Replication of Genome Wide Association Studies on Hepatocellular Carcinoma Susceptibility Loci in a Chinese Population

Kangmei Chen1, Weimei Shi1, Zhenhui Xin1, Huifen Wang2, Xilin Zhu1, Xiaopan Wu1, Zhuo Li3, Hui Li4, Ying Liu1*

1 National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing, P. R. China, 2 Liver Failure Treatment and Research Center, The 302 Hospital of the People’s Liberation Army, Beijing, P. R. China, 3 Department of Infectious Disease, Affiliated Youan Hospital, Capital University of Medical Science, Beijing, P. R. China, 4 Department of Epidemiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing, P. R. China

Abstract

Background: Genome-wide association studies (GWAS) have identified three loci (rs17401966 in KIF1B, rs7574865 in STAT4, rs9275319 in HLA-DQA) as being associated with hepatitis B virus-related hepatocellular carcinoma (HBV-related HCC) in a Chinese population, two loci (rs2596542 in MICA, rs9275572 located between HLA-DQA and HLA-DQB) with hepatitis C virus-related HCC (HCV-related HCC) in a Japanese population. In the present study, we sought to determine whether these SNPs are predictive for HBV-related HCC development in other Chinese population as well.

Method and Findings: We genotyped 4 SNPs, rs2596542, rs9275572, rs17401966, rs7574865, in 506 HBV-related HCC patients and 772 chronic hepatitis B (CHB) patients in Han Chinese by TaqMan methods. Odds ratio(OR) and 95% confidence interval (CI) were calculated by logistic regression. In our case-control study, significant association between rs9275572 and HCC were observed (P = 0.02, OR = 0.73, 95% CI = 0.56–0.95). In the further haplotype analysis between rs2596542 at 6p21.33 and rs9275572 at 6p21.3, G-A showed a protective effect on HBV-related HCC occurrence (P<0.001, OR = 0.66, 95% CI = 0.52–0.84).

Conclusion: These findings provided convincing evidence that rs9275572 significantly associated with HBV-related HCC.

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* E-mail: liuyingpumc@163.com (YL); lihui99360@sohu.com (HL)

Introduction

Liver cancer is the fifth most frequently diagnosed cancer worldwide but the second most frequent cause of cancer death [1]. Hepatocellular carcinoma (HCC) accounts for between 70% and 85% of primary liver cancers, and ranks fifth and sixth as causes of cancer mortality worldwide in men and women, respectively [2–3]. Globally, there are more than 250,000 new cases of HCC and an estimated 500,000–600,000 deaths due to this disease annually [4]. Eastern Asia is the geographic area at highest risk of HCC, and China accounts for 55% of all HCC cases worldwide [1]. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the leading cause of HCC in the world [4]. In Western countries and Japan, infection with HCV is the more common cause of HCC, while in Asia and developing countries, HBV is more common [5]. Furthermore, accumulated evidences in molecular genetics indicate that single nucleotide polymorphisms (SNPs) in immune response and tumorigenesis related genes are associated with susceptibility to HCC [6–9]. Recently, a number of Genome-wide association studies (GWAS) have identified several new loci associated with the risk of HCC, such as SNPs in the gene KIF1B, MICA, HLA-DQA/DQB, STAT4 and HLA-DQA, respectively [10–12].

Two independent GWAS have been performed to identify novel susceptibility loci associated with HBV-related HCC [10,12]. Among these studies, Zhang et al. found one susceptibility locus (rs17401966) in KIF1B at chromosome 1p36.22 [10], Jiang et al. confirmed two other loci, rs7574865 in the STAT4 at 2q32.2–2q32.3 and rs9275319 in HLA-DQA at 6p21.3 [12]. Moreover, a GWAS of Japanese population conducted by Kumar et al. identified two susceptibility loci for HCV-related HCC, with lead SNPs rs2596542 located 4.7 kb upstream of MICA on 6p21.33 and rs9275572 located between HLA-DQA and HLA-DQB on 6p21.32 [11]. Although the mechanism of chronic HBV and HCV infection is not identical, they share some common characteristics to induce HCC [13]. So, we speculated these two SNPs associated with HCV-related HCC patients would be also associated with HBV-related HCC patients.
Given the confirmatory results from several studies in multiple populations, the above hypothesis and the design of TaqMan probe, we focus on four polymorphisms, *KIF1B* rs17401986, *STAT4* rs7574865, *MICA* rs2596542 and *HLA-DQA1/HLA-DQB1* rs9275572, to replicate in a HBV-related HCC case-control study among Chinese Han population.

**Materials and Methods**

**Subjects**

The subjects enrolled in the present study consisted of 506 HBV-related HCC cases and 772 CHB controls. All subjects were recruited from Beijing Youan Hospital (Beijing) and the 302 Hospital of the People’s Liberation Army (Beijing) from Oct 2005 to Jul 2010.

Subjects with CHB were identified with the following diagnostic criteria: liver ultrasonography confirmed; serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) continuously >40 IU/L; HBsAg seropositive for at least 6 months and serum HBV DNA >2000 copies/ml. The diagnosis of HCC was identified based on clinical evidence obtained from liver function tests, serum immunologic marker screening, pathologically confirmed, liver ultrasonography(U/S)/computed tomography(CT) and proved not to have other cancers.

Subjects were considered smokers if they smoked up to 6 months before the date of cancer diagnosis for HCC cases or the date of interview for CHB controls. An alcohol drinker was defined as someone who consumed alcohol at least once per week for at least 6 months.

The subjects were excluded if: (1) there was evidence of past or current infection with other hepatitis viruses or hepatitis not caused by HBV; (2) they were not of Han ethnicity. The study was carried out in accordance with the guidelines of the Helsinki Declaration after obtaining written informed consent from all the subjects and was approved by the ethics committee of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

**SNP Selection and Genotyping**

Genomic DNA was extracted from peripheral blood by using a salting-out protocol [14]. TaqMan assays for four SNPs were purchased from Applied Biosystems (Foster City, CA) and run according to the manufacturer. Briefly, each 10 μL TaqMan reaction contain 40 ng of genomic DNA, primers, probes, and 2×GoldStar TaqMan Mixture (CWBIO, Beijing, China) and was performed with the following procedure: 95°C for 10 min, followed by 40 cycles of 92°C for 15 s, and 64°C for 1 min in the real-time PCR instrument. Primers and TaqMan probes used are listed in Table S1. All the samples were successfully genotyped. To ensure quality control, 5% samples were randomly selected and directly sequenced, and we obtained 100% identical results.

**Statistical Analysis**

We used 2×2 and 2×3 contingency tables for comparing allele and genotype frequencies between subjects with HCC and CHB. Multiple logistic regression models (dominant, recessive, and log-additive) were used for calculating the Odds ratio (OR), 95% confidence interval (CI), and corresponding *P* value, with adjustment for sex and age. Nonsuperiority test was conducted to confirm the absence of association between SNPs and risk of HCC [15]. Multiple comparison adjustment based on the false discovery rate (FDR) principle. After correction, the frequency for allele (P = 0.04) and the association under recessive model (P = 0.04) remained significant at the 5% level, the other effects were no significant (P = 0.08 for genotype, P = 0.08 under additive model, P = 0.08 under dominant model).

Nonsuperiority test was used to confirm the absence of association between rs2596542, rs17401986 and rs7574865 with HBV-related HCC, respectively. The null hypothesis is that the frequency of rs2596542*A, rs17401986*C and rs7574865*A in HCC patients is greater by Δ compared to the frequency in CHB controls. The Δ was set (5% for rs17401986; 3% for rs7574865)

**Table 1.** Clinical features of the subjects included in the study.

|                | HCC   | CHB   | *P*   |
|----------------|-------|-------|-------|
| Number         | 506   | 772   |       |
| Age, years, mean ± SD | 53.9±10.6 | 35.7±11.8 | <0.001* |
| Gender (male/female) | 425/81 | 572/199 | <0.001* |
| Smoking (Yes/No) | 246/260 | 191/499 | <0.001* |
| Drinking (Yes/No) | 261/245 | 217/470 | <0.001* |

* Mann-Whitney U test.
  * Chi-square test.
  * doi:10.1371/journal.pone.0077315.t001
based on the lowest difference of minor allele between HCC and CHB patients in Chinese population in the previous study. There was no reference data for rs2596542 significantly associated with HCC in Chinese population in recent study. The corresponding nonsuperiority P-values for rs17401966 and rs7574865 was 0.0001 and 0.3425, respectively, which support the absence of association between rs17401966 and HBV-related HCC.

**Discussion**

In this study, we attempted to replicate, in a Chinese population, the associations between the 4 SNP loci and the risk of HCC, which were identified in previous GWAS study. The result showed that, rs9275572 between HLA-DQA and HLA-DQB, was significantly associated with HBV-related HCC risk, although it was identified by Kumar et al. to be associated with HCV-related HCC in Japanese patients. However, there was no association between the other 3 SNPs and HCC risk.

The GWAS study by Kumar et al. identified two susceptibility loci for HCV-related HCC, with lead SNPs rs2596542 and rs9275572. Importantly, the frequency of the minor allele A of rs9275572 was reported to be a risk factor for HCV-related HCC [11]. Interestingly, in our study the minor allele A appeared to have a protective impact on HBV-related HCC development, which represented an inverse association as compared to the result in Japanese population. To some extent, this result seemed puzzling, but the following points should be noted.

First, chronic infection with HBV and HCV is one of the most important risk factors of the development of HCC. Although there is certain similarity in clinical manifestations of hepatitis induced by these viruses and creating background for subsequent development for HCC, their molecular organization, replication strategy and functions of constituent proteins are different. HBV and HCV are two different viruses. HBV is a DNA-containing virus, which belongs to hepadnaviruses, whereas HCV is a RNA-containing virus of the flavivirus family [13,19]. It is also reported that HBV and HCV infections tend to suppress each other in dual virus infections. HCV super infection is seen to reduce HBsAg expression and promote its clearance [20]. Thus, on the basis of these findings, different mechanisms of liver carcinogenesis might operate in HBV related and in HCV related chronic inflammation and cirrhosis. It is possible that rs9275572*A involved in two different pathways in HBV and HCV induced HCC, respectively. This may explain our findings rs9275572*A has opposite effect on HBV and HCV related HCC development. Second, extensive allele diversity is observed in HLA locus associations with susceptibility regarding HBV and HCV infections and disease progression in different global ethnic populations. However, the specific HLA associations with HBV and HCV infections are different, agreeing to their differences in viral properties and

### Table 2. Associations between GWAS-identified SNPs and HBV-related HCC in a Chinese population.

| Allele | Genotype, n (%) | Additive model | Dominant model | Recessive model |
|--------|----------------|---------------|---------------|----------------|
| 1/2    | n (%)          | OR (95% CI) P | OR (95% CI) P | OR (95% CI) P |
| A/A    | 284(28.1%)     | 1.11 (0.93–1.33) 0.24 | 2.09 (1.55–2.81) 0.001 | 4.28 (3.19–5.74) 0.001 |
| A/G    | 808(79.8%)     | 0.78 (0.64–0.95) 0.01 | 2.07 (1.53–2.75) 0.001 | 4.15 (3.59–4.78) 0.001 |
| G/G    | 192(19.4%)     | 1.17 (1.00–1.38) 0.05 | 2.49 (2.00–2.99) 0.001 | 6.07 (4.74–7.85) 0.001 |

| Allele | Genotype, n (%) | Additive model | Dominant model | Recessive model |
|--------|----------------|---------------|---------------|----------------|
| 1/2    | n (%)          | OR (95% CI) P | OR (95% CI) P | OR (95% CI) P |
| C/T    | 303(30.1%)     | 0.90 (0.75–1.07) 0.23 | 2.87 (2.37–3.50) 0.001 | 6.52 (5.00–8.65) 0.001 |
| A/K    | 566(56.3%)     | 1.17 (1.00–1.38) 0.05 | 2.58 (2.15–3.09) 0.001 | 6.16 (4.75–7.85) 0.001 |
| G/K    | 184(18.6%)     | 1.08 (0.92–1.27) 0.35 | 2.31 (2.00–2.68) 0.001 | 5.90 (4.57–7.51) 0.001 |

### Table 3. The results of haplotype analysis of rs2596542 and rs9275572.

| Haplotype | HCC (%) | CHB (%) | P     | OR (95% CI) |
|-----------|---------|---------|-------|------------|
| A-A       | 95(9.4%)| 138(9.0%)| 0.72  | 1.05 (0.80–1.38) |
| A-G       | 189(18.7%)| 263(17.0%)| 0.28  | 1.12 (0.91–1.38) |
| G-A       | 109(10.8%)| 239(15.5%)| 0.0007| 0.66 (0.52–0.84) |
| G-G       | 619(61.2%)| 904(58.6%)| 0.19  | 1.11 (0.95–1.31) |
HCC had been reported, nonsuperiority test about this locus cannot be conducted, further studies are needed.

There are some limitations in this study should be acknowledged. First, the sample size of our case-control study was relatively small. Second, the non-HBV control was not involved in the present study, further study will be needed to confirm whether rs9275572 has an association with development of CHB.

In conclusion, our study provided convincing evidence of the genetic involvement of rs9275572 polymorphism in HBV-related HCC susceptibility. Further studies including larger sample sizes and different ethnic populations should be taken to investigate the mechanisms underlying the role of this SNP in both HBV-related HCC and HCV-related HCC.

Supporting Information

Table S1 Primers and Probes used in TaqMan Genotyping.

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Author Contributions

Conceived and designed the experiments: YL HL KMC. Performed the experiments: KMC WMS ZHX. Analyzed the data: KMC. Contributed reagents/materials/analysis tools: HFW XLZ XPW ZL HL YL. Wrote the paper: KMC.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69–90.
2. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP (2006) The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 45: 529–538.
3. Maheswaran S, Carey FA (2012) Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. CA Cancer J Clin 62: 394–399.
4. Arzumanyan A, Reis HM, Feitelson MA (2013) Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. Nat Rev Cancer 13: 123–135.
5. Sarma MP, Asim M, Meishi S, Bharathi T, Diwan R, et al. (2012) Viral genotypes and associated risk factors of hepatocellular carcinoma in India. Cancer Biol Med 9: 172–181.
6. Kong SY, Park JW, Lee JA, Park JE, Park KW, et al. (2007) Association between vascular endothelial growth factor gene polymorphisms and survival in hepatocellular carcinoma patients. Hepatology 46: 446–453.
7. Deng G, Zhou G, Zhang R, Zhai Y, Zhao W, et al. (2008) Regulatory polymorphisms in the promoter of CXCL10 gene and disease progression in hepatocellular cancer. Ann Transl Med 134: 478–716.
8. Clifford RJ, Zhang J, Meereaman DM, Lyu MS, Hu Y, et al. (2010) Genetic variations at loci involved in the immune response are risk factors for hepatocellular carcinoma. Hepatology 52: 2034–2043.
9. Long XD, Ma Y, Zhou YF, Ma AM, Fu GH (2010) Polymorphism in xeroderma pigmentosum complementation group C codon 939 and aflatoxin B1-related hepatocellular carcinoma in the Guangxi population. Hepatology 52: 1301–1309.
10. Zhang H, Zhai Y, Hu Z, Wu C, Qian J, et al. (2010) Genome-wide association study identifies 156322 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. Nat Genet 42: 755–758.
11. Kumar V, Kato N, Tsuchi Y, Takahashi A, Miura Y, et al. (2011) Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. Nat Genet 43: 455–458.
12. Jiang DK, Sun J, Cao G, Liu Y, Lin D, et al. (2013) Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. Nat Genet 45: 72–75.
13. Michalak T1, Pham TN, Murooney-Coons PM (2007) Molecular diagnosis of occult HCV and HBV infections. Future Virology 2: 451–465.
14. Miller SA, Dykes DD, Polesky HF (1988) A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells. Nucleic Acids Research 16: 1215–1215.
15. Gourraud PA, Image (2011) When is the absence of evidence, evidence of absence? Use of equivalence-based analyses in genetic epidemiology and a conclusion for the KIF1B rs10492972*c allelic association in multiple sclerosis. Genetic Epidemiology 35: 568–571.
16. Noble WS (2009) How does multiple testing correction work? Nature Biotechnology 27: 1135–1137.
17. Sole X, Gaño E, Vallé J, Íñesta R, Moreno V (2006) SNPStats: a web tool for the analysis of association studies. Bioinformatics 22: 1928–1929.
18. Shi YY, He L (2005) SHEatS, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 15: 97–98.
19. Gurtsevitch VE (2008) Human oncogenic viruses: Hepatitis B and hepatitis C viruses and their role in hepatocarcinogenesis. Biochemistry (Moscow) 73: 504–513.
20. Liaw YF (1995) Role of hepatitis C virus in dual and triple hepatitis virus infection. Hepatology 22: 1101–1108.
21. Singh R, Kaul R, Kaul A, Khan K (2007) A comparative review of HLA associations with hepatitis B and C viral infections across global populations. World Journal of Gastroenterology 13: 1770–1770.
22. Lange CM, Riberti S, Dubois EF, Cellera C, Cerm A, et al. (2013) Comparative genetic analyses point to HCP5 as susceptibility locus for HCV-associated hepatocellular carcinoma in, J Hepatol.
23. Wang ZC, Gao Q, Shi JY, Yang LX, Zhou J, et al. (2013) Genetic polymorphism of the kinase-like protein KIFIB gene and the risk of hepatocellular carcinoma. PLoS One 8: e62571.
24. Clark A, Gerlach F, Tong H, Hoan NX, Song H, et al. (2013) A trivial role of STAT4 variant in chronic hepatitis B induced hepatocellular carcinoma. Infect Genet Evol 18: 257–261.