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Association of genetic polymorphisms in toll-like receptor 2 (*TLR2*) and susceptibility to hepatocellular carcinoma.

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Abstract

Purpose: Toll-like receptors (*TLR*) act as a vital innate immunity receptors involved in immune response. More and more evidence implies that mutations of *TLR2* gene are correlated with cancers. This retrospective study aimed to identify variants in *TLR2* affecting HCC (Hepatocellular Carcinoma) susceptibility.

Methods: Four tgSNPs (tag SNPs) were chose from HapMap data and genotyped in 274 patients with HCC and 277 healthy controls.

Results: Rs3804099 genotype CT (P=0.0001, OR=2.00) and allele T (P=0.019, OR=1.37) showed significant difference between HCC and HC (Healthy Control). People who carried rs7656411 genotype TT (P=0.002, OR=2.18) and allele T (P=0.002, OR=1.46) had a significant association with increased HCC risk. The results were consistent with previous results in dominant or recessive model (P=0.001, OR=1.81; P=0.014, OR=1.58; P=0.009, OR=1.78).

Conclusions: Rs3804099 polymorphism C/T and rs7656411 polymorphism G/T in *TLR2* were significantly correlated with HCC susceptibility.

Keywords: Toll-like receptor 2 (*TLR2*), Genetic polymorphism, Hepatocellular carcinoma.

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Introduction

Liver cancer is one of the most frequent cancer or the most frequent cause of cancer-related death around the world [1]. About 70% to 85% of the liver cancer is comprised of Hepatocellular Carcinoma (HCC) [1]. East or Southeast Asia and sub-Saharan Africa have the highest HCC incidence rates. The rate varies greatly between males and females. Chronic hepatitis (such as hepatitis B virus (HBV) or hepatitis C virus (HCV) infection), alcohol drinking, aflatoxin B1 exposure, diabetes, cigarette smoking, and some genetic factors are risks for HCC [2]. In China, about 94 million individuals are hepatitis B surface antigen (HBsAg) positive, which result in a high HCC rate [1,3].

Toll-like receptors (*TLR*s) is one of transmembrane receptors that act an important role in immune response against microbial pathogens [4]. *TLR* signals are involved in many liver diseases (hepatitis B or hepatitis C, alcoholic liver disease, primary biliary cirrhosis, non-alcoholic liver diseases, hepatic fibrosis, etc.) [5]. In the liver, various cell types (such as Kupffer cells, hepatocytes and biliary epithelium) can express *TLR2* [5]. In HBsAg seropositive patients, expression of *TLR2* is downregulated in peripheral blood monocytes, Kupffer cells and hepatocytes [6]. However, in HBsAgseronegative patients, expression of tumor necrosis factor-a and *TLR2* is up-regulated [6]. In hepatoma cell lines, stimulating IL-1 receptor or *TLR2* receptors could inhibit HBV replication and result in a signaling cascade [7]. Activation of *TLR2* was correlated with tumorigenesis in liver in a drug-primed mouse model [8]. Accumulating evidences implying that *TLR2* genetic polymorphisms are associated with risk of multiple cancers such as gallbladder cancer, cervical cancer, non-Hodgkin lymphoma, and endometrial cancer [9-12].

We hypothesized that SNPs of *TLR2* may be indeed associated with susceptibility to HCC. In order to verify the hypothesis, we conducted this study to characterize the association between *TLR2* polymorphisms and HCC in Chinese han population.

Materials and Methods

Study populations

In this retrospective study, we got blood samples from unrelated Chinese han persons in our hospital, Chongqing, China between February 2012 and June 2015. A total of 551

individuals including 274 patients with HCC and 277 healthy controls (HC) were studied. HCC was diagnosed by clinical and biological criteria, and confirmed by examination of iconography (computerized tomography and/or magnetic resonance imaging). Clinical classification was conducted by the tumor-node-metastasis staging system (TNM) from International Union Against Cancer (UICC). Child-Pugh score was used to evaluate the severity of liver disease between HCC and HC groups.

We defined HBV carriers as positive for both HBsAg and HBcAb. HCC patients could have the following treatments: transcatheter arterial chemoembolization, chemotherapy, hepatectomy and liver transplantation. During the period of this study, we randomly selected healthy controls who attending hepatitis examination to eliminate the confounding factor of HBV infection. The criteria for healthy controls were no history of any cancers, and its frequency must match to the HCC patients on gender and age. The study protocol was approved by the ethics committee of our hospital. Written informed consents were obtained from all subjects.

Tagging SNP selection

We chose 4 tgSNPs from *TLR2* gene on the International HapMap Project, release27 (http://hapmap.ncbi.nlm.nih.gov) in the CHB population. The exclution criterion: an r2 value<0.8, MAF<0.05. SNPs that seemed to have a function in the literature or are non-synonymous SNP in NCBI were selected firstly.

DNA extraction and SNP analysis

According to manufacturer's instructions of the Wizard® Genomic DNA Purification Kit (Promega, USA), genomic DNA samples were extracted from peripheral blood leukocytes. We used a NanoDrop spectrophotometer to measure DNA samples, diluted to 30 ng/μl, and stored at -80°C for genotyping. We genotyped the five SNPs with IplexGOLD chemistry on SEQUENOM mass spectrometer (SequenomInc, San Diego, USA). A negative control and duplicate samples were set in every 96-well plate for quality control.

Statistical analysis

We evaluated the risk of HCC patients according to genotypes and alleles of *TLR2* in comparison to the HC groups. We used HWE software to test Hardy-Weinberg equilibrium in both groups. Differences in gender, age, and Clinical Characteristics were compared between groups with χ^2 or Mann-Whitney U test. Each genotype was estimated with dominant or recessive genetic models. Binary logistic regression was used for assessing the relative risks of SNPs. Haploview software (v4.2) was used to identified haplotype frequencies. We carried out SPSS 21.0 (SPSS Inc., Chicago, IL, USA) for statistical calculations and P<0.05 were thought to indicate satistical significance.

Results

In HC group, all the four genotyped SNPs of *TLR2* were in accordance with the Hardy-Weinberg equilibrium.

Characteristics of the study population

Demographic and clinical characteristics of the study population are given in Table 1. There were no significant differences in gender, age, HBV carriers and Child-Pugh score between HCC and HC groups (P>0.05).

Table 1. *Demographic and clinical characteristics of the study population.*

Characteristics	HC	HCC	P
	n=277	n=274	
Male/female	225/52	230/44	0.401
Age	55.59+15.38	54.97+14.92	0.631
HBV carriers	221 (79.8%)	225 (82.1%)	0.486
Child-Pugh score			
A	235 (85.8%)	250 (90.3%)	0.105
B	39 (14.2%)	27 (9.7%)	
a-FP level			
>400 ng/ml	104 (38.0%)		
<400 ng/ml,	170 (62.0%)		
UICC classification			
Stage I-II	131 (47.8%)		
Stage III-IV	143 (52.2%)		

HCC: Hepatocellular Carcinoma; HC: Healthy Controls; P: P value.

Correlations of SNPs with HCC risks

We studied the correlation of the four SNPs in *TLR2* between HCC and HC groups. Rs3804099 genotype CT (P=0.0001, OR=2.00) and allele T (P=0.019, OR=1.17) showed significant difference between HCC and HC. People who carried rs7656411 genotype TT (P=0.002, OR=2.18) and allele T (P=0.002, OR=1.21) had a significant association with increased HCC risk (Table 2). In dominant or recessive model, the results were consistent with previous results (P=0.001, OR=1.81; P=0.014, OR=1.26; P=0.009, OR=1.33). The other two SNPs (rs7696323 and rs11938228) showed no significant correlation with HCC risk (Table 3).

Table 2. *Association of TLR2 (rs3804099 and rs7656411) in HCC VS HC.*

Genotype	HCC	HC	P	OR
rs3804099				
CC	157	119	-	-

CT	92	140	0.0001	2.00
TT	25	18	0.875	1.05
C	406	378	-	-
T	142	176	0.019	1.37
Dominant model	-	-	0.001	1.81
Recessive model	-	-	0.407	0.77
rs7656411				
GG	99	73	-	-
GT	134	138	0.089	1.40
TT	41	66	0.002	2.18
G	312	284	-	-
T	236	270	0.002	1.46
Dominant model	-	-	0.014	1.58
Recessive model	-	-	0.009	1.78

HCC: Hepatocellular Carcinoma; HC: Healthy Controls; P: P value; OR: Odds Ratio.

Table 3. *Association of TLR2 (rs11938228 and rs7696323) in HCC VS HC.*

Genotype	HCC	HC	P	OR
rs11938228				
AA	101	100	-	-
AC	116	135	0.394	1.18
CC	57	42	0.233	0.74
A	318	335	-	-
C	230	219	0.410	0.90
Dominant model	-	-	0.853	1.03
Recessive model	-	-	0.086	0.68
rs7696323				
CC	142	149	-	-
CT	104	111	0.925	1.02
TT	28	17	0.096	0.58
C	388	409	-	-
T	160	145	0.262	0.86
Dominant model	-	-	0.644	0.92
Recessive model	-	-	0.083	0.57

HCC: Hepatocellular Carcinoma; HC: Healthy Controls; P: P value; OR: odds ratio.

Finally, we did a haplotype analysis. Rs7696323 and rs11938228 were in high LD. But none of the haplotypes were significantly associated with HCC risk.

Discussion

In this study, we chose four SNPs in *TLR2* to evaluate the risk of HCC in Chinese Han population. We found evidence of variants in *TLR2* (rs3804099 and rs7656411) were on the risk of HCC. Toll-like receptors (*TLR*s) is one of transmembrane receptors that act an important role in immune response against microbial pathogens [4]. *TLR2* is proved to be associated with multiple cancers including CRC [13-16]. Researchers have explored the association between *TLR2* variants and tumors. Microsatellite GT polymorphism in *TLR2* was reported to be associated with sporadic colorectal cancer in Croatian [17]. Carriers with *TLR2* (Delta22) polymorphisms were correlated with an increased risk for gallbladder cancer [18]. Another study showed significantly correlation between *TLR2* (-196 to -174 del) and cervical cancer susceptibility [10]. Subsequently, the frequency of the *TLR2* -196 to -174 del allele was found to be significantly higher in patients with HCV-correlated HCC than those without HCC [19]. Stimulation of monocytes in carriers with the *TLR2* -196 to -174 del allele had significantly lower *TLR2* and IL-8 expression levels than in those with the *TLR2* -196 to -174 ins/ins. The current study found that *TLR2* polymorphisms influence the risk of HCC. In our study, rs3804099 genotype CT (P=0.0001, OR=2.00) and allele T (P=0.019, OR=1.17) showed significant difference between HCC and HC. People who carried rs7656411 genotype TT (P=0.002, OR=2.18) and allele T (P=0.002, OR=1.21) had a significant association with increased HCC risk. In dominant or recessive model, the results were consistent with previous results (P=0.001, OR=1.81; P=0.014, OR=1.26; P=0.009, OR=1.33). The other two SNPs (rs7696323 and rs11938228) showed no significant correlation with HCC risk. Our data implied that variations in *TLR2* may act an important risk role in HCC risk.

TLR2 rs3804099 is a synonymous SNP. Synonymous SNPs have been assumed inconsequential for a long time, as they do not result in change of polypeptide structure. However, this concept change as synonymous mutations is confirmed to be implicated in diseases in many studies over the last decade. It effect on gene function may through the following mechanisms: (1) Perturbations of mRNA splicing [20,21]; (2) The stability of mRNA [22]; (3) mRNA structure [23]; (4) Protein folding [24,25]. Rs7656411 located in in 3'-UTR of *TLR2* with a change from G to T. Rs7656411 was found to be associated with colon cancer [26], Asthma [27]. SNP in 3'-UTR may act its function with miRNA or alone by influence of mRNA splicing or the stability of mRNA, and influence the expression level of *TLR2*. We found that both the rs3804099 and rs7656411 were significantly correlated with HCC susceptibility and might influence the expression level of *TLR2*. However, the exact mechanisms of might be different as they located in different regions of *TLR2*. In collusion, we found rs3804099 polymorphism C/T and rs7656411 polymorphism G/T in *TLR2* were significantly associated with HCC susceptibility. Our study has several limitations: lack of a validation assay and a relatively small sample size. In the

future, we need a prospective longitudinal study to confirm the findings in larger populations.

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