ABSTRACT

This study aimed to explore the relationship between CCNE1 gene single nucleotide polymorphisms (SNP rs1406 and rs3218038) and the incidence of hepatitis B virus-related hepatocellular carcinoma (HCC) in the Chinese Han population in Hubei. A total of 663 subjects, including 173 HCC patients, 172 HBV-related liver cirrhosis (LC) patients, 162 asymptomatic HBV carriers (AsC), and 156 healthy controls, participated in the study. Genotyping of CCNE1 rs1406 and rs3218038 polymorphisms was done by illumina second generation sequence method. Our findings showed that rs1406 G>T variant decreased the risk of HCC (OR 0.710, \(P=0.035\) G vs T), and no significant differences were found between rs3218038 SNP and HCC risk using the \(\chi^2\) test. Furthermore, stratified analysis revealed that differences in genotype frequencies were related to gender. Women who carried the CCNE1 GT genotype were significantly associated with a decreased risk of HCC, compared with healthy controls carrying the GG genotype (additive model, OR 0.378, \(P=0.030\)). The results suggest that the rs1406 G allele and CCNE1 rs1406 polymorphism produce an increased risk of HCC in comparison with the T allele. Whereas, the GT genotype is a protective factor in the development of HCC in female patients.

Keywords: single nucleotide polymorphisms, hepatocellular carcinoma, second generation sequence

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy and a leading cause of cancer death worldwide. An estimated 240 million people worldwide are chronically infected with the hepatitis B virus (HBV), placing them at increased risk of developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma. Although most chronically HBV-infected subjects will not go on to develop hepatic complications, 15%–40% will give rise to serious sequelae during their lifetime\(^{[1,2]}\). The progression of HCC is a multiple process which is affected by multiple genetic and environmental factors such as alcoholism, aflatoxin or chemical carcinogens, hepatitis B or C viruses. Many factors contribute to HBV associated HCC, including...
HBV integration and mutation, and host susceptibility. Chronic HBV infection remains the major aetiologial factor of HCC worldwide with more than one half of HCC patients being chronic carriers. In recent years, single nucleotide polymorphisms (SNPs) have been extensively studied in order to identify host genetic factors affecting HCC pathogenesis, and accumulated evidences in molecular genetics have indicated the correlation of genetic polymorphisms and HCC.

The protein encoded by the CCNE1 gene, belongs to the highly conserved cyclin family, which forms a complex and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition. The cell cycle is composed of a tightly regulated sequence of events whose main purpose is to ensure that genomic material is ready to be replicated, faithfully copied, and properly segregated into daughter cells. During HBV infection, viruses express proteins that perturb cellular DNA repair and cell cycle pathways, promoting tumorigenesis in their quest for cellular domination. Viruses manipulate progression through the cell cycle and alter checkpoint signaling in order to provide a favorable environment for their own replication. In so doing, they can predispose the infected cell to replicate DNA whose fidelity may be compromised, impairing the cell’s ability to repair damaged DNA, which together promote genomic instability. Alterations in the regulation of the cell cycle are strongly linked to tumorigenesis, so genetic variants in genes critical to control of this cycle are good candidates to have their association with susceptibility to HCC assessed.

Recent human somatic cell reprogramming study demonstrated that a pluripotency specific spliced form of CCNE1 specific to humans significantly enhanced reprogramming. In addition, SNP expression analysis revealed that monoallelic gene expression was induced in the intermediate stages of reprogramming, while biallelic expression was recovered upon completion of reprogramming. Another study, using advanced sequencing technologies revealed that recurrent HBV DNA integration sites in hepatoma cells and the susceptible CCNE1 gene played an important role in the pathogenesis of liver cancer. The G/T single-nucleotide variation polymorphism locus rs1406 and rs3218038 on human chromosome 19 was obtained from Hapmap. Haploview software was used to conduct linkage disequilibrium and tagging algorithm.

Despite different studies revealing that polymorphisms in the CCNE1 gene are associated with carcinogenesis, there has been limited data regarding the association of CCNE1 SNP polymorphisms and HBV-related HCC. In the present study, we evaluated the relationship between CCNE1 gene SNPs (rs1406 and rs3218038) polymorphisms and the incidence of HBV-related HCC.

MATERIALS AND METHODS

Study subjects

A total of 507 subjects including 173 HBV-related hepatocellular carcinoma (HCC) patients, 172 HBV-related liver cirrhosis (LC) patients, and 162 asymptomatic HBV carriers (AsC) were recruited from the outpatient clinics and hospitalization wards at Hubei Provincial Hospital of TCM, Wuhan, China, between May 2012 and February 2016. The clinical criteria for HCC is defined by HBsAg and anti-HBcAb being positive for at least 6 months, confirmed by biopsy, sonography, computed tomography or magnetic resonance imaging, with or without elevated alphafetoprotein. The clinical criteria for LC is defined by patients being found HBsAg and anti-HBcAb positive for at least 6 months, with clinical presentation of gastrointestinal varices or a history of bleeding, ascites, edema, or encephalopathy, or serum albumin < 35 g/L, or total bilirubin > 35 μmol/L, confirmed by biopsy, sonography, computed tomography, or magnetic resonance imaging. The clinical criteria for AsC is defined by the presence of positive HBsAg over a period of six months, while their levels of alanine aminotransferase (ALT) remain within the normal range.

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HapMap project.

DNA extraction and genotyping of the CCNE1 SNPs

Genomic DNA from whole blood was extracted from peripheral blood leukocytes using a TIANamp blood DNA kit (Tiangen Biotech [Beijing] Co. Ltd., Beijing, China). The concentration and purity of the DNA samples were detected by NanoDrop spectrophotometer. We excluded 25 samples that were genotyped unsuccessfully or were not in accordance with standard conditions (1.6<DNA purity<1.9). DNA samples were diluted to 4ng/L and distributed in 96-well plates (DNA panels), each of which contained 94 samples and 2 DNA-free control water. The analysis of CCNE1 polymorphism rs1406 was performed by illumina second generation sequence method. The accuracy of the genotyping results was assessed using the ABI PRISM 3730 to examine the representative PCR-amplified DNA samples(Fig. 1 and 2). The SNP primer sequences and the necessary reaction conditions were listed in Table 1.

Table 1  Primer sequence and the reaction condition for genotyping CCNE1 polymorphisms

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer sequence</th>
<th>Annealing temperature (℃)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1406</td>
<td>F: 5’–TCCAGCCTTGGTGACAGA – 3’</td>
<td>50.7</td>
<td>79 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’–TCTCCTGGCAGGTITCT – 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S : 5’–GGCATTGTACACTGTAAGCATG – 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A:5’–AGCGTTGTGCAGAGCCCATA – 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rs3218038</td>
<td>F: 5’– AGCCATTGTGCAACAACTC – 3’</td>
<td>48.2</td>
<td>81 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’–TTAGGGACCAGTGGATGA – 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S: 5’–ATGAGGAGTGGAGTGGTGGAGCA – 3’</td>
<td>48.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A: 5’–TACACCAAGTTTCAAAGCAAGAT – 3’</td>
<td>54.8 ± 22.5</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Population characteristics

Detailed patient demographics for all groups including gender, age, and HBV-DNA serum levels were listed in Table 2. The mean age of the healthy controls, asymptomatic HBV carrier group, HBV-related liver cirrhosis group, and HBV-related HCC group were 46, 44, 55, and 55, respectively. In these retrospective analyses, we found that there was no significant difference in age between the healthy controls and AsC group \((P = 0.172)\), however we observed a significant difference among the healthy controls and the LC and HCC groups. As for gender distribution from the subject profiles, we noted that there were higher numbers of men than women in each group. HBV-DNA serum levels did not dif-
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The genotype and allele frequencies of CCNE1 gene polymorphisms among the AsC group and HBV-related LC group and healthy controls were shown in **Table 3**. In the AsC group, the frequencies of GG, GT, and TT rs1406 genotypes were 38.27%, 48.76%, and 12.96%, respectively. While the frequencies of GG, GT, and TT rs3218038 genotypes were 61.73%, 34.57%, and 3.70%, respectively. In the HBV-related LC group, the frequencies of GG, GA, and AA rs1406 genotypes were 39.53%, 48.25%, and 12.21%, respectively, and in the healthy controls, they were 36.53%, 45.51%, and 17.94%, respectively. In the HBV-related LC group, the frequencies of GG, GA, and AA rs3218038 genotypes were 56.40%, 38.95%, and 4.65%, respectively, and in the healthy controls, they were 62.18%, 35.26%, and 2.56%, respectively. No significant effects were observed between genotype and allele frequencies of the CCNE1 gene rs1406 and
rs3218038 polymorphisms using the $\chi^2$ test. SNP distribution was tested using the Hardy-Weinberg equilibrium (HWE) test, finding rs1406 and rs3218038 SNP distribution among the controls was consistent ($P = 0.592$, $P = 0.590$, respectively).

**HBV-related HCC patients versus healthy controls**

The genotype and allele frequencies of the CCNE1 gene polymorphisms between HBV-related HCC patients and healthy controls are shown in Table 3. Using the $\chi^2$ test, we found that rs1406 G $>$ T variant decreased the risk of HCC (OR 0.710, 95% CI 0.516–0.910, $P = 0.035$ G vs T), but no significant differences between rs3218038 SNP and HCC risk (Table 3). Furthermore, we tested the Hardy-Weinberg equilibrium (HWE) for SNP, finding the distribution of rs1406 SNP among the controls was consistent ($P = 0.572$).

**Stratified analysis**

We next investigated whether the differences in genotype and allele frequencies were related to gender. Significant differences in the distributions of CCNE1 gene polymorphisms between HBV-related HCC patients and control groups were indeed observed (Table 4). Women who carried the CCNE1(rs1406) GT genotype (but not the TT genotype) were significantly related to a decreased risk of HCC, after adjusting for age using binary logistic regression analyses (OR = 0.378, 95% CI 0.157–0.910), compared with patients carrying the GG genotype ($P = 0.030$).

The frequencies of genotype and SNP rs1406 alleles in our control group were compared with those from the HapMap Project (HapMap) Project (http://www.ncbi.nlm.nih.gov/snp/). As seen in Table 5, we observed differences between polymorphisms in healthy controls in the present study and other ethnicities’ healthy controls included in the HapMap project, namely allele frequencies in the CEU cohort (Utah residents with northern and western European ancestry). The frequencies of genotype in JPT (Japanese) and CHB (Chinese Han in Beijing) were also different from those in the present study. There was a lower detection rate of the GG allele (29.06%) and a higher detection rate of the GT allele (54.65%) at the rs1406 site. However, there were no significant differences between SNPs in the present study and CHB (Chinese Han in Beijing).

**DISCUSSION**

HBV is a small enveloped DNA virus which primarily infects hepatocytes and causes persistent liver disease. Recent genetic studies indicated that HBV–
related HCC displays a distinctive profile with a high rate of chromosomal alterations\textsuperscript{[16-17]}. HBVs are streamlined organisms that lack many of the proteins required for genome replication, such as DNA polymerases. They rely on the host cell for these resources, which accumulate during the S phase of the cell cycle to replicate cellular DNA. Viral activation of DDR pathways can lead to checkpoint signaling that stalls the cell cycle at G1/S\textsuperscript{[18]}. During an HBV infection, host cell cycle regulatory proteins and cyclin dependent kinase with different SNP sites are believed to have different roles in mediating cell DNA damage repair and gene instability.

The CCEN1 gene was found to associate with, and be involved in the phosphorylation of NPAT protein (nuclear protein mapped to the ATM locus), which participates in cell-cycle regulated histone gene expression, plays a critical role in promoting cell-cycle progression in the absence of pRB5\textsuperscript{[19-20]}, and forms a complex with CDK2, whose activity is required for cell cycle G1/S transition. Activation of cyclin E1 (a key regulator of the G1/S cell-cycle transition) has been implicated in many cancers including HCC. Although much is known about the regulation of cyclin E1 expression and stability, its post-transcriptional regulation mechanism remains incompletely understood since different SNP sites are likely to have different effects on mRNA expression and CCNE1 gene viral integration risks. It was found that the cell cycle CCNE1 gene was one of the high frequency integration gene sites of the DNA virus. HBV and AAV2 are both DNA viruses associated with oncogenic insertional mutagenesis in human HCC. Nault JC \textit{et al.} reported that AAV2 integrations occurred in the CCNE1 gene, leading to an over expression of target genes\textsuperscript{[21]}. Zhu BH \textit{et al.} identified CCNE1 integration sites in the host genome for the HBV-encoded X protein (HBx) in HCC biopsies that were positive for HBsAg\textsuperscript{[22]}. Sung WK. \textit{et al.} surveyed HBV integration in liver cancer genomes, identifying recurrent HBV integration events that were validated by RNA sequencing (RNA-seq) and Sanger sequencing at the known and putative cancer-related CCNE1 genes\textsuperscript{[22]}. Alterations in the regulation of the cell cycle are strongly linked to tumorigenesis, so genetic variants in genes critical to control of the cycle are good candidates to have their associations to all kinds of cancer assessed. Many studies have indicated that the rs1406 polymorphism in CCNE1 gene is likely to contribute to an increased cancer risk. Amininia S \textit{et al.} investigated the effects of single nucleotide polymorphisms in the CCNE1 gene relating to the risk of breast cancer (BC) in an Iranian population in southeast Iran, concluding that the rs1406 C/A polymorphism increased the risk of BC in codominant and dominant inheritance models\textsuperscript{[23]}. Murali A \textit{et al.} found that a significant risk of oral cancer was also evident for individual polymorphisms of cyclin E (at rs1406)\textsuperscript{[24]}.

\begin{table}[h]
\centering
\caption{Stratified analysis of CCNE1 polymorphisms in healthy controls and HBV-related HCC patients}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\multirow{2}{*}{\textbf{Genotypes}} & \textbf{Healthy controls} & \textbf{HCC} & \textbf{OR(95\%CI)*} & \textbf{p Value} & \textbf{Healthy controls} & \textbf{HCC} & \textbf{OR(95\%CI)*} & \textbf{p Value} \\
\hline
\multirow{3}{*}{\textbf{Female}} & G/G & 24 & 25 & 1 & 33 & 55 & 1 \\
\cline{2-8}
 & G/T & 34 & 16 & 0.378 (0.157,0.910) & 0.030 & 37 & 56 & 1.13 (0.577,2.146) & 0.749 \\
\cline{2-8}
 & T/T & 11 & 6 & 0.407 (0.122,1.359) & 0.144 & 17 & 15 & 0.659 (0.269,1.617) & 0.362 \\
\hline
\multirow{3}{*}{\textbf{Male}} & G & 82 & 66 & 1 & 103 & 166 & 1 \\
\cline{2-8}
 & G/T & 28 & 28 & 0.621 (0.356,1.085) & 0.097 & 71 & 86 & 0.752 (0.304,1.120) & 0.184 \\
\cline{2-8}
 & T/T & 56 & 28 & 1 & 1 & 1 & 1 & 1 & 1 \\
\hline
\end{tabular}

\textsuperscript{1}Logistic regression models were used for calculating the odds ratios (95\% confidential intervals) and corresponding \textit{p} values, controlling for age as covariates. Significant associations (\textit{p}<0.05) are in bold. HCC, HBV-related hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.
\end{table}

\begin{table}[h]
\centering
\caption{Comparison of genotype and allele frequencies in the healthy control subjects of the present study with examples from the HapMap project}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{SNP rs1406} & \textbf{Samples} & \textbf{Genotype frequency, n(%)} & \textbf{P values} & \textbf{Allele frequency, n(%)} & \textbf{P values} \\
\hline
\textbf{Present Study} & N & GG & GT & TT & REF & 185 (59.29) & 127 (40.71) & REF \\
\hline
\textbf{HCB} & 86 & 57 (36.53) & 71 (45.51) & 28 (17.94) & 1 & 104 (64.46) & 68 (35.53) & 0.047 \\
\hline
\textbf{CHB} & 82 & 30 (46.34) & 30 (46.34) & 14 (17.97) & 0.137 & 106 (64.63) & 58 (35.36) & 0.277 \\
\hline
\textbf{JPT} & 172 & 50 (29.06) & 94 (54.65) & 28 (16.27) & 0.107 & 194 (56.39) & 150 (43.60) & 0.477 \\
\hline
\textbf{YRI} & 226 & 110 (48.67) & 88 (38.93) & 20 (8.39) & 0.055 & 308 (64.14) & 141 (35.85) & 0.014 \\
\hline
\textbf{CEU} & 294 & 126 (45.25) & 64 (21.90) & 64 (22.75) & 0.007 & 336 (75.00) & 112 (25.00) & 0 \\
\hline
\end{tabular}

\textsuperscript{HapMap, Haplotype Map; HCB, Chinese Han in Beijing, China; CHB, Chinese in Metropolitan Denver, Colorado; JPT, Japanese in Tokyo, Japan; CEU, Utah residents with northern and western European ancestry; and YRI, Yoruba in Ibadan, Nigeria.}
\end{table}
Our findings showed that the rs1406 G > T variant decreased the risk of HCC (OR 0.710, 95% CI 0.516–0.976, \( P = 0.035 \) G vs T) using the \( \chi^2 \) test. However, rs1406 SNP was not significantly associated with susceptibility to HCC after adjusting for age and sex using binary logistic regression analyses. Stratified analysis revealed that the differences in genotype frequencies were related to gender. Women who carried the CCNE1 GT genotype were significantly associated with a decreased risk of HCC compared with healthy controls carrying the GG genotype (additive model, OR 0.378, 95% CI 0.157, 0.910, \( P = 0.030 \)). In conclusion, this study indicated that the rs1406 G allele increased the risk of HCC in comparison with the T allele. The GT genotype was a protective factor in the development of HCC in female patients, and the CCNE1 rs1406 polymorphism is likely to contribute to HCC risk.

References

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