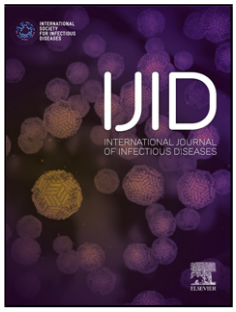

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CYP24A1 genetic variants in the vitamin D metabolic pathway are involved in the outcomes of hepatitis C virus infection among high-risk Chinese population

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Abstract

Background and aims: It has been demonstrated that 1,25-hydroxyvitamin-D3-24-hydroxylase, encoded by CYP24A1 gene, is a key enzyme that neutralizes the active vitamin D3 metabolite 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] in response to hepatitis C virus (HCV) infection. This study aimed to investigate whether CYP24A1 genetic variation is associated with HCV infection outcomes.

Methods: 848 HCV chronically infected subjects, 507 natural clearance subjects, and 1017 uninfected controls were enrolled. Nine single nucleotide polymorphisms (SNPs) in the CYP24A1 gene were genotyped using the Sequenom MassARRAY platform.

Results: After adjusting for age, gender, and routes of infection, logistic regression analyses showed that rs6013897-A was associated with an elevated risk of HCV infection ($P<0.05$). In addition, this study has also demonstrated that rs6068816-T significantly reduced the risk of chronic HCV infection, while rs3787557-C, rs6022999-G, and rs2248359-T significantly increased the risk of chronic HCV infection (all $P<0.05$). Haplotype analysis suggested that, compared to the most frequent $T_{rs6068816}T_{rs3787557}A_{rs6022999}C_{rs2248359}$ haplotype, the CTGT haplotype (adjusted OR=1.376, 95% CI=1.092-1.735, P=0.007) and CCAC haplotype (adjusted OR=1.483, 95% CI=1.139-1.929, P=0.003) were associated with an increased risk of chronic HCV infection.

Conclusion: These findings indicate that SNPs in CYP24A1 gene may contribute to the risk of HCV infection and chronic HCV infection among high-risk Chinese population.

Abbreviations

1,25(OH)2D3, 1,25-dihydroxyvitamin D3; HCV, hepatitis C virus; SNPs, single nucleotide polymorphisms; WHO, World Health Organization; HCC, hepatocellular carcinoma; 25(OH)D3, 25-hydroxyvitamin D3; HIV, human immunodeficiency virus; G1, genotype 1; CHC, chronic hepatitis C; SVR, sustained virologic response; Peg-IFN, pegylated-interferon; CYP, cytochrome P450; CYP2R1, CYP family 2 subfamily R member 1; CYP27A1, CYP family 27 subfamily A member 1; CYP27B1, CYP family 27 subfamily B member 1; CYP24A1, CYP family 24 subfamily A member 1; DHCR7, 7-dehydrocholesterol reductase; VDR, vitamin D receptor; GWAS, Genome-wide association study; HD, hemodialysis; PBD, paid blood donors; CHB, Han Chinese in Beijing; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; ALT, alanine aminotransferase; AST, aspartate aminotransferase; tSNPs, tagging SNP; PCR, polymerase chain reaction; DAVID, the Database for Annotation, Visualization and Integrated Discovery; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CC, cellular component; BP, biological process; MF, molecular function; eQTL, expression quantitative trait loci; TF, transcription factor; OR, odds ratio; 95% CI, 95% confidence interval; FDR, false discovery rate; LD, linkage disequilibrium; IQR, interquartile range; ID, inject drugs; ERAD, endoplasmic reticulum-associated

degradation; PPAR, peroxisome proliferator-activated receptor; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; NHL, non-Hodgkin lymphoma; CAC, coronary artery calcification; PD, parkinson's disease; FN-BMD, bone mineral density at the femoral neck.

Keywords: hepatitis C virus, CYP24A1, vitamin D, metabolism, polymorphism

Introduction

Globally, hepatitis C virus (HCV) infection is an important public health issue, with an estimated population of 185 million infected people and 71 million chronically infected patients (Blach et al., 2017; WHO, 2017). In addition, approximately 55-85% of HCV-infected individuals fail to clear the virus and develop chronic infections and progress to more serious conditions, such as liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC), and even death (WHO, 2018; Torres et al., 2017). There are many factors influencing the outcome of HCV infection, which are mainly related to the virological characteristics of HCV and host factors. Among them, innate and adaptive immune of host is considered to be a crucial mechanism in the natural history of HCV infection (Georgel et al., 2010; Neumann-Haefelin et al., 2008; Thimme et al., 2001).

Studies have increasingly found that vitamin D not only affects the stability of calcium and bone, but also plays a key role in regulating the immune system, inflammatory response and fibrogenesis (Terrier et al., 2011). The activated hormonal form of vitamin D is 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], and the production and degradation of 1,25$(OH)_2D_3$ has been found to be an important signal component in the innate and adaptive immune system (Liu, 2006; von Essen et al., 2010). As an important immune modulator, vitamin D and its two metabolites, 25-hydroxyvitamin D3 [25$(OH)D_3$] and 1,25$(OH)_2D_3$, are found to be associated with cancer, autoimmune diseases, and viral infections such as respiratory tract infections, and human immunodeficiency virus (HIV) (Beard et al., 2011; Chesney, 2010; Garland et al., 2006; Vanamerongen et al., 2004).

In a treatment cohort study of patients with chronic hepatitis C (CHC), low serum levels of 25(OH)D_3 were associated with severe lesions of liver fibrosis and low sustained virologic response (SVR) based on pegylated-interferon (Peg-IFN) therapy (Petta et al., 2010). Studies have found that vitamin D supplementation was associated with improved SVR rates in patients with CHC treated with Peg-IFN and ribavirin, regardless of viral genotype (Abumouch et al., 2011; Yokoyama et al., 2014). In particular, vitamin D deficiency has been associated with increased higher rates of infections and mortality in patients with HCV-related cirrhosis (Buonomo et al., 2017; Buonomo et al., 2019). In vitro studies have found that vitamins D2, D3 and 1,25$(OH)_2D_3$ have anti-HCV activity and may inhibit HCV replication (Gutierrez et al., 2014).

Vitamin D3 needs to be converted into the activated form of vitamin D, 1,25$(OH)_2D_3$ through two hydroxylation reactions (Kitson, 2012). Firstly, vitamin D3 is hydrolyzed in the liver to synthesize 25(OH)D_3, which is catalyzed by 25-hydroxylase encoded by cytochrome P450 (CYP) family 2 subfamily R member 1 (CYP2R1) and CYP family 27 subfamily A member 1 (CYP27A1) genes. Subsequently, 25(OH)D_3 is hydroxylated in the kidney and synthesized 1,25$(OH)_2D_3$ in other tissues, which is catalyzed by the 1α-hydroxylase encoded by CYP family 27 subfamily B member 1 (CYP27B1) gene. However, 1,25$(OH)_2D_3$ can be decomposed by 1,25-hydroxyvitamin-D3-24-hydroxylase encoded by CYP family 24 subfamily A member 1 (CYP24A1) gene, and then excreted through bile to maintain the balance of vitamin D metabolism in vivo. Studies have reported that SNPs involved in the pathway of vitamin D synthetic,

including CYP2R1, CYP27B1, 7-dehydrocholesterol reductase (DHCR7), and vitamin D receptor (VDR) genes, are associated with complications of HCV infection or Peg-IFN treatment response (de Azevedo et al., 2017; Lange et al., 2011; Thanapirom et al., 2017). 24-hydroxylase is a rate-limiting enzyme in the metabolism of vitamin D and plays an important role in maintaining the balance of vitamin D metabolism. Genome-wide association studies (GWAS) found that multiple genetic variants were associated with vitamin D status, among which SNP rs6013897 near CYP24A1 was associated with 25(OH)D_3 concentration (Wang et al., 2010). This study attempted to explore the correlation of the potential functional SNPs in CYP24A1 gene with the risk of HCV infection and chronic HCV infection among HCV-infected high-risk population.

Materials and Methods

Study participants and design

A total of 1017 uninfected controls, 507 HCV natural clearance subjects and 848 HCV chronically infected subjects from 2008 to 2017. All of these participants were from nine hospital hemodialysis (HD) centers (Nanjing, Jiangsu, China), Nanjing Compulsory Detoxification Center (Nanjing, Jiangsu, China), and six former paid blood donation (PBD) aggregated villages (Zhenjiang, Jiangsu, China). The definitions of the three groups were as follows: (1) uninfected control group was defined as an anti-HCV antibody seronegative and HCV-RNA seronegative; (2) natural clearance group was defined as anti-HCV antibody seropositive and HCV-RNA seronegative; (3) chronically infected group was defined as anti-HCV antibody seropositive and HCV-RNA seropositive. The exclusion criteria for participants were as following: (1) no clear

history of multiple HD, intravenous drug (IVD) or PBD; (2) history of antiretroviral therapy; (3) history of other liver viruses or HIV infections; (4) severe dysfunction of important organs (such as heart, lung, or kidney); (5) liver cirrhosis and other liver diseases (including metabolic, autoimmune, or alcoholic liver disease, etc.); (6) history of cancer. All patients were diagnosed by experienced physicians based on clinical interviews, laboratory results and international standards. In particular, the diagnostic criteria for cirrhosis are as follows: (1) FibroScan score <12.5 kPa; (2) FibroTest score $\leqslant$0.72; or (3) AST/PLT $\leqslant$2. The current study protocol in accordance with the Declaration of Helsinki, and was approved by the Institutional Ethics Review Committee of Nanjing Medical University (Nanjing, China). Written informed consent was obtained from all participants.

Data and Blood Sample Collection

The demographic and clinical characteristics of all participants were collected from self-administered questionnaire and electronic medical record system. A baseline blood sample (~10 mL) was collected from all participants using standard procedures. 5 mL of blood was extracted by phenol-chloroform method for further genotyping. The remaining blood was used to detect HCV indicators (anti-HCV antibodies, viral load, and viral genotype), liver function indicators (alanine aminotransferase, ALT; aspartate aminotransferase, AST), and other indicators (anti-HAV, HBsAg, anti-HEV, and HIV antibody). The detection of HCV indicators has been described in our past studies (Wu et al., 2016). It should be emphasized, the HCV genotyping were performed by the murex HCV serotyping 1–6 assay ELISA kit (Abbott, Wiesbaden, Germany)

corresponding to type-specific antibodies.

SNP selection and genotyping

The detailed process of SNP selected was shown in the Figure S1. Frist of all, the genotype database of CYP24A1 gene in Han Chinese in Beijing (CHB) was downloaded from the 1000 Genomes Project database (http://www.1000genomes.org/). Secondly, the genotype database was introduced into the Haploview software (version 4.2; Broad Institute, Cambridge, MA, USA), and 126 sites of CYP24A1 gene were found. Set Hardy-Weinberg (H-W) P-value cutoff to 0.05 and minor allele frequency (MAF) to 0.05, and then selected 83 SNPs sites that meet the Hardy-Weinberg equilibrium (HWE) and high frequency. Then, $r^2 \geq 0.8$ was used as the cut-off criterion during tagging SNP (tSNPs) selection, which generated 33 tSNPs. Finally, we performed literature search for these tSNPs, and screening according to the following criteria: (1) conducted relevant research in the Chinese population; (2) has been found to be associated with other diseases, especially hepatic diseases, viral infections, inflammatory diseases, immunological diseases, etc. Based on this, 8 disease-related tSNPs of CYP24A1 gene were selected, including rs4809957 (G>A), rs2762934 (G>A), rs927650 (C>T), rs6068816 (C>T), rs3787557 (T>C), rs2296241 (G>A), rs6022999 (A>G), and rs2248359 (C>T). In addition, we also included rs6013897 (T>A) as our candidate SNP based on previous publications (Wang et al., 2010). Therefore, we eventually nine candidate SNPs for further study.

All SNPs were genotyped using the Sequenom MassARRAY platform (Sequenom, CA, USA), and the primers and probes information were shown in Table S1. The

concentration of extracted DNA was adjusted to 50 ng/μl and transferred to 384-well plate. After multiplex polymerase chain reaction (PCR) amplification, the product was treated with alkaline phosphatase for further single-base extension. Lastly, after purified by clean resin, the final products were transferred to SpectroCHIP arrays, and genotype data were analyzed using MassARRAY TYPER 4.0 software. The following measures were implemented to control the data quality: (1) blind method were adopted in genotyping, that all laboratory personnel were unclear about the clinical data of the subjects; (2) randomly select 10% of the samples for repeated experiments with repeatability of 100%. Genotyping success rate of all SNPs was higher than 95%.

In silico analysis

To further explore the potential functional significance of CYP24A1 gene variants in HCV infection, we performed bioinformatics analysis using online database. First, we constructed the protein interaction network of CYP24A1 using STRING (https://string-db.org/). The Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) were used to annotate the Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of CYP24A1, where the GO function includes cellular component (CC), biological process (BP), and molecular function (MF). Then, the RegulomeDB online database (http://regulome.stanford.edu/) was used to assess the RegulomeDB score of SNPs, which ranges from 1 to 6, and to predict whether there are potential functions such as expression quantitative trait loci (eQTL), transcription factor (TF) binding, matched TF motifs, any motifs, matched DNase footprints, DNase footprint, and DNase peaks. HaploRegV4.1

(https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) was used to investigate the impact of non-coding variants on clinical phenotypes and normal variation.

Statistical Analyses

All statistical analyses were performed using the SPSS 25.0 (SPSS, Chicago, IL, USA). Distributions of demographic and clinical characteristics among uninfected controls, natural clearance and persistent infection subjects were evaluated by one-way Kruskal-Wallis test (for non-normal continuous variables) and χ^2-test (for categorical variables). HWE in the control group was assessed by the goodness of fit χ^2-test. Logistic regression analysis was used to calculate odds ratio (OR) and 95% confidence interval (95% CI) for assessing the associations of genotypes and alleles of selected SNPs with the risk of HCV infection and chronic HCV infection. And the analysis was adjusted for age, gender, and routes of infection to eliminate the possible confounding effects. Each SNP was analyzed using codominant, dominant, recessive, and additive genetic models. The codominant model considers mutant homozygous type versus wild type and heterozygous type versus wild type, respectively. The dominant model considers the mutant homozygous type and heterozygous type together versus the wild type. The recessive model considers the heterozygous type versus the mutant homozygous type and wild type together, and the additive model considers the heterozygous type versus the mutant homozygous type versus the wild type. False discovery rate (FDR) correction was used to correct for multiple comparisons. Subgroup analysis was performed for statistically significant SNPs and Q test was performed to calculate the

heterogeneity between subgroups. The Haploview (version 4.2) software was used to calculate the linkage disequilibrium (LD) parameters (D' and r^2). The PHASE software (version 2.1) was used to predict for haplotype construction based on the observed genotype. All statistical analyses were two sided and $P<0.05$ was considered statistically significant.

Results

Basic characteristics of participants

Of the 2372 participants, the median age was 50.0 years (interquartile range, IQR=42.0-60.0), and female accounted for 63.2%. The distributions of demographic and clinical characteristics among the three study groups were summarized in Table 1. The ages and gender of 1017 uninfected control group, 507 natural clearance group and 848 chronically infected group were similar (all $P>0.05$). The constituent ratio of HCV infection among HD patients, IVD users, and PBD subjects was 22.1%, 60.9%, and 77.4%, respectively. There was significant difference in the distribution of routes of infection among the three study groups ($P<0.05$). The abnormal rates of ALT in the three groups were 5.3%, 25.1%, and 42.7%, and the abnormal rates of AST were 5.8%, 23.7%, and 41.6%, respectively, with significant differences ($P<0.05$). In addition, there was no significant difference in the distribution of HCV genotypes between natural clearance group and chronically infected group ($P=0.693$). As can be seen from Table S1, the MAF for all nine SNPs were greater than 5% in uninfected control, similar to that reported in the NCBI dbSNP database. Moreover, the genotype frequencies of all SNPs were in HWE (all $P>0.05$).

Associations between CYP24A1 SNPs and the outcomes of HCV infection

The genotype distributions of the nine SNPs among the three study groups and the results of the logistic regression analysis were shown in Table 2. To determine the association between the CYP24A1 SNPs and the risk of HCV infection, we combined natural clearance group and chronically-infected group into the HCV-infected group, and compared that with uninfected group. The allelic frequencies of candidate SNPs were first compared between HCV-infection group and uninfected control group (Table 2). After adjusting for gender, age and routes of infection, co-dominant genetic models showed that rs6013897-TA/AA genotypes significantly increased the risk of HCV infection, compared to the TT genotype (adjusted OR=1.519, 95% CI=1.209-1.907, $P=3.257\times10^{-4}$ for TA; adjusted OR= 2.220, 95% CI= 1.207-4.081, P=0.010 for AA). In addition, other genetic models have shown that rs6013897-A was associated with an increased risk of HCV infection (dominant model: adjusted OR=1.577, 95% CI=1.266-1.965, $P=4.734\times10^{-5}$; recessive model: adjusted OR=1.992, 95% CI=1.088-3.646, P=0.025; additive model: adjusted OR=1.509, 95% CI=1.247-1.826, $P=2.400\times10^{-5}$).

An analysis of the risk of chronic HCV infection was performed in a comparison between natural clearance group and chronically infected group (Table 2). After adjusting for gender, age and routes of infection, we found that the minor allele of rs6068816 (dominant model: adjusted OR=0.754, 95% CI=0.598-0.951, P=0.017; additive model: adjusted OR=0.809, 95% CI=0.686-0.954, P=0.012), rs3787557 (dominant model: adjusted OR=1.272, 95% CI=1.011-1.601, P=0.040; additive model: adjusted OR=1.243, 95% CI=1.028-1.502, P=0.025), rs6022999 (recessive model: adjusted OR=1.885, 95% CI=1.107-3.208, P=0.020), and rs2248359 (recessive model: adjusted OR=1.492, 95% CI=1.039-2.143, P=0.030) were significantly associated with the risk of chronic HCV infection. However, none of these polymorphisms differed significantly between the two groups after correcting for multiple comparison (all P_{FDR}>0.05, shown in Table S2). In addition, co-dominant genetic models showed that rs6068816-TT (adjusted OR=0.669, 95% CI=0.470-0.953, compared to the CC genotype), rs6022999-GG (adjusted OR=1.932, 95% CI=1.125-3.318, compared to the AA genotype), and rs2248359-TT (adjusted OR=1.541, 95% CI=1.046-2.269, compared to the CC genotype) genotypes were also significantly associated with the risk of chronic HCV infection.

Further stratification analyses of rs6013897 were conducted to eliminate age, gender, ALT status, AST status, and route of infection. The additive genetic models were applied to calculate OR and 95% CI of each stratification. As shown in Table 3, the association of rs6013897 with an elevated risk of HCV infection remained in the

stratification of age and gender (all adjusted P<0.05). Analysis of other stratification factors found that the association of rs6013897 with the risk of HCV infection was more pronounced among subjects with ALT≤40 U/L (adjusted OR=1.557, 95% CI= 1.265-1.917, P= 3.022×10^{-5}), AST≤40 U/L (adjusted OR=1.538, 95% CI=1.250-1.892, P= 4.748×10^{-5}), HD (adjusted OR=1.454, 95% CI=1.065-1.987, P=0.019), and PBD (adjusted OR=1.712, 95% CI=1.264-2.319, P=0.001). Moreover, the heterogeneity test found that the association between rs6013897 and the risk of HCV infection did not have significant heterogeneity among different stratification (all P>0.05).

For further analysis, we combined rs6068816, rs3787557, rs6022999, and rs2248359 into one SNP set and performed haplotype analysis (Table 4). LD analyses of these four SNPs were conducted before the haplotype analysis. As shown in Figure 1, there was strong LD between them in chronicity analysis. Haplotype analyses of these SNPs were performed in a comparison between natural clearance group and chronically-infected group. Compared to the most frequent $T_{rs6068816}T_{rs3787557}A_{rs6022999}C_{rs2248359}$ haplotype, the CTGT haplotype (adjusted OR=1.376, 95% CI=1.092-1.735, P=0.007) and CCAC haplotype (adjusted OR=1.483, 95% CI=1.139-1.929, P=0.003) were associated with an increased risk of chronic HCV infection.

Notes: The LD between the SNPs is measured as r2 and shown (×100) in the diamond at the intersection of the diagonals from each SNP. r2 = 0 is shown as white, 0< r2<1 is shown in pink and r2 =1 is shown in red. The analysis track at the top shows the SNPs

according to chromosomal location.

Bioinformatics analysis of CYP24A1 SNPs

The protein interaction network of CYP24A1 was shown in Figure 2. The top three functional partners are CYP2R1, CYP27B1, and VDR, with scores of 0.941, 0.922, and 0.869, respectively. GO function analysis found that CYP24A1 involved in the vitamin D metabolic process (BP ontology), 1,25$(OH)_2D_3$ 24-hydroxylase activity (MF ontology), and mitochondrial inner membrane (CC ontology) (https://david.ncifcrf.gov/data/download/tr_8735183BF51F1536902986935.txt). The RegulomeDB scores for rs6068816, rs3787557, rs6022999, and rs2248359 were 2b, 4, 4, and 4, respectively. It suggested that rs6068816 may be related to TF binding, any motif, DNase Footprint, and DNase peak, while rs3787557, rs6022999, and rs2248359 may be related with TF binding, and DNase peak. HaploRegV4.1 data shown that rs6013897, rs6068816, rs3787557, rs6022999, and rs2248359 contained H3K4me1 and H3K27Ac in multiple tissues or cell lines, and appear to change known motifs (Table S3). H3K4me1 and H3K27Ac are the predominant histone modifications found in nucleosomes around enhancer elements, and associated with transcriptional regulation of genes.

Abbreviations: CYP24A1, Cytochrome P450, family 24, subfamily A, polypeptide 1; CYP2R1, Cytochrome P450, family 2, subfamily R, polypeptide 1; CYP27B1, Cytochrome P450, family 27, subfamily B, polypeptide 1; VDR, Vitamin D receptor;

ECSIT, ECSIT homolog; PTH, Parathyroid hormone; FGF23, Fibroblast growth factor 23; CA7, Carbonic anhydrase VII; CA6, Carbonic anhydrase VI; CA5A, Carbonic anhydrase VA; DNAJC17, DnaJ (Hsp40) homolog, subfamily C, member 17.

Notes: Associations are meant to be specific and meaningful, i.e. proteins jointly contribute to a shared function.

Discussion

In the current study, the associations of CYP24A1 genetic polymorphisms with the risk of HCV infection and chronic HCV infection among HCV-infected high-risk population were investigated. Our results showed that the TA/AA genotypes and A allele of rs6013897 were significantly associated with an increased risk of HCV infection. In addition, for the risk of chronic HCV infection, the CTGT haplotype and CCAC haplotype of rs6068816, rs3787557, rs6022999, and rs2248359 expressed a significant risk effect (compared to $T_{rs6068816}T_{rs3787557}A_{rs6022999}C_{rs2248359}$ haplotype).

CYP24A1, a vitamin D target gene, is transactivated by the VDR. The 24 hydroxylase encoded by CYP24A1 mediates the first step in the catabolism of calcitriol, which is directly related to the concentration of calcitriol. Through bioinformatics analysis, we found that CYP24A1 was involved in $1,25(OH)_2D_3$ 24-hydroxylase activity and played an important role in vitamin D metabolic process. The three most important functional partners co-expressed with CYP24A1 were CYP2R1, CYP27B1 and VDR. Some studies suggested that the inhibition of CYP24A1 expression rather than the promotion of CYP27B1 expression had a great effect on the maintenance of high levels of $1,25(OH)_2D_3$ in response to HCV infection (Gal-Tanamy et al., 2011). Furthermore,

SNPs in genes involved in vitamin D metabolic process could be able to affect vitamin D status. Our previous studies have found that the vitamin D level and VDR SNPs can directly affect HCV infection outcomes (Wu et al., 2016). The associations of CYP2R1 and CYP27B1 gene polymorphisms with HCV-associated HCC development and HCV therapy outcomes have also been reported (Cusato et al., 2014; Lange et al., 2011; Lange et al., 2013).

CYP24A1 polymorphisms have been partially determined and have been reported to be associated with a variety of diseases, including colorectal cancer (CRC), non-small cell lung cancer (NSCLC), non-Hodgkin lymphoma (NHL), and coronary artery calcification (CAC) (Chen et al., 2017; Dong et al., 2009; Kelly et al., 2012; Shen et al., 2010; Wu et al., 2016). In this study, based on the analyses of four genetic models, the stable results of individuals with rs6013897-A allele being greater risk of HCV infection were found. Moreover, our report showed that the risk effects of the rs6013897 variant were statistically significant in most subgroups, and there was no significant heterogeneity within each subgroup variable, which suggested that these variables did not modify this association. Some previous researches have also reported that rs6013897 can affect the risk of parkinson' s disease (PD), aggressive prostate cancer, bone mineral density at the femoral neck (FN-BMD) by affecting the 25(OH)D_3 concentration (Dimitrakopoulou et al., 2017; Larsson et al., 2017; Larsson et al., 2018). This study also demonstrated that CYP24A1 rs6068816, rs3787557, rs6022999, and rs2248359 SNPs were associated with the risk of chronic HCV infection. Logistic regression analyses found that the effects of these SNPs were weak, and the power of

test was relatively low when performing multiplicity correction (Moskvina and Schmidt, 2008). To reduce the impact of type I errors induced by multiple comparisons of SNPs, we performed haplotype analysis of rs6068816, rs3787557, rs6022999, and rs2248359. Haplotype analyses showed that these four SNPs might have a joint effect on the risk of chronic HCV infection, and the CTGT haplotype and CCAC haplotype were associated with an increased risk of chronic HCV infection, which were consistent with the single SNP effects. Similarly, previous studies reported that rs6068816 and rs2248359 can affect 25(OH)D_3 concentration and were associated with the risk of NSCLC, breast cancer, multiple sclerosis and other diseases (Pérez-Pérez et al., 2018; Reimers et al., 2015; Wu et al., 2016). However, relatively few studies have reported the relationship of rs3787557 and rs6022999 with diseases, and only found that they may be associated with prostate cancer (Holt et al., 2010; Oh et al., 2014).

Bioinformatics analysis found that SNPs rs6013897 (T>A), rs6068816 (C>T), rs3787557 (T>C), rs6022999 (A>G), and rs2248359 (C>T) were located on intergenic region (BCAS1||CYP24A1), exon, intron, intron, and promotor region of CYP24A1 gene, respectively. Rs6068816 was a synonymous mutation, encoding a protein residue that was threonine (Thr), whereas other SNPs do not encode protein residue. Based on the RegulomeDB database, the RegulomeDB score of rs6068816, rs3787557, rs6022999, and rs2248359 are 2b, 4, 4, and 4, respectively. This meant that these SNPs may regulate the expression of the CYP24A1 gene by changing multiple regulatory motifs and interfere with protein binding activity. Moreover, based on another web-based analysis tool, HaploRegV4.1, we found that these SNPs (or SNPs in high LD)

were located at multiple regulatory elements, including promoter histone marks, enhancer histone marks, and DNase hypersensitivity. This suggested that these SNPs may be involved in the transcriptional regulation of CYP24A1 genes (Kundaje et al., 2015). Totally, these SNPs may affect the $1,25(OH)_2D_3$ concentration by regulating the transcription and expression of the CYP24A1 gene, and ultimately affect HCV infection.

There are some inevitable limitations of the present study that should be discussed. Firstly, we failed to measure the $1,25(OH)_2D_3$ level of participants, which made it impossible to assess whether the selected SNPs were indeed related to circulating vitamin D levels in our study population. Secondly, since some of the biochemical tests were performed independently by the hospital and HCV genotyping was not a mandatory test in this study, only a subset of HCV-infected individuals was genotyped. This data has been included in the analysis and shown in Table 1. No significant differences in HCV genotypes between natural clearance group and chronically infected group were found. Finally, we had limitation of only performing bioinformatics analyzes of SNPs to predict the possible biological functions, it should be necessary for conducting further genetic and functional studies to verify the contribution of these CYP24A1 polymorphisms to the outcomes of HCV infection.

In conclusion, our findings supported the associations between SNPs in CYP24A1 gene and the risk of HCV infection and chronic HCV infection in a high-risk Chinese Han population. Specifically, rs6013897-A was associated with an increased risk of HCV infection, while rs6068816-C, rs3787557-C, rs6022999-G, and rs2248359-T were

associated with an increased risk of chronic HCV infection. Our results could provide clues for further functional studies and reveal the underlying biological mechanisms of HCV infection and progression.

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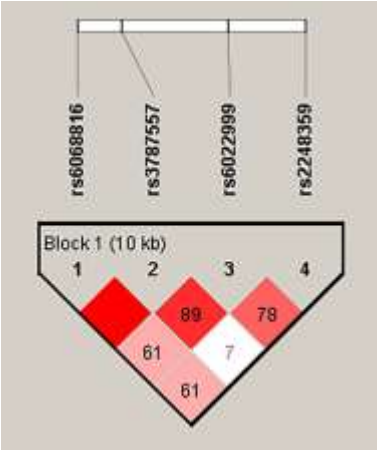

Figure 1. Linkage disequilibrium (LD) information of rs6068816, rs3787557, rs6022999, and rs2248359.

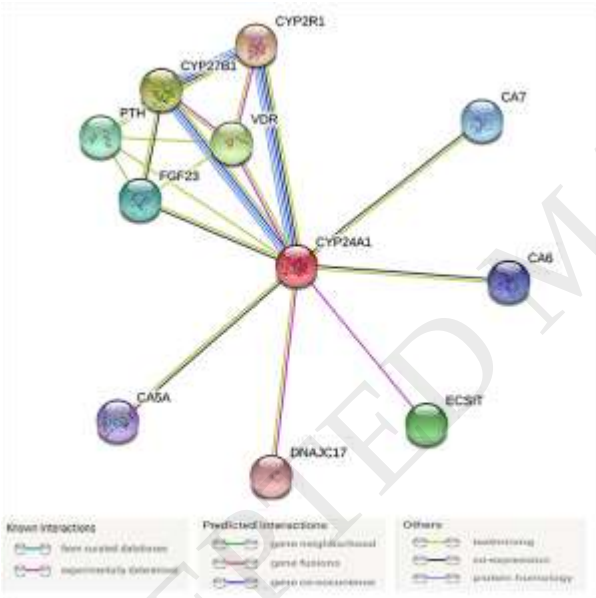

Figure 2. The protein interaction network of CYP24A1.

Table 1. Demographic and clinical characteristics of the three study groups

Variables	Uninfected control	Natural clearance	Chronically infected	P
	(N=1017)	(N=507)	(N=848)	
Median age, year (IQR)	50.0 (40.0-61.0)	52.0 (41.0-61.0)	50.00 (44.0-59.0)	0.764*
Gender, n (%)				0.843**
Male	378 (37.2)	190 (37.5)	306 (36.1)	
Female	639 (62.8)	317 (62.5)	542 (63.9)	
Routes of infection, n (%)				**< 0.001****
HD	591 (58.1)	92 (18.1)	76 (9.0)	
IVD	146 (14.4)	118 (23.3)	109 (12.9)	
PBD	280 (27.5)	297 (58.6)	663 (78.1)	
HCV genotype, n (%)				0.693**
1	-	43 (43.4)	213 (45.6)	
Non-1	-	56 (56.6)	254 (54.4)	
ALT (U/L), n (%)				**< 0.001****
<40	947 (94.7)	347 (74.9)	445 (57.3)	
≥40	53 (5.3)	116 (25.1)	331 (42.7)	
AST (U/L), n (%)				**< 0.001****
<40	942 (94.2)	347 (76.3)	448 (58.4)	
≥40	58 (5.8)	108 (23.7)	319 (41.6)	

Abbreviations: IQR, interquartile range; HD, hemodialysis; IVD, intravenous drug; PBD, paid blood donation; Non-1, genotypes 2 and 3 and co-infected with genotypes 1, 2 and 3; ALT, alanine transaminase; AST, aspartate aminotransferase.

Notes: Bold type indicates statistically significant results.

* One-way Kruskal-Wallis test among three groups.

** χ^2-test among three groups.

Table 2. Genotype distributions of CYP24A1 gene polymorphisms and association analyses of these SNPs and HCV infection outcomes

Genotypes	Uninfected control	Previously infected	Chronically infected	OR (95% CI)*	P*	OR (95% CI) **	P**
rs6013897-TT	758 (75.6)	350 (69.6)	579 (69.2)	1	-	1	-
TA	221 (22.0)	134 (26.6)	233 (27.9)	**1.519 (1.209–1.907)**	**3.257×10^{-4}**	1.111 (0.858–1.439)	0.425
AA	24 (2.4)	19 (3.8)	25 (2.9)	**2.220 (1.207–4.081)**	**0.010**	0.750 (0.403–1.396)	0.365
Dominant model				**1.577 (1.266–1.965)**	**4.734×10^{-5}**	1.063 (0.830–1.361)	0.630
Recessive model				**1.992 (1.088–3.646)**	**0.025**	0.729 (0.393–1.351)	0.315
Additive model				**1.509 (1.247–1.826)**	**2.400×10^{-5}**	1.008 (0.817–1.245)	0.938
rs4809957-GG	395 (39.0)	193 (38.1)	339 (40.3)	1	-	1	-
GA	480 (47.4)	234 (46.2)	392 (46.6)	1.013 (0.825–1.244)	0.901	0.993 (0.777–1.269)	0.954
AA	137 (13.5)	80 (15.7)	111 (13.1)	1.155 (0.857–1.557)	0.345	0.818 (0.579–1.157)	0.256
Dominant model				1.044 (0.859–1.268)	0.668	0.948 (0.752–1.195)	0.653
Recessive model				1.147 (0.869–1.513)	0.332	0.822 (0.597–1.131)	0.228
Additive model				1.058 (0.921–1.217)	0.425	0.925 (0.785–1.090)	0.351

rs2762934-GG	803 (79.3)	393 (78.0)	662 (78.6)	1	-	1	-
GA	195 (19.3)	105 (20.8)	175 (20.8)	1.110 (0.873–1.411)	0.393	0.992 (0.750–1.311)	0.953
AA	14 (1.4)	6 (1.2)	5 (0.6)	0.596 (0.237–1.501)	0.272	0.426 (0.128–1.425)	0.166
Dominant model				1.075 (0.851–1.359)	0.544	0.959 (0.729–1.261)	0.762
Recessive model				0.584 (0.232–1.468)	0.253	0.427 (0.128–1.425)	0.166
Additive model				1.033 (0.832–1.284)	0.768	0.927 (0.716–1.200)	0.563
rs927650-CC	559 (55.1)	263 (53.0)	466 (55.3)	1	-	1	-
CT	384 (37.8)	197 (39.6)	326 (38.7)	1.154 (0.944–1.411)	0.164	0.999 (0.786–1.269)	0.994
TT	72 (7.1)	37 (7.4)	50 (6.0)	0.955 (0.650–1.401)	0.813	0.761 (0.480–1.208)	0.247
Dominant model				1.121 (0.925–1.358)	0.243	0.960 (0.764–1.207)	0.727
Recessive model				0.900 (0.619–1.309)	0.580	0.761 (0.485–1.195)	0.236
Additive model				1.057 (0.907–1.233)	0.478	0.931 (0.775–1.119)	0.449
rs6068816-CC	418 (41.3)	192 (38.3)	372 (44.3)	1	-	1	-
CT	450 (44.5)	231 (46.1)	367 (43.7)	0.958 (0.780–1.176)	0.679	0.782 (0.611–1.001)	0.051
TT	143 (14.2)	78 (15.6)	100 (11.9)	0.896 (0.667–	0.464	**0.669 (0.470–**	**0.026**

				1.203)		**0.953)**	
Dominant model				0.943 (0.777–1.143)	0.548	**0.754 (0.598–0.951)**	**0.017**
Recessive model				0.916 (0.696–1.206)	0.532	0.760 (0.548–1.055)	0.102
Additive model				0.949 (0.827–1.089)	0.459	**0.809 (0.686–0.954)**	**0.012**
rs3787557-TT	562 (55.5)	301 (59.8)	460 (54.6)	1	-	1	-
TC	397 (39.2)	176 (35.0)	329 (39.0)	0.958 (0.785–1.169)	0.672	1.231 (0.969–1.564)	0.089
CC	53 (5.3)	26 (5.2)	54 (6.4)	1.232 (0.804–1.888)	0.338	1.579 (0.947–2.632)	0.080
Dominant model				0.989 (0.816–1.198)	0.909	**1.272 (1.011–1.601)**	**0.040**
Recessive model				1.254 (0.825–1.907)	0.290	1.455 (0.880–2.406)	0.143
Additive model				1.025 (0.875–1.201)	0.755	**1.243 (1.028–1.502)**	**0.025**
rs2296241-GG	322 (32.1)	174 (34.7)	259 (30.9)	1	-	1	-
GA	514 (51.2)	255 (50.8)	429 (51.1)	0.983 (0.793–1.219)	0.878	1.126 (0.875–1.451)	0.356
AA	167 (16.7)	73 (14.5)	151 (18.0)	1.016 (0.761–1.357)	0.912	1.415 (0.999–2.005)	0.051
Dominant model				0.991 (0.808–1.216)	0.934	1.190 (0.935–1.514)	0.158
Recessive model				1.027 (0.794–	0.839	1.316 (0.963–	0.085

				1.328)		1.799)	
Additive model				1.004 (0.872–1.155)	0.957	1.177 (0.996–1.391)	0.056
rs6022999-AA	562 (55.5)	290 (57.9)	456 (54.1)	1	-	1	-
AG	388 (38.3)	191 (38.1)	327 (38.8)	1.000 (0.819–1.222)	0.997	1.063 (0.838–1.347)	0.615
GG	63 (6.2)	20 (4.0)	60 (7.1)	0.961 (0.640–1.445)	0.849	**1.932 (1.125–3.318)**	**0.017**
Dominant model				0.995 (0.821–1.205)	0.959	1.143 (0.910–1.437)	0.251
Recessive model				0.961 (0.645–1.432)	0.846	**1.885 (1.107–3.208)**	**0.020**
Additive model				0.991 (0.847–1.158)	0.906	1.197 (0.990–1.446)	0.063
rs2248359-CC	360 (36.0)	199 (39.7)	304 (36.6)	1	-	1	-
CT	490 (49.0)	251 (50.1)	413 (49.7)	0.885 (0.718–1.091)	0.252	1.058 (0.829–1.350)	0.649
TT	150 (15.0)	51 (10.2)	114 (13.7)	0.782 (0.578–1.060)	0.113	**1.541 (1.046–2.269)**	**0.029**
Dominant model				0.862 (0.706–1.052)	0.143	1.137 (0.900–1.436)	0.282
Recessive model				0.840 (0.636–1.108)	0.217	**1.492 (1.039–2.143)**	**0.030**
Additive model				0.885 (0.767–1.020)	0.093	1.180 (0.992–1.403)	0.061

Abbreviations: SNPs, single nucleotide polymorphisms; HCV, hepatitis C virus; OR, odds ratio; 95% CI, 95% confidence interval.

Notes: Bold type indicates statistically significant results, deriving from logistic regression analyses with adjustment for age, gender, and routes of infection.

* HCV infection group versus uninfected control group.

** HCV persistent infection group versus natural clearance group.

Table 3. Stratified analyses of the association between rs6013897 and the risk of HCV infection

Subgroups	Uninfected control n (TT/TA/AA)	Natural clearance n (TT/TA/AA)	Chronically infected n (TT/TA/AA)	OR (95% CI) *	P*	P**
Age						
<50	358/104/9	153/54/9	264/118/11	**1.545(1.154-2.068)**	**0.003**	0.770
≥50	400/117/15	197/80/10	315/115/14	**1.457(1.130-1.878)**	**0.004**	
Gender						
Male	289/77/8	116/63/7	206/87/8	**1.695(1.242-2.313)**	**0.001**	0.399
Female	469/114/16	234/71/12	373/146/17	**1.420(1.114-1.811)**	**0.005**	
ALT (U/L)						
≤40	708/205/21	238/95/12	305/120/17	**1.557(1.265-1.917)**	**3.022×10^{-5}**	0.173
>40	32/12/3	77/32/5	228/88/8	1.064(0.607-1.865)	0.829	
AST (U/L)						
≤40	703/204/22	238/93/13	312/116/18	**1.538(1.250-**	**$4.748\times10^{-}$**	0.49

				1.892)	**5**	8
>40	42/13/2	72/31/4	215/89/7	1.228(0.649-2.324)	0.528	
Route of infection						
HD	436/127/23	57/34/1	46/26/2	**1.454(1.065-1.987)**	**0.019**	0.457
IVD	104/39/1	81/30/4	74/26/4	1.229(0.800-1.887)	0.346	
PBD	218/55/0	212/70/14	459/181/19	**1.712(1.264-2.319)**	**0.001**	

Abbreviations: HCV, hepatitis C virus; OR, odds ratio; 95% CI, 95% confidence interval; ALT, alanine transaminase; AST, aspartate aminotransferase; HD, hemodialysis; IVD, intravenous drug; PBD, paid blood donation.

Notes: Bold type indicates statistically significant results.

* HCV infection group versus uninfected control group, deriving from dominant model of logistic regression analyses with adjustment for age, gender, and routes of infection (the stratified factor in each stratum was excluded).

** P-value for the heterogeneity test.

Table 4. Haplotypes analyses of rs6068816, rs3787557, rs6022999, and rs2248359 to predict HCV infection chronicity

Haplotype	Natural clearance	Chronically infected	OR (95% CI) *	P*
TTAC	314(31.0)	440(25.9)	1	1
CTGT	194(19.1)	374(22.1)	**1.376(1.092-1.735)**	**0.007**
CTAC	184(18.1)	288(17.0)	1.102(0.867-1.402)	0.427
CCAC	129(12.7)	258(15.2)	**1.483(1.139-1.929)**	**0.003**
CCAT	99(9.8)	173(10.2)	1.275(0.951-1.710)	0.105
Others†	94(9.3)	163(9.6)	1.234(0.915-1.666)	0.169

Abbreviations: HCV, hepatitis C virus; OR, odds ratio; 95% CI, 95% confidence interval.

Notes: 1) Haplotypes analysis of rs6068816, rs3787557, rs6022999, and rs2248359 in sequence. 2) Bold type indicates statistically significant results.

* HCV persistent infection group versus natural clearance group, deriving from logistic regression analyses with adjustment for age, gender, and routes of infection.

† Haplotypes with a frequency less than 5% in all three groups were combined as others.