Association between the rs9131 and rs3806792 polymorphisms of the CXCL2 gene and the risk of HBV-related hepatocellular carcinoma in a Guangxi population

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Abstract
Background: Genetic polymorphisms in the CXCL2 may participate in the progress of HBV-related HCC. However, no researches have evaluated the association between them.

Methods: To figure out the effects of CXCL2 gene polymorphisms on the risk of HBV-related HCC, two major variants of CXCL2 and their association with chronic hepatitis B (CHB), HBV-related liver cirrhosis (LC), and HCC were conducted in a Guangxi population. CXCL2 polymorphisms rs9131 and rs3806792 were examined in 147 healthy controls, 138 CHB patients, 137 HBV-related LC patients, and 150 HBV-related HCC patients, using the SNaPshot™ genotyping technique.

Results: No significant differences were found regarding the CXCL2 rs9131 and rs3806792 polymorphisms among the case groups (including CHB, LC, and HCC) and the healthy controls, no matter in comparisons of alleles, genotypes, or haplotypes. Similar insignificant results were also observed when subgroup analyses were performed in different gender. However, when compared the frequencies of allele and genotype in the healthy individuals of our research and those from the 1000 Genomes Project, CC and C for rs9131, and TT and T for rs3806792 of CXCL2 in our healthy controls were only similar with those in Han Chinese in Beijing, but significantly higher than other ethnicities; this indicates that these two polymorphisms of CXCL2 may be not associated with the pathogenesis of HBV-related HCC in Chinese population, but may play a role in other ethnicities.

Conclusion: Our observation suggests that SNPs rs9131 and rs3806792 of CXCL2 gene might not contribute to the development of CHB, HBV-related LC, and HCC in a Guangxi population.

Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; CEU, Utah residents with northern and western European ancestry; CHB, chronic hepatitis B; CI, confidence intervals; CT, computed tomography; CXCL2, C-X-C motif chemokine ligand 2; GIH, Gujarati Indian from Houston, Texas; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; JPT, Japanese in Tokyo, Japan; LC, liver cirrhosis; LD, linkage disequilibrium; ORs, odds ratios; PCR, polymerase chain reaction; SNPs, single nucleotide polymorphisms; YRI, Yoruba in Ibadan, Nigeria.

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1 | INTRODUCTION

Hepatitis B virus (HBV) infection is a well-known global public health issue. According to the WHO, more than 2 billion people worldwide have been infected with HBV; among them, approximately 2.57 billion individuals have developed chronic hepatitis for persistent infection with HBV, and 780 000 have died annually from HBV-related diseases.\(^1\) Of these diseases, hepatocellular carcinoma (HCC) is the most lethal disease caused by HBV infection. China has the highest HCC incidence rate worldwide, containing about 19% of the world population but accounting for more than half of all newly confirmed HCC cases and deaths.\(^2\) Guangxi, a province in western China, has the highest HCC incidence and mortality rate in China, as well as a relatively high prevalence of HBV.\(^3\) It has been widely accepted that chronic HBV infections progress to chronic hepatitis B (CHB), then develop into liver cirrhosis (LC), and finally result in HCC.\(^3\) However, the underlying mechanisms of this progression are still not fully understood. The clearance of HBV depends on an effective host immune response\(^4\); thus, immune system-related cytokines and chemokines are believed to have important roles in HBV-related HCC development.

C-X-C motif chemokine ligand 2 (CXCL2), an antimicrobial gene that is part of a chemokine superfamily, is an immune-related chemokine expressed at sites of inflammation and participates in various inflammatory and immunoregulatory processes. Recent research has proven that CXCL2 plays multiple roles in various cancers, including HCC, cervical cancer, and bladder cancer.\(^5\) However, the results remain controversial. A study by Zhang H et al\(^5\) showed that the expression of CXCL2 was elevated in patients with bladder cancer, co-elevation of the levels of CXCL1 and CXCL8, both of which are NF-kB-dependent chemokines. Zhang W et al\(^6\) found that A-kinase–interacting protein 1 is critical in angiogenesis and the growth of cervical cancer. Our previous study also suggested that CXCL2 has a higher expression in their three-dimensional co-culture system as well as clinical HCC tissues, and could significantly increase the migration and invasion ability of SMMC7721 cells.\(^7\) Conversely, a recent study by Ding et al\(^8\) demonstrated a significant down-regulation of CXCL2 expression in 264 clinical HCC samples. Subat et al\(^9\) also found that the expression of CXCL2 was significantly downregulated in HCC tumor tissues in which the regulation mechanism may be controlled by DNA methylation. However, after treatment with a DNA demethylating agent, the expression of CXCL2 in HCC cell lines was significantly elevated; furthermore, tumors with lower CXCL2 expression have significantly fewer multiple tumors than tumors with higher CXCL2 expression.

As reported, numerous studies have suggested that gene polymorphisms play pivotal roles in the development of HBV-related HCC. For instance, Dai et al\(^10\) found that the rs187238 GG genotype of IL-18 may increase the risk of HCC in a healthy population and the risk of LC in CHB carriers; Wang et al\(^11\) suggested that a polymorphism in the liver fatty acid binding protein (rs1545224) might increase HCC risk in LC patients; and several SNPs of interferon genes combined with interferon receptor genes, including IFNL4, IFNLR1, IFNA, and IFNAR2, were also proven associated with HBV-related liver disease in a Han Chinese population.\(^12\) Rs9131 and rs3806792 are two major polymorphisms of CXCL2, with rs9131 being a 3 prime UTR variant and rs3806792 being an upstream transcript variant (https://www.ncbi.nlm.nih.gov/snp/?term=CXCL2).\(^13\) So far, the association between SNPs of CXCL2 and the risk of CHB, HBV-related LC, and HCC has not been indentified, but the relationship between CXCL2 expression and HCC has been reported. Therefore, the aim of the present study was to evaluate whether the rs9131 and rs3806792 gene polymorphisms of CXCL2 in males and females of Guangxi are associated with the risks of CHB, HBV-related LC, and HCC.

2 | MATERIAL AND METHODS

2.1 | Participants

All included participants were Chinese individuals from Guangxi province. The present study was approved by the ethics committee of the First Affiliated Hospital of Guangxi Medical University (Guangxi, China), and we confirmed that all research was performed in accordance with relevant guidelines, with written informed consent obtained from each patients. A total of 425 patients were enrolled in the present study, which comprised of 150 patients with HBV-related HCC, 137 patients with HBV-related LC, and 138 patients with CHB. The patients were all enrolled from the First Affiliated Hospital of Guangxi Medical University from June 2017 to February 2018. All patients had confirmed chronic HBV infections for at least 6 months according to seropositive HBsAg, HBeAb, and HBeAg or HBeAb. In addition, CHB was further confirmed with elevated alanine aminotransferase (ALT, >40 IU/mL) or aspartate aminotransferase (AST, >40 IU/mL) levels. LC was confirmed according to the typical morphologic results from ultrasonography or computed tomography (CT), combined with laboratory findings. Regarding the patients with HCC, they were diagnosed according to typical cytologic or histological results, or elevated AFP levels (over 400 ng/mL) together with a positive findings on CT or ultrasonography, and only newly diagnosed HCC patients without any other type of cancer were included. For comparison, 147 healthy volunteers who underwent a routine physical examination at the Health Examination Center of the same hospital were randomly selected. None of the healthy subjects had an HBV infection, and all had normal liver functions.

**KEYWORDS**
chronic hepatitis B, CXCL2, gene polymorphism, hepatocellular carcinoma, liver cirrhosis
Peripheral white blood cell samples from all participants were used in the extraction of DNA with an AxyPrep Blood Genomic DNA Miniprep Kit (Axygen). After extraction, a polymerase chain reaction (PCR) was used to amplify the DNA, with the kit in a total reaction volume of 20 μL. The primers for PCR were TGATAGGCTAGGAAATCCAAGA (rs9131-forward), gggACAGTACAAATAGACACACATAC (rs9131-reverse), CGCCGCTTCTAGAGTAGCCCG (rs3806792-forward), and CCCTAATCTACATACCTCGGT (rs3806792-reverse).

After amplification, SNPs rs9131 and rs3806792 were genotyped using the SNaPshot™ Multiplex Kit (Applied Biosystems) with single-base extension primers TCTTTACAGTTACAAAAT AGACACACATACATTTCCCTGCGTCACTTGATCT and tCCCTA ACTTTCATCCAACACTGAAATGTCTTCCAGAGAA GTACTCCCCCGTGA, respectively. Data were collected and analyzed with GeneMarker software (GeneMarker V2.2.0).

2.3 | Statistical analysis

Statistical differences in continuous variables such as age among all groups were compared by analyses of variance (ANOVA). Then, chi-square tests and Fisher’s exact tests were used to analyze categorical variables. The Hardy-Weinberg equilibrium (HWE) among all groups was first checked with the goodness-of-fit chi-square test. Allele and genotype frequencies of CXCL2 SNPs were calculated in different comparison groups using the chi-square test or Fisher’s exact test, when appropriate. Later, binary logistic regression was further performed to evaluate the odds ratios (ORs) and 95% confidence intervals (CIs), and both age and sex status were adjusted to assess the relative risk derived from a particular genotype or allele. As is known to all, HCC rates in males are 2-4 times higher than females; gender was therefore treated as a possible confounder.

Subgroup analyses in different ethnicities from the 1000 Genomes Project (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). According to the results in Table 4, excluding Han Chinese in Beijing (HCB), the distributions of both rs9131 and rs3806792 in our study are dramatically different from that in Utah residents with northern and western European ancestry (CEU), Gujarati Indian from Houston, Texas (GIH), Japanese in Tokyo, Japan (JPT), and Yoruba in Ibadan, Nigeria (YRI) (all P < .05). For rs9131, the distributions of the C allele and CC genotype in CEU, GIH, and JPT individuals were statistically lower than those in our healthy subjects, while the frequencies of allele T and genotype TT were significantly higher. As for rs3806792, significantly lower frequencies of the allele T and genotype TT, as well as

### TABLE 1 Basic characteristic of the study population

| Variable          | Healthy controls (n = 147) | CHB patients (n = 138) | P-value | LC patients (n = 137) | P-value | HCC patients (n = 150) | P-value |
|-------------------|----------------------------|------------------------|---------|-----------------------|---------|------------------------|---------|
| Age (y, mean ± SD)| 48.23 ± 11.37              | 39.71 ± 11.69          | <.001   | 47.04 ± 11.30         | <.371   | 50.53 ± 10.35          | .076    |
| Gender, N (%)     |                            |                        |         |                       |         |                        |         |
| Male              | 121 (0.82)                 | 108 (0.78)             | .390    | 108 (0.79)            | .458    | 127 (0.85)             | .585    |
| Female            | 26 (0.18)                  | 30 (0.22)              |         | 29 (0.21)             |         | 23 (0.15)              |         |
higher frequencies of the allele C and genotype CC in the CEU, GIH, and JPT populations, were found when compared with our control participants.

### 3.3 | Haplotype analysis of the CXCL2 SNPs and HBV-related diseases risk

Haplotype analyses were carried out in all four patient and healthy groups with the SHEsis software, and all four possible haplotypes were observed. Overall, in all three comparisons (ie, the CHB group vs the healthy group, the LC group vs the healthy group, and the HCC group vs the healthy group), a strong linkage disequilibrium was found between the alleles of the rs9131 and rs3806792 SNPs, D’ = 0.991, D” = 0.991, D’’ = 0.992, respectively. According to the results, the C<sup>9131</sup>C<sup>3806792</sup> haplotype was the major haplotype and accounted for >65% in all four groups, and the T<sup>9131</sup>T<sup>3806792</sup> was the second highest haplotype, accounting for 28%-32% among all groups. However, after haplotype analyses were conducted, significant differences in the distribution of these haplotypes were not observed in any group. The C<sup>9131</sup>T<sup>3806792</sup> and T<sup>9131</sup>C<sup>3806792</sup> haplotypes were rare, accounting for <2% in all groups, so further analyses were therefore not conducted between them. Data are showed in Table 5.

### 4 | DISCUSSION

HBV infection, for which China accounts for the highest incidence worldwide, is the most common risk factor for the progression of HCC. However, the clinical manifestations of HBV infections vary significantly among individuals, most of them are transient infections, and then are asymptomatic carriers with normal liver histology, the most severe symptoms are persistent carriers with chronic liver diseases such as LC and HCC. And among the subjects persistently infected with HBV, only 10%-30% will develop LC and HCC. The mechanisms of these highly diverse disease outcomes can only be partially explained by the differences in HBV viral infection, dietary aflatoxin exposure, or other environmental risk factors. Thus, individuals’ genetic background and immunological status may play pivotal roles in such progression.

Chemokines, which are structurally related molecules, are members of a subfamily of homologous proteins. Through chemical interactions, they can regulate the trafficking of multiple types of
leukocytes. Furthermore, by recruiting and activating leukocytes, as well as regulating the roles played by T lymphocytes, chemokines have long been considered as crucial mediators in the homeostasis, development, and function of the innate and adaptive immune system.\textsuperscript{22} As a member of this chemokines family, CXCL2 has also been shown to be essential for the pathogenesis of HBV-related HCC;

| Polymorphisms | Healthy controls, N = 121 | CHB patients, N = 108 | LC patients, N = 108 | HCC patients, N = 127 | CHB patients vs Healthy controls | OR (95% CI) \textsuperscript{a} | LC patients vs Healthy controls | HCC patients vs Healthy controls |
|---------------|---------------------------|----------------------|---------------------|---------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|
| rs9131        |                           |                      |                     |                     |                               |                                 |                               |                               |
| CC            | 63 (0.52)                 | 54 (0.50)            | 52 (0.48)           | 58 (0.46)           |                               | 1.00                           | 1.00                          | 1.00                          |
| CT            | 47 (0.39)                 | 47 (0.44)            | 46 (0.43)           | 54 (0.43)           |                               | 1.22 (0.66-2.27)               | 1.08 (0.62-1.90)              | 1.25 (0.74-2.13)              |
| TT            | 11 (0.09)                 | 7 (0.06)             | 10 (0.09)           | 15 (0.12)           |                               | 1.04 (0.34-3.19)               | 1.09 (0.42-2.81)              | 1.50 (0.63-3.53)              |
| Dominant model\textsuperscript{b} | 58 (0.48)                 | 54 (0.50)            | 56 (0.52)           | 69 (0.54)           |                               | 1.19 (0.66-2.16)               | 1.09 (0.64-1.85)              | 1.30 (0.79-2.14)              |
| Recessive model\textsuperscript{c} | 110 (0.91)                | 101 (0.94)           | 98 (0.91)           | 112 (0.88)          |                               | 0.95 (0.32-2.78)               | 1.05 (0.42-2.62)              | 1.35 (0.59-3.07)              |
| C allele      | 173 (0.71)                | 155 (0.72)           | 150 (0.69)          | 170 (0.67)          |                               | 1.00                           | 1.00                          | 1.00                          |
| T allele      | 69 (0.29)                 | 61 (0.28)            | 66 (0.31)           | 84 (0.33)           |                               | 1.10 (0.70-1.74)               | 1.06 (0.70-1.60)              | 1.25 (0.85-1.83)              |

\textsuperscript{a}Adjusted by age.
\textsuperscript{b}Dominant model: TT + CT vs CC.
\textsuperscript{c}Recessive model: TT vs CT + CC.

| Polymorphisms | Samples, N | Genotype frequency, n | P values | Alleles frequency, n | P values |
|---------------|------------|-----------------------|----------|----------------------|----------|
| rs9131        |            |                       |          |                      |          |
| Present study | 147        | 77 (0.52)             | 57 (0.39) | 13 (0.09)            | 211 (0.72) | 83 (0.28) |
| CEU           | 99         | 12 (0.12)             | 49 (0.49) | 38 (0.38)            | <.001    | 73 (0.37) | 125 (0.63) |
| HCB           | 103        | 45 (0.44)             | 46 (0.45) | 12 (0.12)            | .382     | 136 (0.66) | 70 (0.34) |
| GIH           | 103        | 11 (0.11)             | 57 (0.55) | 35 (0.34)            | <.001    | 79 (0.38) | 127 (0.62) |
| JPT           | 104        | 26 (0.25)             | 57 (0.55) | 21 (0.20)            | <.001    | 109 (0.52) | 99 (0.48) |
| YRI           | 108        | 80 (0.74)             | 24 (0.22) | 4 (0.04)             | .002     | 184 (0.85) | 32 (0.15) |

| rs3806792     |            |                       |          |                      |          |
| Present study | 147        | 18 (0.12)             | 53 (0.36) | 76 (0.52)            | 89 (0.30) | 205 (0.70) |
| CEU           | 99         | 39 (0.39)             | 49 (0.49) | 11 (0.11)            | <.001    | 127 (0.64) | 71 (0.36) |
| HCB           | 103        | 13 (0.13)             | 45 (0.44) | 45 (0.44)            | .425     | 71 (0.34) | 135 (0.66) |
| GIH           | 103        | 36 (0.35)             | 59 (0.57) | 8 (0.08)             | <.001    | 131 (0.64) | 75 (0.36) |
| JPT           | 104        | 21 (0.20)             | 57 (0.55) | 26 (0.25)            | <.001    | 99 (0.48) | 109 (0.52) |
| YRI           | 108        | 4 (0.04)              | 24 (0.22) | 80 (0.74)            | .001     | 32 (0.15) | 184 (0.85) |

Abbreviations: CEU, Utah residents with northern and western European ancestry; GIH, Gujarati Indian from Houston, Texas; HCB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo, Japan; YRI, Yoruba in Ibadan, Nigeria.

Bold indicates P <.001.
however, it remains a question whether the SNPs of CXCL2 play roles in such progression.

Our present study answered this question by evaluating the association between two major SNPs of CXCL2 and subjects with CHB, LC, and HCC, respectively. However, according to our results, no associations between CXCL2 genotypes, alleles, or haplotypes and the risk of CHB, LC, or HCC were observed; similarly, significant associations were not found when subgroup analyses were conducted in males and females. All these findings indicated that the SNPs of CXCL2 may not be associated with the pathogenesis of HBV-related HCC. In fact, little research has been conducted on correlations between CXCL2 and cancer, although numerous studies have suggested an association between the polymorphisms occurring in other chemokine genes and cancers. For instance, in CXCL10, a chemokine that serve as a T lymphocyte recruiter, the distributions of the G allele and GG genotype in the rs4508917 polymorphism of CXCL10 were dramatically higher in patients with breast cancer (BC) than in healthy volunteers.23 And CCL22, acting as a chemoattractant, mediated the intratumoral Treg migration, and the distribution of C allele and CC genotype at rs223818 of this gene was significantly higher in individuals with BC when compared to healthy controls.24 A CXCL12 G801A polymorphism was also found to be a low penetrance risk factor for the progression of colorectal cancer and was further confirmed to be associated with the T stage of colorectal cancer.25 It is noteworthy that in our study, when we compared the genotype and allele distributions in healthy subjects to those from the 1000 Genomes Project, a significant difference was found in the CXCL2 genetic background among those ethnicities. In our study, the frequencies of the genotype and allele (CC and C for rs9131 and TT and T for rs3806792) of the two SNPs of CXCL2 in our healthy controls were only similar with those in HCB, but were significantly higher than individuals of other ethnicities, including CEU, GIH, and JPT populations. These data indicate that SNPs rs9131 and rs3806792 of CXCL2 may not be associated with the pathogenesis of HBV-related HCC in the Chinese population but may play a role in other ethnicities. Another possible explanation for the current nonsignificant results is the limited sample size, given that the strength of an association between a certain polymorphism and a disease partially depends on the sample size achieved. Therefore, further investigations with larger populations with various ethnicities are warranted.

In sum, the present study did not find any effect of genetic polymorphisms between the CXCL2 gene and CHB, HBV-related LC, or HCC in a Guangxi population. However, the current study had some limitations. First, the present study used a relatively small sample and limited population to assess the relationship between CXCL2 SNPs and HBV-related diseases; second, only two sites of CXCL2 polymorphisms were assessed; and third, whether the SNPs of rs9131 and rs3806792 reflect the expression of CXCL2 remains unclear, and that may be the possible mechanism for the current nonsignificant results. Therefore, further larger and multicenter research with other SNPs of CXCL2 and their serum levels should be conducted to confirm our findings.
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AUTHOR CONTRIBUTIONS
YL drafted the article; ZH carried out the molecular genetic studies; LL participated in the sequence alignment; JZ and SY participated in the sequence alignment; JQ and XQ conceived of the study, participated in its design and coordination, and helped to draft the article.

DATA AVAILABILITY STATEMENT
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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