRs, n=10; NLRs, n=22                                                                      |
| Adaptive immunity            | HLA class 1 genes, n=32; MHC class 1 genes, n=32; T-cell inhibitors, n=18; T-cell activators, n=28 |
| Others                       | GPCRs, n=54; natural killer cell targets, n=37, others, n=16;                              |

Abbreviation: GPCR, G-protein-coupled receptor.

receptor genes, 18 tumor necrosis factor genes, and 29 tumor necrosis factor receptor genes. The innate genes included 10 TLR genes, and 22 NLRs family genes. The adaptive immunity genes comprised 27 MHC-related genes, 18 T-cell inhibition and co-inhibition related genes, 28 T-cell activation and co-stimulation genes. Other immune response related genes were: 37 natural killer cell target genes, 54 G-protein-coupled receptor genes, and 16 other genes.

Targeted region sequencing performance

TRS yielded 10.84M clean reads (averaged read length was 143.65 bp) or 1577.94M clean bp for each sample, the average base quality was 40.26 and Q30 of call clean reads was 95.64%; the coverage rate of 499.968K region was 99.48%, duplication rate was 39.84%, and align rate was 99.23%; the capture rate was 50.53%. The target average depth was 919.55X, with target 10X rate was 99.31% and target 20X rate was 99.18%.

Variants and genes related to HBV associated with HCC

As shown in flowchart (Figure 1), both HC and self-healing groups were used as controls to identify variants and genes associated with HCC. The results of variant and gene level association test for each subgroup were listed in Table 3.

A total of 39 significantly different variants and 11 genes were identified. Thirty four of the variants were from eight HLA genes, including HLA-B, HLA-C, HLA-DPA1, HLA-DQA1, HLA-DQB1, HLA-DQB2, HLA-DRB1, and HLA-DRB5. We also identified five non-HLA variants in retinoic acid early transcript 1E (RAET1E), NLR protein 11 (NLRP11), and HCAR2. NM_139165:exon5:c.623-2->TT was located in RAET1E. The variants identified were significantly enriched in HCC patients, and rare or no variants were found enriched in controls. rs368692979 and NM_145007:c.*131_130delTG were located in NLRP11, and were two of the top ten most significant variants (P-values were 6.72E-14 and 7.08E-11 in HCC compared with HC and HCC compared with self-healing, respectively). Previous studies showed that NLRP11 was related to inflammation [41]. These variants were also significantly enriched in HCC patients, and rare or no variants were found enriched in controls. rs579876 and rs579877 were located in 3'-UTR of HCAR2.

Discussion

The present study found that 39 variants in 11 genes were strongly associated with HBV associated with HCC (HBV-HCC). Amongst them, 34 variants from eight genes in HLA region were previously described and our data were consistent with previous studies [30-35]. The novelties of our study are: five variants (rs579876, rs579877, rs368692979, NM_145007:c.*131_130delTG, NM_139165:exon5:c.623-2->TT) from three genes (REAT1E, NLRP11, HCAR2) were found to be strongly associated with HBV-HCC. Our study provided fundamental data which needs further study to confirm the roles of these new variants and genes.

HBV infection is a serious public health problem [1]. Patients with HBV infection have different outcomes, such as self-healing, HBV carrier, chronic hepatitis, or HCC [4]. It has been found that the host genotype may be closely related to the different outcomes. Some HCC-related loci have been identified based on GWAS [10-14,28]. However, these studies are mainly based on the common variants, and most of the variants are located in the non-gene region. It is difficult to verify the gene function and explore the mechanism of disease. Given that HTS methods such as whole exome sequencing (WES) or whole genome sequencing (WGS) focus on not only common but also rare variants, HTS has been widely used in complex disease genetics studies and shows promising results. WES has been successfully applied in HCC genetics research [29].

Large sample size is necessary for HCC studies. However, WES-based GWAS is very expensive. TRS, which targets the specific gene families or pathway genes, provides relatively cost-effective solution. The present study investigated
the cytokines and immune genes associated with HCC. We selected 404 genes that may be associated with HBV infection. These genes are mainly involved in antigen recognition, processing and presentation, immune regulatory cytokines and receptors, as well as those included in innate immune system, such as TLRs and NLRs. We have also studied some genes that are directly related to immune checkpoint.

We included SH cases because we speculated that they may carry less risk mutations or carry more protective mutations. Previous study demonstrated that the performance of gene level associated/burden test is reliable in WES/WGS study even in a small sample size in WES/WGS study [27]. The present study performed a comprehensive variants and gene level association analysis between both HCC compared with HC and HCC compared with SH. We found that 39 variants and 11 genes were strongly associated with HBV-HCC.

Not surprisingly, 34 variants from eight genes in HLA region were found strongly associated with HBV-HCC. Our data were consistent with previous studies [30-35]. We found five new variants from three genes that are also strongly related to HBV-HCC.

RAET1E is an MHC class I related gene from the RAET1 family, which functions as a ligand for the NKG2D receptor. NKG2D receptors are expressed on the surface of several types of immune cells and are involved in both innate and adaptive immune responses [36]. Previous studies showed that reduced NKG2D ligand expression in HCC correlates with early recurrence [37], and that the immunoreceptor NKG2D promotes tumor growth in a model of HCC [38]. Consistent with these studies, we found that a splicing variant in RAET1E, NM_139165:exon5:c.623-2->TT, might lead to malfunction of RAET1E, a ligand for the NKG2D receptor. As NKG2D receptor blockade is an attractive target for HCC therapy [39], further research will be necessary to study how RAET1E may be involved in HBV-associated HCC and develop candidate drugs.
### Table 3 Association of variants and genes with HBV associated with HCC

| Gene symbol | Associated SNVs | Variant level OR | Variant level P-value (FDR adjusted) | Numbers of SNVs | SKAT-O gene level P-value (FDR adjusted) |
|-------------|----------------|------------------|--------------------------------------|----------------|------------------------------------------|
| HLA-B       | rs2596493 Inf   | Inf              | 2.25E-18                             | 106            | 1.7232E-16                               |
| HLA-B       | rs1131285 Inf   | Inf              | 1.7E-13                              | 106            | 1.7232E-16                               |
| HLA-B       | rs1065386 Inf   | Inf              | 6.97E-09                             | 106            | 1.7232E-16                               |
| HLA-B       | rs1050570 Inf   | Inf              | 1.87E-08                             | 106            | 1.7232E-16                               |
| HLA-B       | rs105056 Inf    | Inf              | 9.00000019                           | 106            | 1.7232E-16                               |
| HLA-C       | rs1131215 Inf   | Inf              | 7.92E-12                             | 108            | 5.33706E-07                               |
| HLA-C       | rs41556617 Inf  | Inf              | 6.79E-12                             | 108            | 5.33706E-07                               |
| HLA-DPA1    | rs1042174 Inf   | 16.63            | 28.69                                | 33             | 1.4521E-08                               |
| HLA-DPA1    | rs1126543 Inf   | Inf              | 2.9E-09                              | 33             | 1.4521E-08                               |
| HLA-DPA1    | rs2389930 Inf   | Inf              | 6.97E-09                             | 33             | 1.4521E-08                               |
| HLA-DPA1    | rs2389929 Inf   | Inf              | 6.97E-09                             | 33             | 1.4521E-08                               |
| HLA-DPA1    | rs1126542 Inf   | Inf              | 1.87E-08                             | 33             | 1.4521E-08                               |
| HLA-DQA1    | rs9272896 Inf   | Inf              | 9.00000011                           | 81             | 3.33767E-07                               |
| HLA-DQB1    | rs1049055 Inf   | 36.19            | 75.53                                | 110            | 9.04603E-10                               |
| HLA-DQB1    | rs1130430 Inf   | 19.53322682      | 11.3                                 | 110            | 9.04603E-10                               |
| HLA-DQB1    | rs1049057 Inf   | Inf              | 4.67E-08                             | 110            | 9.04603E-10                               |
| HLA-DQB1    | rs1063345 Inf   | Inf              | 4.000000465                          | 110            | 9.04603E-10                               |
| HLA-DQB1    | rs1140316 Inf   | Inf              | 0.00000623                           | 110            | 9.04603E-10                               |
| HLA-DQB2    | rs9276572 Inf   | Inf              | 2.05E-15                             | 21             | 5.04185E-06                               |
| HLA-DRB1    | rs17211105 Inf  | 18.48            | 18.48                                | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs9270299 Inf   | 12.56            | 16.04                                | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs17683134 Inf  | 44.09            | 20.97                                | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs8270303 Inf   | 20.97            | 20.97                                | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs2038759 Inf   | 353.69           | 214.66                               | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs17211091 Inf  | 18.04            | 96.28                                | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs113734598 Inf | 48.46            | 15.62                                | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs9269693 Inf   | 136.13           | 9.53E-13                             | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs1064697 Inf   | Inf              | 0.000000124                          | 126            | 1.08223E-14                               |
| HLA-DRB1    | rs1071752 Inf   | Inf              | 0.00000623                           | 126            | 1.08223E-14                               |
| HLA-DRB5    | rs7785982 Inf   | Inf              | 0.000000801                          | 83             | 4.77962E-10                               |
| HLA-DRB5    | rs701884 Inf    | Inf              | 0.00000245                           | 83             | 4.77962E-10                               |
| HLA-DRB5    | rs1064857 Inf   | Inf              | 0.0000058                           | 83             | 4.77962E-10                               |
| HCAR2       | rs57987 Inf     | Inf              | 0.0000227                           | 11             | 2.03163E-06                               |
| HCAR2       | rs579876 Inf    | Inf              | 0.0000227                           | 11             | 2.03163E-06                               |
| NLRP11      | NM_145007:     | Inf              | 7.92E-12                             | 22             | 6.83802E-07                               |
|             | c."131_130delTG" | Inf              | 6.95927E-07                         | 22             | 6.83802E-07                               |
| NLRP11      | rs68869297 Inf  | 17.84443693      | 32.53501457                        | 22             | 6.83802E-07                               |
| RAET1E      | NM_139165:     | Inf              | 16.8900097                          | 21             | 3.07916E-05                               |
|             | exon5:c.623-2-> | TT                |                                      |                |                                          |

**Abbreviations:** Inf, infinite; HCAR2, hydroxy-carboxylic acid receptor 2; OR, odds ratio; NLRP11, NLR protein 11; RAET1E, retinoic acid early transcript 1E; SH, self-healing cases with previous HBV infection.

**NLRP11** is a member of the NLR gene family, which is reported to be closely related to antiviral immunity [40]. Although little is known about whether NLRP11 is involved in HBV infection, another member of the NLR family, NLRP3, is involved in danger signal inflammatory responses [41]. Furthermore, the four variants identified, including rs368692979 and NM_145007:c."131_130delTG in NLRP11, together with rs579876 and rs579877 in HCAR2, a gene closely related to immune activation [42], were all from the 3' UTR region, which suggests that the expression of NLRP11 and HCAR2 might be under the regulation of miRNA.
Conclusion
Our comprehensive investigation of cytokine- and immune-related gene mutations in HBV-HCC patients and controls showed that 39 different variants and 11 genes were significantly related to HBV-HCC. Our data implied that chronic hepatitis B patients who carry these variants need intensive monitoring.

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Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Author contribution
Fengxue Yu, Xiaolin Zhang and Mingbang Wang conducted the research design; Suzhai Tian, Lianxia Geng, Chaojun Zhang, Lina Guo and Wenting An performed DNA extracting; Weili Xu, Yuan Jia, Xuechen Liu, Junji Ma and Yuan Quan collected the samples; Fengxue Yu, Ning Ma, Xiaolin Zhang, Mingbang Wang and Dianwu Liu analyzed the data.

Abbreviations
BAM, Binary Alignment/Map; FDR, false discovery rate; GWAS, genome-wide association study; HbsAg, Hepatitis B surface antigen; HBeAg, Hepatitis B e antigen; HBcAb, Hepatitis B core antibody; HBeAb, Hepatitis B e antibody; HBV, hepatitis B virus; HBV-HCC, HBV associated hepatocellular carcinoma; HC, healthy control; HCAR2, hydroxy-carboxylic acid receptor 2; HCC, hepatocellular carcinoma; HGNC, HUGO Gene Nomenclature Committee; HLA, human leukocyte antigen; HTS, high-throughput sequencing; IFN, interferon; IL, interleukin; NLR, NOD-like receptor; NLRP11, NOD-like receptor protein 11; NOD, nucleotide-binding oligomerization domain; RAET, retinoic acid early transcript; RAET1E, retinoic acid early transcript 1E; SH, self-healing case; SNV, Single Nucleotide Variant; TLR, toll-like receptor; TRS, target region sequencing; QC, quality control; WES, whole exome sequencing; WGS, whole genome sequencing.

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